



UNIVERSITÀ DI PARMA

ARCHIVIO DELLA RICERCA

University of Parma Research Repository

External and internal EGFR-activating signals drive mammary epithelial cells proliferation and viability

This is the peer reviewed version of the following article:

Original

External and internal EGFR-activating signals drive mammary epithelial cells proliferation and viability / Morato, Alessia; Martignani, Eugenio; Miretti, Silvia; Baratta, Mario; Accornero, Paolo. - In: MOLECULAR AND CELLULAR ENDOCRINOLOGY. - ISSN 0303-7207. - (2020), pp. 1-9. [10.1016/j.mce.2020.111081]

Availability:

This version is available at: 11381/2899512 since: 2022-03-26T21:28:29Z

Publisher:

Elsevier

Published

DOI:10.1016/j.mce.2020.111081

Terms of use:

Anyone can freely access the full text of works made available as "Open Access". Works made available

Publisher copyright

note finali coverpage

(Article begins on next page)

1 **External and internal EGFR-activating signals drive**
2 **mammary epithelial cells proliferation and viability**

3 Alessia Morato ^a, Eugenio Martignani ^a, Silvia Miretti ^a, Mario Baratta ^a and Paolo Accornero ^{a*}

4 1 Department of Veterinary Science, University of Turin, Grugliasco (TO), Italy

5

6 **Corresponding author:** * Paolo Accornero, Department of Veterinary Science, University of Turin,
7 Largo Braccini 2, 10095, Grugliasco (TO), Italy; Tel. +39 011 6709326; Fax. +39 011 6709138;
8 email: paolo.accornero@unito.it

9

10 **Highlights**

- 11 • EGFR autocrine signaling drive mammary epithelial cell proliferation and cell cycle
12 • An EGFR-dependent Erk 1/2 phosphorylation is active in cells cultured in growth factor
13 deprived medium
14 • Krt14 negative / Krt18 positive mammary cells depend on EGFR activation for survival
15 • Mammary cells express high mRNA levels of two or more EGFR ligands

16

17 **Abstract**

18 During puberty, the mammary gland undergoes an intense growth, dependent on the interplay between the
19 Epidermal Growth Factor Receptor (EGFR) in the stroma and different mammary epithelial receptors. **We**
20 **hypothesize that EGFR expressed in the mammary epithelium also has a role in puberty and the epithelial**
21 **cells can self-sustain by EGFR-mediated autocrine signaling.** We adopted mammary cell lines from different
22 species, as *in vitro* model for the epithelium, and we observed that EGFR-signaling positively affects their
23 survival and proliferation. Once deprived of external growth factors, mammary cells still showed strong Erk
24 1/2 phosphorylation, abolished upon EGFR inhibition, coupled with a further reduction in survival and
25 proliferation. Based on gene expression analysis, three EGFR-ligands (AREG, EREG and HBEGF) are
26 likely to mediate this autocrine signaling. In conclusion, internal EGFR-activating signals sustain mammary
27 epithelial cell proliferation and survival *in vitro*.

28

29 **Keywords:** Mammary gland; EGFR; EGFR-ligands; autocrine; Erk 1/2; keratin 14/18

30

31 **Abbreviations:** Epidermal Growth Factor Receptor = EGFR; amphiregulin = AREG; epiregulin = EREG;
32 heparin-binding EGF-like growth factor = HBEGF; Keratin 14 = Krt14; Keratin 18 = Krt18.

33

34 **Acknowledgments:** We are grateful to Cristina Cecere for technical assistance.

35

36 **Funding:** This work was supported by national grants from Ministry of Instruction, University and
37 Research and by local grants from Università di Torino. The funders had no role in study design, data
38 collection and analysis, decision to publish, or preparation of the manuscript.

39

40 **1. Introduction**

41 The mammary gland develops through a complex sequence of events, which start in the embryonic period
42 and culminate towards the end of pregnancy. In the prepubertal period, the mammary morphogenesis is
43 based on a close interaction between the epithelial and the stromal compartments, and the ductal tree grows
44 isometrically with the rest of the body. After puberty, endocrine stimuli are the main drivers of mammary
45 development and the ductal tree undergoes a robust allometric growth. In the murine species, specific club-
46 shaped structures, known as Terminal End Buds (TEBs), make way to the progression of the ductal tree into
47 the surrounding fat pad. Ovarian estrogens trigger this impressive elongation via Estrogen Receptor α (ER α)
48 expressed in a subset of epithelial cells: ER α -positive cells release amphiregulin (AREG), via
49 TACE-shedding (tumor necrosis factor- α -converting enzyme also known as ADAM17), which binds EGFR
50 in the stromal compartment. Activated stromal cells release other growth factors (mainly FGFs) which in
51 turn stimulate both ER-positive and ER-negative epithelial cells (Ciarloni et al., 2007; Macias & Hinck,
52 2012; Zhang et al., 2014). Since only the stromal fraction of EGFR was proved essential for the ductal
53 elongation, ER α -negative cells are thought to proliferate through the **interplay with** the stroma (Sternlicht et
54 al., 2005; Wiesen et al., 1999).

55 The ErbB tyrosine kinase receptor (RTK) family comprises four members: Erb-B1 (EGFR), Erb-B2, Erb-B3
56 and Erb-B4. During the pubertal development of the murine mammary gland, EGFR is highly expressed in
57 the stroma, and to a lesser extent in the epithelial TEBs and ducts. Erb-B2 is mostly present in the
58 epithelium, but being an orphan receptor, requires a heterodimerization shedding for its activation. Erb-B3 is
59 only detectable when the mammary gland is mature, while Erb-B4 can be detected during pregnancy and
60 lactation (Schroeder & Lee, 1998; Spivak-Kroizman et al., 1992). EGFR can be activated upon the binding
61 of seven different peptides of the EGF-family: amphiregulin (AREG), betacellulin (BTC), epidermal growth
62 factor (EGF), epigen (EPGN), epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF) and
63 transforming growth factor α (TGF α) (Harris et al., 2003). Overall AREG is more expressed during puberty
64 than in the following stages of development (D'Cruz et al., 2002; Schroeder & Lee, 1998) and displays a
65 significantly higher expression in the TEBs and in the ducts compared to the stroma, in contrast to other
66 EGFR-ligands that are variably expressed in the mammary epithelium (DiAugustine et al., 1997; Sternlicht
67 et al., 2005).

68 Elegant studies performed on knock-out mice have shed light on many mechanisms of the pubertal
69 mammary morphogenesis, concluding that epithelial EGFR and EGF-related peptides different from AREG
70 have a dispensable role during this period of growth (Ciarloni et al., 2007; Luetteke et al., 1999; Sternlicht et
71 al., 2005). Good evidence exists that the interaction between epithelial EGFR and EGF-like ligands has also
72 a direct (i.e not mediated by stroma) effect on the epithelial cells themselves: immature mammary epithelial
73 organoids undergo a prominent branching in the presence of EGF, TGF α , HBEGF and AREG (Camacho
74 Leal et al., 2012; Jenkins et al., 2012; Simian et al., 2001; Sisto et al., 2017). Although EGFR is primarily a
75 stromal receptor, it is also present in the epithelial compartment, especially in the cap cells of the end buds
76 and in the myoepithelial cells of the mammary ducts (Coleman et al., 1988; DiAugustine et al., 1997). All
77 that considered, it could be speculated that the expression of AREG (or other EGF-like growth factors) is
78 also induced locally (independently from any systemic hormones). It is also possible that these growth
79 factors act locally, activating EGFR expressed by the epithelium itself. In other words, an autocrine activity
80 within the mammary epithelium can be hypothesized (graphical abstract, right panel), whereby mammary
81 epithelial cells can sustain their own proliferation by releasing EGF-like growth factors that activate EGFR
82 expressed locally. An autocrine signaling, though dispensable for the growth of the mammary tree, might
83 contribute to its normal development, together with the previously mechanism (graphical abstract, left
84 panel), based on a stromal-epithelial interplay, that other authors previously described (Ciarloni et al., 2007;
85 Sternlicht et al., 2005).

86 Another open question is whether the information achieved in the murine species can be translated to other
87 animal models. Although not much is known about the distribution of ErbB and growth factors in the
88 mammary gland of other species, their pattern of expression could substantially diverge from what described
89 for the mouse. For instance, in the pubertal rat the TEBs, more than other structures, express EGFR, as well
90 as Erb-B4 which is commonly considered a receptor of the pregnant mammary gland, in the mouse (Darcy et
91 al., 1999, 2000). One might therefore speculate that also the relative importance of ErbB and growth factors
92 in the normal development of the pubertal mammary gland could differ, in other species. Still, most of the
93 studies on the morphogenesis of the mammary gland have been performed on murine models. This
94 represents a substantial limit when data about the endocrine regulation of mammary development are needed
95 in other species.

97 In the present study we sought to test our hypothesis. We chose to work *in vitro* with 4 different cell lines
98 (human, murine, bovine), as a model for the mammary epithelium, for multiple reasons. First, to perform our
99 experiments on a homogeneous population of epithelial cells, without any stromal interference. **Second**, to
100 offer **evidence** valid in different species. Here we investigated the ability of our lines to sustain their own
101 proliferation and to remain viable, even in the absence of external stimuli, in an EGFR-dependent way
102 (autocrine activity). Since the Erk 1/2 pathway is known to have a pivotal function in cell viability and
103 proliferation (Kerpedjieva et al., 2012; Thiel & Cibelli, 2002), downstream **of** EGFR (Camacho Leal et al.,
104 2012; Fata et al., 2007; Kariagina et al., 2010), we subsequently verified whether the activation state of this
105 pathway is involved in the autocrine activity of our cell lines, also investigating other pathways potentially
106 activated in this scenario. To identify potential mediators of the hypothesized autocrine model, we finally
107 looked which EGFR ligands are transcribed by the cells and whether EGFR-dependent signaling regulate
108 this expression.

109

110 **2. Materials and Methods**

111 **2.1. Materials.** All materials, unless otherwise stated, were from Merck KGaA (Darmstadt, Germany).
112 DMEM, DMEM/F12, FBS and Hoechst 33342 were from Thermo Fisher Scientific (Waltham, MA, USA).
113 Epidermal Growth Factor (EGF) was from Immunotools (Friesoythe, Germany). AG1478 and UO126 were
114 from LC Laboratories (Woburn, MA, USA). Rabbit anti-Erk 1 (1:1000; sc-94), anti-EGFR (**for murine cell**
115 **lines 1:1000**; sc-03-G) and anti-STAT5 (1:1000; sc-835) antibodies were from Santa Cruz Biotechnology
116 (Santa Cruz, CA, USA); mouse anti-phospho-Erk 1/2 (1:5000; M 8159), mouse anti-Keratin 18 (Krt18;
117 1:200; K5-B) and mouse anti- α -tubulin (1:10000; T5168) antibodies were from Merck KGaA; rabbit anti-
118 Keratin 14 (Krt14; 1:500 for immunohistochemistry and 1:2000 for western-blot; Poly19053) antibody was
119 from BioLegend (Dedham, MA, USA); mouse anti-Erb-B2 (1:1000; CB11, MA1-35720) and rabbit anti-
120 AREG (1:7500; PA5-16621) were from ThermoFisher Scientific; rabbit anti-Her2/Erb-B2 antibody (1:1000;
121 A0485) was from Dako (Glostrup, Denmark); **rabbit anti-EGFR (for human and bovine cell lines; 1:1000; #**
122 **2232)**; rabbit anti-phospho-EGFR (tyr-1068; 1:1000; # 2234), anti-phospho-Akt (ser-473; 1:1000; # 9271),

123 anti-phospho-STAT3 (tyr-705; 1:1000; # 9131), anti-phospho-STAT5 (tyr-694; 1:1000; # 9351), anti-Akt
124 (1:2000; # 9272) and anti-STAT3 (1:2000; # 9132) antibodies were from Cell Signaling Technologies
125 (Danvers, MA,USA). Alexa-fluor-488 goat-anti-rabbit and Alexa-fluor-594 goat-anti-mouse secondary
126 antibodies were from Invitrogen Corporation (Carlsbad, Ca, USA).

127 **2.2. Cell culture.** HC11 murine mammary epithelial cell line (ATCC n. CRL-3062) was cultured in
128 DMEM with 10% FBS, 10 ng/mL EGF and 5µg/mL insulin. NMuMG murine mammary epithelial cell line
129 (ATCC n. CRL-1636) was kindly provided by Dr. Montesano R. (University of Geneva Medical School,
130 CH) and cultured on collagen coated plates in DMEM with 10% FBS. MCF-10A human mammary epithelial
131 cell line (ATCC n. CRL-10317) was cultured in DMEM/F12 with 5% Horse Serum (HS), 20 ng/mL EGF, 10
132 µg/mL insulin, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin. The BME-UV bovine mammary
133 epithelial cell line was kindly provided by Dr. Politis I. (Agricultural University of Athens, Athens, GR) and
134 cultured in DMEM with 10% FBS, 10 ng/mL EGF and 1:100 ITS liquid media supplement.

135 **2.3. 3D collagen assay.** Collagen gels were obtained preparing a gel solution by mixing, in an ice bucket,
136 3,15 parts of H₂O, 1 part of DMEM 10x, 0,25 parts of Hepes (1 M, pH 7,4), 1 part of NaHCO₃ (22 g/liter), 1
137 part of Fetal Bovine Serum, 0,6 parts of NaOH (0,1N) and 3 parts of Rat type I collagen (final approximate
138 concentration of 1,5 mg/mL). 250 µL of solution were let gel at the bottom of each well (24 well plate) at
139 37° C for 20 min. Cells were trypsinized, resuspended in complete medium and counted. Each well was
140 filled with 0,5 ml collagen solution ($2,5 \times 10^3$ cells) and let gel at 37° C for 25 min. Then, 1 mL of complete
141 medium was slowly added to each well. Cells were allowed to grow for 4 days, then the medium was
142 delicately replaced with new medium containing the indicated inhibitors/factors. After 48 h gels were
143 photographed with a Leica AF6000 LX inverted microscope.

144 **2.4. Flow cytometry.** Cells were seeded on 6 cm dishes at a density of 300.000 cells/well in their specific
145 growth medium. Following 6 h of culture, the medium was replaced with the treatment medium as indicated
146 and cells were let grow for different timepoints depending on the cell line and the experiment. Cells were
147 washed 3 times with PBS and added with 500 µL of trypsin. Following cell detachment, 1,5 mL of DMEM
148 with 10% FBS was added and cells were spinned at 250 x g for 5 min. Medium was carefully removed, 2 mL
149 of PBS were added and cells were resuspended. Cells were then fixed by adding 2 mL of ethanol drop by
150 drop and incubating 1 h at 4°C. Samples were then spinned at 500 x g for 7 min, resuspended in 1 mL of

151 PBS with 5 $\mu\text{g}/\text{mL}$ DAPI and stained overnight at 4°C. The samples were analysed using an Attune Acoustic
152 Focusing Cytometer (Invitrogen Corporation) equipped with a 405 nm (violet) excitation laser and a 405/40
153 nm (blue) emission filter. For each sample 25.000 to 50.000 events were analyzed and each experiment was
154 repeated 3 or more times. The percentages of cells in the G0/G1 phase of their cycle were calculated using
155 the Attune Cytometric Software version 2.1 (Invitrogen). For the gating procedure see Supplementary Fig.
156 S1.

157 For the analysis of cell death, following culture, all the supernatant was collected and mixed with the
158 trypsinized cells. The next steps were identical as above. At least 50.000 events were acquired and
159 experiments were repeated 3 or more times. The percentage of Sub G0/1 was calculated by dividing the
160 number of Sub G0/G1 events by number of FSC/SSC gated events. For the gating procedure see
161 Supplementary Fig. S2.

162 **2.5. Nuclei counting and MTT assay.** Cells were seeded in 6 well-plates at a density of 300.000
163 cells/well, in growth medium (G). After 6 h, G was changed with the medium for each experimental
164 condition. After 48 h (24 h for BME cells), the medium was removed and replaced with fresh culture
165 medium, in preparation to the following experiments.

166 For the nuclei counting, Hoechst stain was added at a concentration of 5 $\mu\text{g}/\text{mL}$ and pictures were taken with
167 Nikon Eclipse Ti2, equipped with a DS-Qi2 digital camera, controlled by NIS-Elements software version
168 5.21. The microscope was programmed to scan 20 fields per condition. Cell profiler software ver. 3.1.8
169 (<https://cellprofiler.org/>) was used to count the nuclei (IdentifyPrimaryObject Module) and the average of 20
170 fields was calculated. Each experiment was repeated 3 times.

171 For the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a
172 concentration of 500 $\mu\text{g}/\text{mL}$ and the plates were incubated at 37° C for 2 h. The cells were finally lysed with
173 a solution of SDS 10% and HCl 0.04 M, and the absorbance of the lysate was measured at 570 nm with a
174 reference at 655 nm. Each experiment was repeated 3 times.

175 **2.6. Gene expression analysis.** For RNA extraction cells of each line were seeded in 6 cm dishes in
176 growth medium for 6 h, then cultured for 16 h (HC11, NMuMG, MCF-10A) or 4 h (BME-UV) under the
177 indicated experimental conditions. Cells were then lysed in 1 mL Tryzol and DNA-free total RNA was
178 isolated following the manufacturer's protocol. 500 ng of RNA were reverse transcribed with iScriptTM

179 cDNA Synthesis Kit (Bio-Rad Laboratories). Reverse transcribed samples were diluted 1:20 in RNase free
180 water. For quantitative rt-PCR cDNA (7.5 ng) was amplified with a CFX Connect real-time PCR system
181 (Bio-Rad Laboratories, Hercules, Ca, USA), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad
182 Laboratories) following the manufacturer's protocol. Primers were concentrated 250 nM. Primers sequences
183 and efficiencies are indicated in Supplementary Table S1. The expression of each analysed gene was
184 normalized to hypoxanthine phosphoribosyl-transferase 1 (HPRT-1) and glyceraldehyde-3-phosphate
185 dehydrogenase (GADPH) mRNA expression, which are constant in the cell lines under our experimental
186 conditions except in the MCF-10A experiments in which only GAPDH was used because HPRT was not
187 stable. Each amplification curve was corrected for efficiency of the corresponding gene, calculated by
188 standard dilution curves. CFX Manager Software 3.1 (Bio-Rad Laboratories) was used for both gene
189 expression analysis and efficiency calculation/correction.

190 **2.7. Protein expression analysis.** For protein extraction, cells were seeded in 6 cm dishes (1×10^6 cells
191 per dish), for 6 h in G, then cultured under the indicated experimental conditions for 16 h (4 h for BME-UV).
192 Dishes were washed with ice-cold PBS and lysed for 10' on ice, in 300 μ l of a lysis solution (20 mM Tris pH
193 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 1 mM glycerolphosphate), Protease
194 Inhibitor Cocktail (1:100), 1 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride. Samples were
195 scraped (Orange Scientific), collected, and centrifuged at 4°C for 10' at 13.000 rpm. Supernatants were
196 quantified with a DC Protein Assay (Biorad Laboratories). For Western-Blot, samples (20-100 μ g of total
197 protein) were resolved on SDS-PAGE gels and transferred to 0,45 μ m hybridization nitrocellulose filters
198 (Millipore, Burlington, MA, USA). Membranes were blocked at RT for 1 h in a 10% BSA Tris-buffered
199 saline (TBS, 10 mM Tris and 150 mM NaCl, pH 7.4) then incubated for 16 h at 4°C with the indicated
200 primary antibodies. Membranes were washed in TBS-Tween then incubated for 1 h at room temperature
201 with 1:10.000 diluted HRP-conjugated secondary antibodies (Thermo Fisher Scientific). The membranes
202 were in TBS-Tween and incubated for five min at room temperature with Clarity Western ECL Substrate
203 (Biorad Laboratories). The proteins were visualized by briefly exposing the membrane to an
204 autoradiographic CL-XPosure Film (Thermo Fisher Scientific). Western Blot results were then acquired with
205 an Epson scanner.

206 **2.8. Immunocytochemistry.** MCF-10A cells seeded in 6-well plates (200.000 cells per well) in G for 6 h
207 then cultured under different conditions for 48 h. Culture medium was then removed and cells were fixed for
208 40'' in acetone/methanol solution (1:1). Culture dishes were washed with Tris Buffered Saline (Tris 0,1 M,
209 NaCl 8g, pH 7,6) then incubated for 1 h with goat serum 10% in Antibody Dilution Buffer (ADB; TBS with
210 BSA 1%, Na-azide 0,1%). Cells were then incubated with diluted Krt14 (1:500) and Krt18 (1:200)
211 antibodies for 1 h at RT. Dishes were washed with TBS and incubated with Alexa-fluor-488 goat-anti-rabbit
212 and Alexa-fluor-594 goat-anti-mouse secondary antibodies (5 µg/mL); after 60 minutes cells were washed in
213 TBS and DAPI (5 µg/mL) was added for 15 minutes. Dishes were washed twice with TBS and pictures were
214 acquired with a Leica AF6000 LX (Leica Microsystems, Wetzlar, Germany) fluorescent microscope
215 equipped with a Leica DFC350FX digital camera controlled by the LAS AF software (Leica Microsystems).
216 For every culture condition 16 randomly selected fields were acquired and every experiment was repeated
217 three times. ImageJ software was used to determine the Krt14Area, the area positive for Krt14, by using the
218 ImageJ Image_Adjust_Color threshold... command with a Brightness value set at constant value followed by
219 the Analyze_Histogram command. The whole area occupied by cells (TOTarea) was determined using the
220 area occupied by the Krt18 stained cells (all MCF-10A cells are positive). For this purpose, we used the
221 ImageJ Adjust_Brightness/Contrast... to highlight cell boundaries, then the Wand tool to select the area of
222 the plate covered by the cells (with the Freehand selections when isolated cells or group of cells needed to be
223 selected manually), then the Edit_Selection_Make Inverse to select the area covered by cells and finally the
224 Analyze_Measure tool to quantify this area. The average percentage value was obtained by dividing
225 Krt14Area by the TOTarea. For an outline of the procedure see Supplementary Fig. S3. An example of the
226 images used to produce the composites of Fig. 5 is present in Supplementary Fig. S10.

227 **2.9. Statistics.** The analysis was performed with SPSS 25.0 Statistical Software. Data are expressed as
228 mean ± sem of samples measured in triplicate or more. One-way univariate ANOVA with contrasts was used
229 to test for significant differences between culture conditions. When normality and/or homoscedasticity of the
230 dependent variable were not verified, a Kruskal-Wallis test or the combination of Welch and Brown-
231 Forsythe tests were used for the same purpose. Consequently, multiple comparisons were performed with an
232 appropriate post hoc test (Bonferroni following Kruskal Wallis, Games-Howell following Welch and Brown-
233 Forsythe).

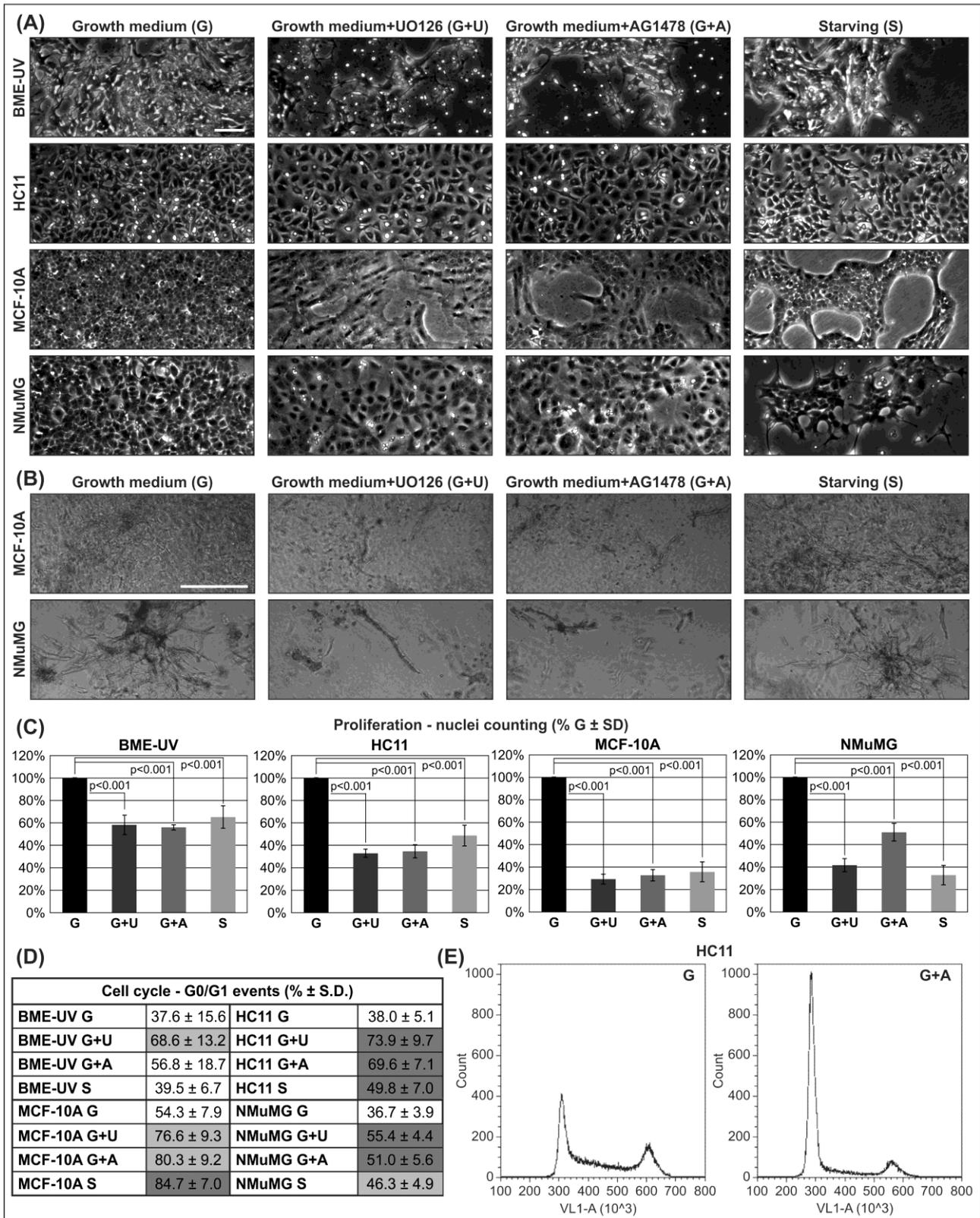
234

235 **3. Results**

236 **3.1. Mammary cell lines of different species are sensitive to EGFR inhibition.** We first analysed
237 the relative addition of different mammary epithelial cell lines to EGFR signaling by adding to the growth
238 medium (G) a highly specific tyrosine kinase inhibitor (AG1478; G+A) at nanomolar concentrations (300
239 nM). AG1478 was chosen out of three selective EGFR-inhibitors (AG1478, Erlotinib 1 μ M and Gefitinib
240 1 μ M), after comparing their effect in both 2D and in 3D culture, since no differences were seen, either
241 qualitatively (phase contrast 2D and 3D images: Supplementary Fig. S4A and S4B) or quantitatively (nuclei
242 counting and MTT assay: Supplementary Fig. S4C and S4D; p values in Supplementary Tables S2 and S3).
243 As a positive control for inhibition, we used a selective inhibitor of both MEK1 and MEK2, UO126 at 20
244 μ M (G+U). We also used the starving condition (S) to test whether deprivation of all external growth factors
245 influenced cell growth. At 48 h post-treatment cells were recorded (Figure 1A and 1B). Given a high
246 mortality beyond 24 h, BME-UV were recorded only at this timepoint. All cell lines exhibited a clear
247 modification in morphology. BME-UV and MCF-10A, that normally grow as compact colonies in 2D
248 culture, showed a reduction in size and a change in shape; HC11 and NMuMG, that grow as spared cells,
249 showed a decrease in cell number. In collagen, MCF-10A growth did not show organized structures while
250 NMuMG showed extensive 3D branching structures. Both Erk- or EGFR-inhibition abolished growth in 3D.
251 On the other side structures were still visible in the S condition (Figure 1B). BME-UV and HC11 cells did
252 not generate 3D structures in collagen. Cell proliferation, measured by nuclei counting, showed a
253 significant/highly significant reduction in all cell lines cultured with AG1478, UO126 or starved, when
254 compared to control cells in growth medium (Figure 1C and Supplementary Table S2), consistently with a
255 significant decrease of cellular metabolic activity, as confirmed by the MTT assay (Supplementary Fig. S4D;
256 p values in Supplementary Table S3). Similar results were obtained when analyzing the percentage of cells in
257 the G0/G1 phase of the cell cycle. At 16 h post-treatment all lines showed a significant increase *vs* the G
258 control group (Figure 1D; outlined in grey; p values are shown in Supplementary Table S4). A representative
259 example of G0/G1 increase by AG1478 treatment is shown in Fig. 1D, right panel. G0/G1 percentage in the
260 BME-UV line was very variable due to the high level of cell mortality after 16 h of incubation (Figure 1A
261 and 1D and Supplementary Table S4). Inhibiting the PI3K pathway with Wortmannin (100 nM) showed

262 minor alterations in proliferation (Supplementary Fig. S4C and Supplementary Table S2) and metabolism
263 (Supplementary Fig. S4D and Supplementary Table S3). These data indicate that all mammary epithelial cell
264 lines are highly sensitive to EGFR inhibition. In this setting we could not discriminate whether this response
265 depended on the presence of externally added (in the medium; three out of four cell lines have EGF added to
266 the medium) or internally produced (by the cells) growth factors.

FIGURE 1



267

268 **Figure 1. EGFR inhibition or starving decrease proliferation and block cell cycle in mammary**
 269 **epithelial cells of different origin. A)** Phase contrast images of BME-UV (bovine), HC11 and NMuMG
 270 (murine) and MCF-10A (human) mammary epithelial cells cultured for 48 h (24 h for BME-UV) in growth

271 medium (G), growth medium added with AG1748 (G+A), with UO126 (G+U) or in starving medium (S)
272 (see Methods). Bar = 100 μ m. **B)** 3D collagen images of NMuMG and MCF-10A mammary epithelial cells
273 treated for 48 h as in Fig. 1A. Bar = 500 μ m. **C)** Mammary cells treated as in Fig. 1A were counted (nuclei
274 counting) at 48 h (24 h for BME-UV) post treatment. P values vs G are indicated (exact p values in
275 Supplementary Table S2). **D)** Left panel: the percentage of cells in G0/G1 was calculated by FACS analysis
276 after 16 h of treatment. In grey: $p < 0.05$ vs G. In dark grey: $p < 0.001$ vs G. Exact p values are indicated in
277 Supplementary Table S4. Right panel: an example of the G0/G1 increase in AG-treated HC11 cells. N/D
278 indicates not done because of high level of mortality after 16 h of treatment.

279

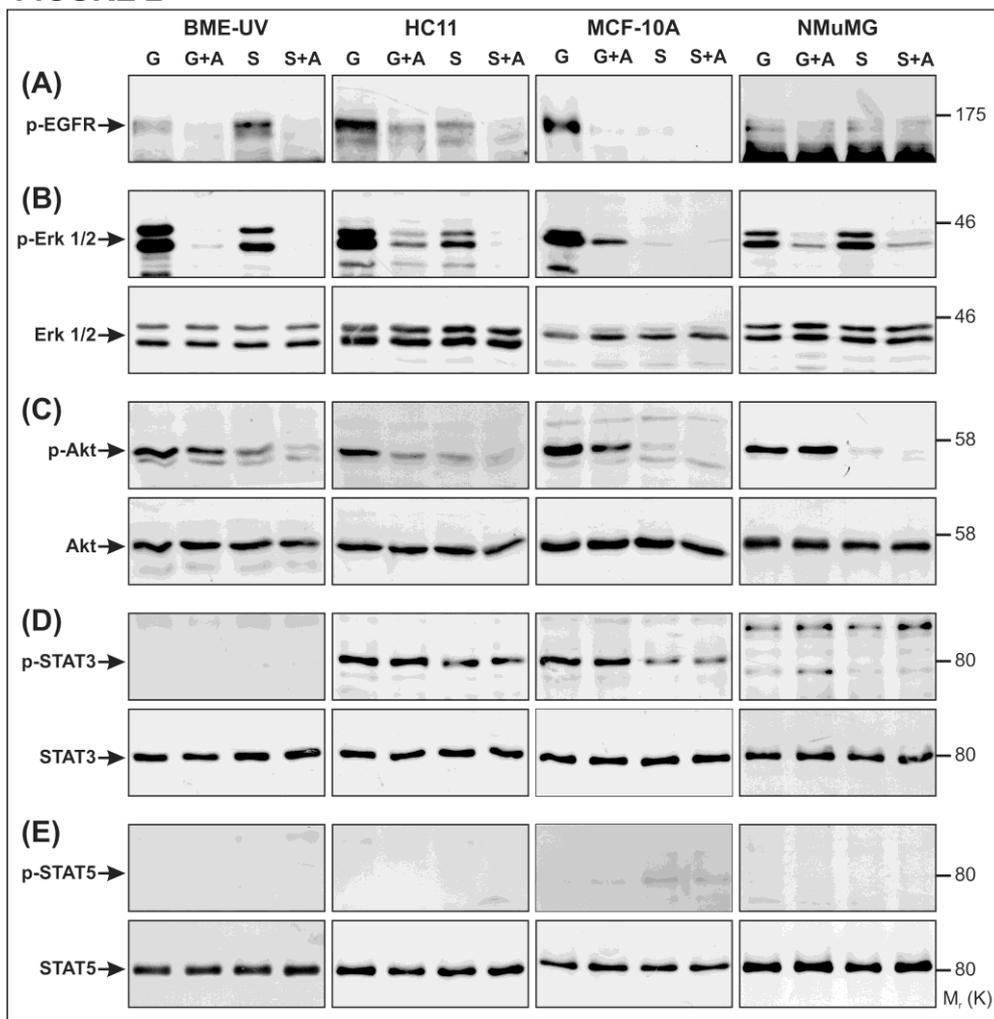
280 **3.2. Mammary cells maintain EGFR-dependent Erk 1/2 activation following external growth**
281 **factor deprivation.** Since UO126 inhibited proliferation and cell cycle in all mammary cell lines to a
282 higher level than serum / growth factors starved cells (S), we decided to verify the Erk 1/2 activation status
283 under the different growth condition and other pathways potentially involved in an EGFR-dependent
284 signaling. We also treated starved cells with AG1478 (S+A), in order to inhibit all external and potential
285 internal EGFR-activating ligands. The selectivity of AG1478 was supported biochemically in MCF-10A
286 cells, by comparison with other inhibitors (Supplementary Fig. S5A and S5B).

287 EGFR was variably phosphorylated in all the lines (Figure 2A, G) and strongly abolished by AG1478
288 (Figure 2A, G+A). In the starving condition, when all external growth factors were removed, EGFR was still
289 phosphorylated (Figure 2A, S). AG1478 almost totally abolished this residual EGFR phosphorylation
290 (Figure 2A, S+A). In MCF-10A the activation of EGFR seemed to be mostly dependent on EGFR-ligands
291 present in the medium. The dephosphorylation of EGFR, under S and S+A conditions, is even more
292 significant, if the levels of total EGFR are considered (Figure 4C). The trend of p Erk 1/2 was substantially
293 consistent with p EGFR.

294 The phosphorylation level of Akt was also negatively affected by EGFR-inhibition (Figure 2, G+A), in all
295 the lines but NMuMG. The phosphorylation of Akt was mainly dependent on external stimuli, since Akt in
296 the starving condition was only slightly dephosphorylated upon AG1478 treatment (Figure 2C, S+A). We
297 finally investigated the phosphorylation of STAT3 and STAT5 upon starving and/or EGFR inhibition.
298 STAT5 did not show phosphorylation under any condition (Figure 2E). STAT3 was phosphorylated in HC11

299 and MCF-10A lines. The activation of STAT3 did not decrease following EGFR-inhibition (Figure 2D, S
 300 and S+A). Since the activation of STAT3 seemed not to depend on EGFR-signaling, and based on the fact
 301 that JAK is the activator of STAT signal transducers, we confirmed our evidence in MCF-10A line with a
 302 time course of inhibition, by investigating the activation of p STAT3 and p Erk 1/2 in the presence of
 303 Ruxolitinib (specific JAK inhibitor). As expected, Ruxolitinib (R) abolished STAT3 but not p Erk 1/2
 304 phosphorylation (Supplementary Fig. S6).
 305 Taken together, these data demonstrate biochemically that an EGFR-dependent signaling is still active in
 306 almost all our cell lines, when external stimuli are withdrawn, suggesting that the cells can express EGFR-
 307 ligands in starving conditions.

FIGURE 2



308

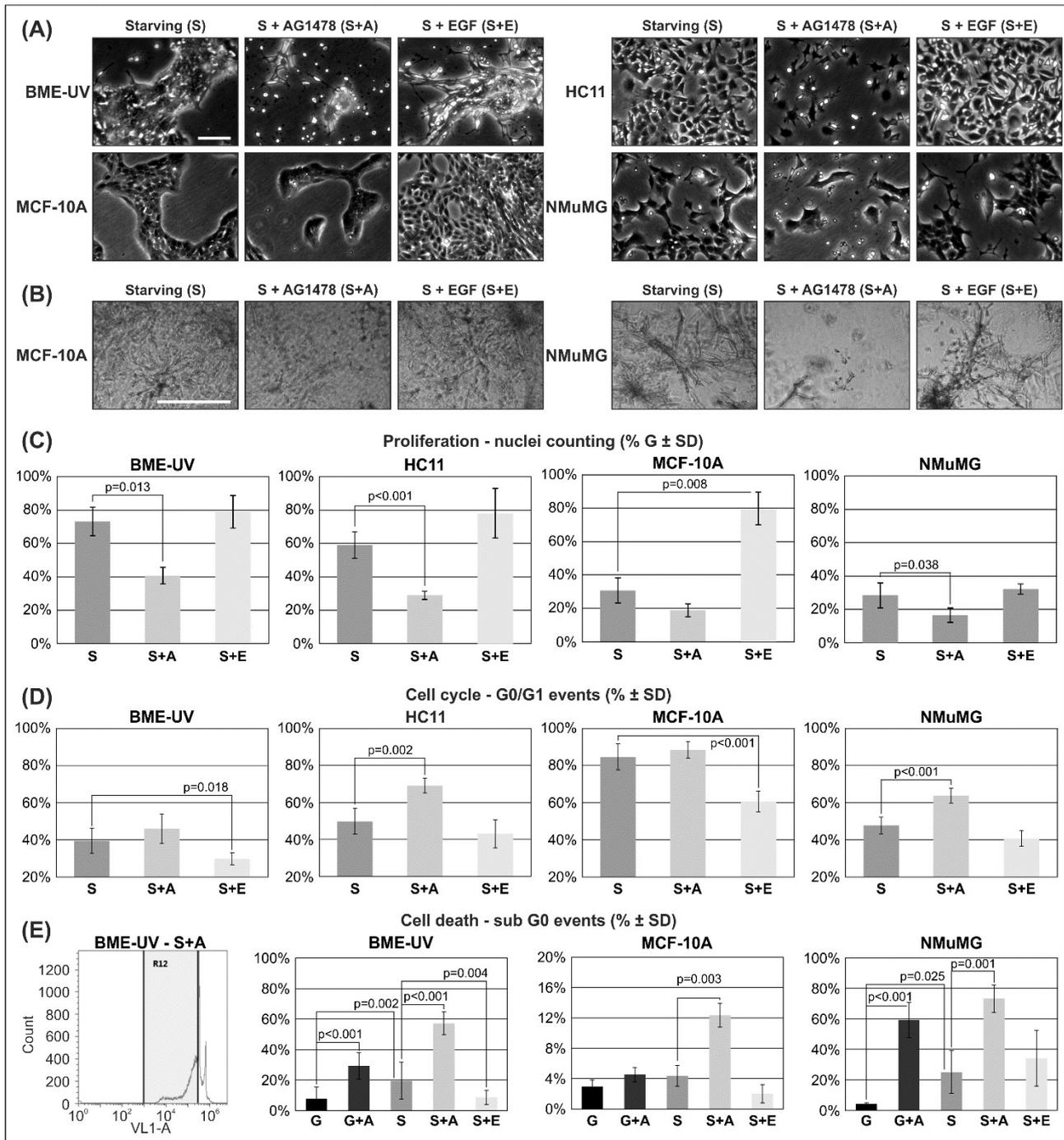
309 **Figure 2. EGFR signaling maintains Erk 1/2 activation in growth factor deprived mammary cells.**
 310 Western-blot analysis of EGFR (A), Erk 1/2 (B), Akt (C), STAT3 (D) and STAT5 (E) phosphorylation in
 311 mammary epithelial cells cultured under the indicated conditions for 16 h or 4 h (for BME-UV, because of

312 high mortality at 16 h). 30 μ g (Erk 1/2, STAT3, STAT5) or 100 μ g (EGFR, Akt) of total protein were run on
313 10% SDS-acrylamide gels. EGFR, Erk 1/2, Akt, STAT3, STAT5 total proteins were used to show
314 comparable protein loading (EGFR total protein is shown in Figure 4C).

315

316 **3.3. External, but also internal stimuli are responsible for EGFR-induced cell proliferation**
317 **and survival.** We thus analysed whether proliferation, still present in the S condition and possibly
318 determined by internally produced ligands (there are 7 known EGFR ligands), was further reduced upon
319 EGFR inhibition. We also tested whether EGF treatment could recover proliferation/cell cycle in starved
320 cells. No significant differences in the S condition were found, between cells inhibited with AG1748 and cell
321 inhibited with Erlotinib/Gefitinib, either qualitatively (phase contrast 2D and 3D images: Supplementary Fig.
322 S7A and S7B) or quantitatively (nuclei counting and MTT assay: Supplementary Fig. S7C and S7D; p values
323 in Supplementary Tables S5 and S6). Under the S+A condition, when compared to S, BME-UV, HC11 and
324 NMuMG cell lines had a significant reduction in cell number (Figure 3C, Supplementary Table S5),
325 consistent with a decrease of metabolic activity (Supplementary Fig. S7D, Supplementary Table S6), and a
326 significant increase in the G0/G1 population (for the murine lines; Figure 3D and Supplementary Table S7).
327 EGFR- and Erk-inhibition abolished 3D structures in collagen (Figure 3B and Supplementary Fig. S7). EGF
328 treatment recovered proliferation in starved MCF-10A, cell cycle and metabolic activity in MCF-10A and
329 BME-UV (Figures 3C and 3D, Supplementary Fig. S7D, Supplementary Tables S5, S6 and S7). BME-UV,
330 MCF-10A and NMuMG, photographed at 48 h, displayed objects in suspension (Figure 3A), a possible
331 indication of cell death. Abundant debris was also visible among NMuMG and MCF-10A 3D structures in
332 S+A condition (Figure 3B). Analysis of sub-G0 events by FACS showed that EGFR inhibition (G+A vs G)
333 significantly increased cell death in BME-UV and NMuMG, while the S+A condition, compared to S, was
334 significantly increased, in three cell lines out of four. A recovery from cell death after treatment with EGF
335 was apparent in BME-UV cells (Figure 3E and Supplementary Table S8). These data indicate that internal
336 EGFR-activating signals are active **in** mammary cells and sustain proliferation or survival.

FIGURE 3



337

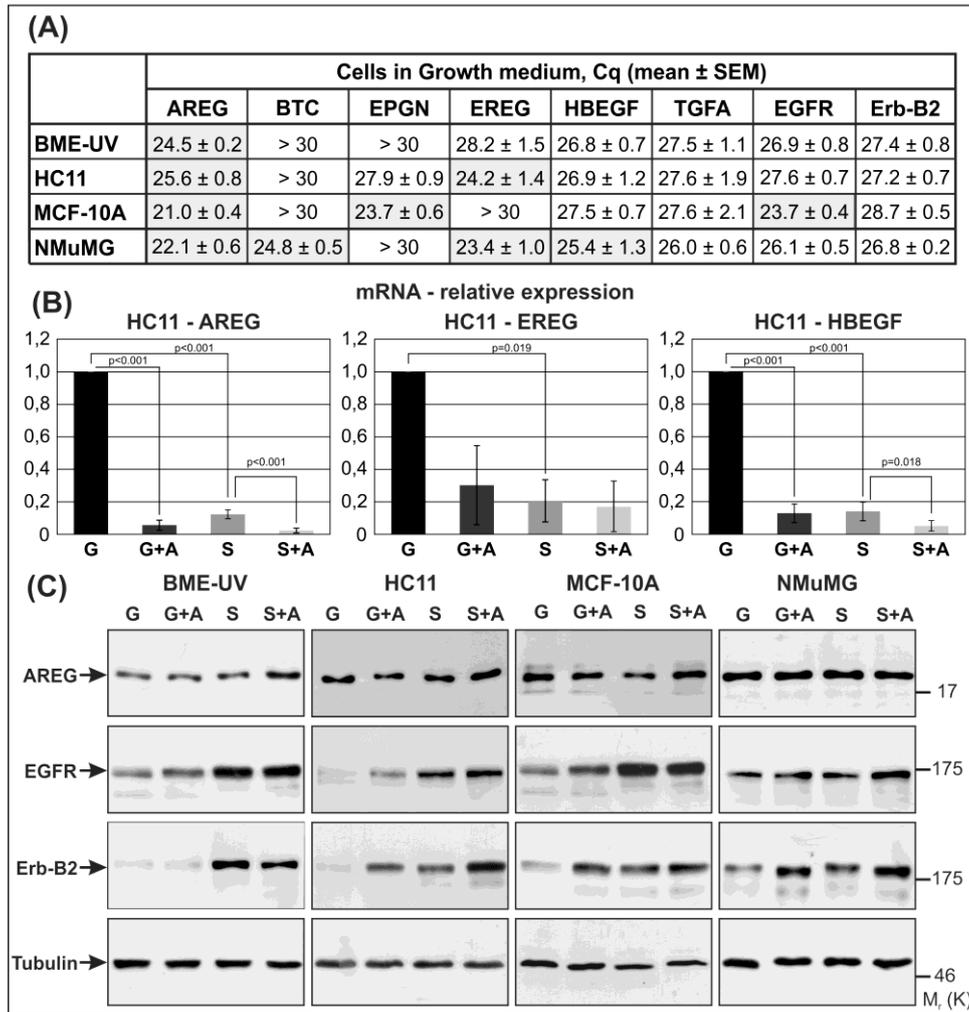
338 **Figure 3. Mammary epithelial cells possess an autocrine EGFR signaling activity sufficient to sustain**
 339 **proliferation, cell cycle and cell viability. A)** Phase contrast images of mammary epithelial cells cultured
 340 for 48 h in ST and treated with the EGFR inhibitor AG1478 or EGF (10 ng/mL). Bar = 100 μm. **B)** Phase
 341 contrast images of NMuMG and MCF-10A mammary epithelial cells cultured in 3D collagen and treated as
 342 in Fig. 3A for 48 h. Bar = 500 μm. **C)** Absolute number of cells mammary cells treated as in Fig. 3A. P
 343 values vs S are indicated (exact p values in Supplementary Table S5). **D)** G0/G1 percentages of cells treated
 344 for 16 h as in Fig. 3A. P values vs S are indicated (exact p values in Supplementary Table S7). **E)** Left panel:

345 an example of the sub-G0 population in BME-UV cultured for 16 h in S+A. Right panels: percentages of
346 sub-G0 population determined by FACS analysis after 16 h (BME-UV) or 48 h (MCF-10A and NMuMG). P
347 values *vs* G and *vs* S are indicated (exact p values in Supplementary Table S8).

348

349 **3.4. Expression and modulation of EGFR ligands, EGFR and Erb-B2 in mammary cells.** We
350 thus tested by real-time PCR, the levels of expression of all seven EGFR ligands, EGFR and Erb-B2 (the
351 preferential EGFR hetero-dimerization partner) in cells cultured in growth medium. The mean Cq cycles are
352 indicated in Fig. 4A. Setting an arbitrary cut-off threshold to < 26 Cq, we found that AREG showed a high
353 expression in all cell lines and three of them had two or more “highly” expressed ligands (outlined in gray in
354 Fig. 4A). EGF is not shown because **none of the cell lines** expressed EGF mRNA (Cq>30). It was then tested
355 whether EGFR ligands, EGFR and Erb-B2, with a Cq<30, were regulated under the different culture
356 conditions. We observed that AREG, EREG and HBEGF were often significantly downregulated by the
357 AG1478 or starving treatments (Figure 4B, Supplementary Figure S8 and Supplementary Table S9).
358 AG1478 treatment in starved cells often significantly reduced the expression of these ligands when
359 compared to the starving treatment alone (Figure 4B and Supplementary Table S9). Given the crucial role of
360 AREG in the development of the ductal tree, we sought to confirm its expression at a protein level. Although
361 AREG was present in all the cell lines (Supplementary Fig. S9), no clear variations were apparent in any of
362 them, upon either starving or EGFR-inhibition (Figure 4C). **However, possible variations of AREG shedding**
363 **were not evaluated in the present study.** EGFR and Erb-B2 receptors were not modulated at the mRNA level,
364 to the exception of a slight but significant modulation in MCF-10A (EGFR and Erb-B2; Supplementary Fig.
365 S8 and Supplementary Table S9) and BME-UV cells (Erb-B2 only; Supplementary Figure S8). On the other
366 hand, EGFR and Erb-B2 receptors were upregulated at the protein level, after the G+A, S or S+A treatments
367 (Figure 4C), **indicating that the cells respond to both EGFR-inhibition and EGFR-hypoactivation,**
368 **upregulating EGFR and Erb-B2 post-transcriptionally.**

FIGURE 4



369

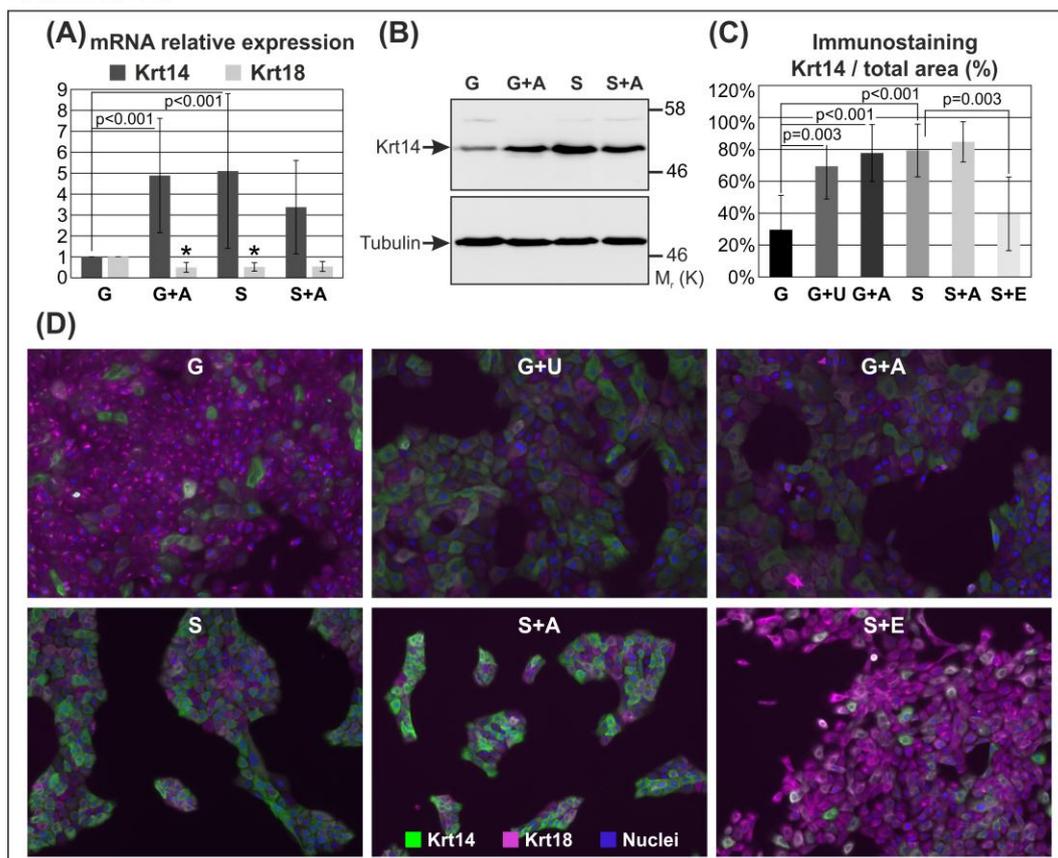
370 **Figure 4. Expression and modulation of EGFR ligands, EGFR and Erb-B2 in mammary epithelial**
 371 **cells. A)** Cq mean values ± SEM of six EGFR-ligands, EGFR and Erb-B2 determined by real-time PCR. 7,5
 372 ng of cDNA were used for each amplification reaction. Grey = Cq<26. **B)** Modulation of AREG, EREG and
 373 HBEGF in the HC11 cell line cultured for 16 h under the indicated conditions. 7,5 ng of cDNA were
 374 processed for each reaction. P values vs G and vs S are indicated (exact p values in Supplementary Table S9).
 375 **C)** Western-blot analysis of AREG, EGFR, and Erb-B2 in mammary epithelial cells cultured under the
 376 indicated conditions for 4 h (BME-UV) or 16 h. 30 µg of total protein were run. Tubulin was used to show
 377 comparable protein loading.

378

379 **3.5. EGFR signaling downmodulation reduces the Krt14 negative / Krt18 positive cell**
 380 **population of MCF-10A human mammary cells.** EGFR inhibition induced cell death primarily in
 381 BME-UV and NMuMG cell lines. Both lines express Krt18, the mouse mammary luminal marker, but not

382 Krt14, the basal marker. MCF-10A cells, on the other side, express both Krt18 (100% positive expression)
 383 and Krt14 (only a cell subpopulation expresses this marker; Supplementary Figure S11 and Qu et al., 2015).
 384 We observed that MCF-10A cells treated with G+A or S showed an increase in Krt14 expression both at the
 385 mRNA (Figure 5A and Supplementary Table S10) and protein (Figure 5B) levels compared to the untreated
 386 G control. When we analyzed Krt14 and Krt18 expression by immunostaining, we found that G+A, G+U, S
 387 and S+A treatments increased the area of the well covered by Krt14 cell versus the area covered by all the
 388 cells (Krt14/total area; Figures 5C, 5D and Supplementary Table S11). These data indicated that the Krt14
 389 negative population did not grow under these treatments. This effect was reversed upon EGF treatment: in
 390 the S+E condition the Krt14/total area ratio returned to G levels (Figure 5D, S+E and Fig. 5C, S+E and
 391 Supplementary Table S11). These data indicate that Krt14 negative / Krt18 positive population is highly
 392 sensitive to EGFR inhibition.

FIGURE 5



393
 394 **Figure–5. EGFR inhibition in MCF-10A cells reduces the Krt14 negative / Krt18 positive cell**
 395 **population. A)** Krt14 and Krt18 mRNA expression in MCF-10A cells cultured under the indicated
 396 conditions for 16 h. 7,5 ng of cDNA were used for each amplification reaction. Significant p values vs G for

397 Krt14 are indicated. * denotes significant p values vs G for Krt18. Exact p values in Supplementary Table
398 S10. **B)** Krt14 protein expression in MCF-10A cells cultured under the indicated conditions for 16 h. 20 µg
399 of total protein lysates were run. Tubulin shows comparable protein loading in all samples **C)** Krt14 / total
400 area ratio (%) obtained by immunostaining of MCF-10A cells cultured under the indicated conditions for 48
401 h (see Methods). P values vs G and vs S are indicated (exact p values in Supplementary Table S11). **D)**
402 Example of composites of immunostaining images stained for Krt14 (green), Krt18 (magenta; adapted for
403 color blindness) and nuclei (blue) in human MCF-10A mammary cells cultured for 48 h under the indicated
404 conditions. Bar = 100 µm. Single images used for the composites and adapted for color blindness, are visible
405 in Supplementary Fig. S10.

406

407 **4. Discussion**

408 The mammary gland consists of an epithelial cell bilayer surrounded by a mesenchymal stroma, mainly
409 represented by adipocytes in the murine species (Wang & Scherer, 2019). Our current knowledge of this
410 gland reveals that, during the pubertal phase of growth, an endocrine signaling coming from the ovaries
411 induces estrogen receptor positive epithelial cells to release amphiregulin (Ciarloni et al., 2007). This
412 hormone, in turn, activates EGFR in the stromal compartment, enhancing the expression of other growth
413 factors (fibroblast growth factors are the main candidates) that talk back to the epithelial compartment
414 promoting cell proliferation (Sumbal & Koledova, 2019). Both EGFR^{-/-} and AREG^{-/-} mice display arrest of
415 ductal growth and a normal lobuloalveolar development (Ciarloni et al., 2007; Sternlicht et al., 2005). Those
416 and other studies did not however **deny** the possibility that EGFR expressed also in the epithelial
417 compartment (possibly interacting with Erb-B2) plays an albeit minor role during the ductal development
418 (Andrechek et al., 2005; Jackson-Fisher et al., 2004; Sternlicht et al., 2005), for instance in terms of growth
419 rate or morphology of the mature mammary gland. Nor can be ignored the chance that EGF-like ligands are
420 expressed and act locally, targeting EGFR in the epithelium itself. In summary, an autocrine activity within
421 the epithelial compartment could be hypothesized. In the present work, we adopted different epithelial cells
422 lines, as *in vitro* models for the epithelial compartment, and we sought to demonstrate that these cells can
423 sustain their own proliferation, even in the absence of external stimuli, by activating EGFR (i.e autocrine
424 activity).

425 Although we observed differences in the responses of the various cell lines, a common behaviour was
426 apparent. The EGFR tyrosine kinase inhibitor tyrphostin (AG1478; IC50 3nM) was used at 300 nM thereby
427 maintaining a unique selectivity over other kinase receptors like Erb-B2 or PDGFR (IC50 100µM) (Fry et
428 al., 1994; Levitzki & Gazit, 1995). This treatment allowed us to properly isolate EGFR-dependent signaling
429 from other non-specific ones. The choice of AG1478 was justified both biologically and biochemically, by
430 comparing its potency with other selective EGFR-receptor, Gefitinib/Iressa (Baselga & Averbuch, 2000) and
431 Erlotinib/Tarceva (Akita & Sliwkowski, 2003). No significant differences were observed **between the**
432 **inhibitory effects** of these molecules on proliferation, **either qualitatively measured by nuclear counting and**
433 **MTT assay, or qualitatively evaluated as 2D and 3D growth.** AG1478 affected all cell lines by interfering
434 with proliferation, as evidenced by reduced cell count, block of cells in the G0/G1 phase of the cell cycle and
435 **impaired** 3D-growth. The BME-UV (after 16 h) and NMuMG (after 48 h) cell lines also exhibited an
436 increase in the sub-G0 population, indicative of cell death. At this point we could not discriminate whether
437 EGFR activation was elicited by EGFR-ligands coming from the medium (EGF is present in the culture
438 medium for BME-UV, HC11 and MCF-10A) or by the cells themselves. The cell lines used in the present
439 work are moreover variably responsive to steroid hormones. HC11 cells were found Estrogen Receptor
440 positive (ER+) both at mRNA and protein level (Hedengran Faulds et al., 2004; Sornapudi et al., 2018;
441 Williams et al., 2009), BME-UV cells are responsive to both estrogen and progesterone, although their
442 molecular signature has not been elucidated (Sobolewska et al., 2011), nor NMuMG line was described with
443 that respect. MCF-10A were reported to be ER- and Progesterone Receptor positive (PR+) only in
444 microarray analysis (Charafe-Jauffret et al., 2006; Hevir et al., 2011; Moran et al., 2000). We partially
445 confirmed these previous **findings** by gene expression analysis, except for a low but detectable level of ERα
446 mRNA in MCF-10A cells, as well as in the NMuMG line (data not shown). Therefore, the chance that
447 external factors present in the growth media induce the cells to proliferate, potentially activating ER or other
448 receptors, cannot be excluded. This is relevant especially because ERα affects the **synthesis** of AREG (and
449 potentially other EGF-like factors) in the mammary epithelium. All that considered, we deprived the cells of
450 all ligands and hormones present in the medium and added AG1478. Comparing these treatments, we
451 observed a further reduction in cell number in HC11, NMuMG and BME-UV, and an increase in the G0/G1
452 events in HC11 and NMuMG. We also detected an increase in the sub-G0 population in BME-UV, MCF-

453 10A and NMuMG. Adding EGF alone to the starving condition either increased cell number (in MCF-10A
454 and to a lesser extent HC11), reduced G0/G1 events (in BME-UV and MCF-10A) or reduced cell death (in
455 BME-UV and MCF-10A). Therefore, all cell lines show that EGFR signaling activated by factors either
456 present in the medium or produced by the cells themselves, plays a role in proliferation or survival.

457 Different pathways are known to be variably activated downstream of Erb-B receptors, but a good evidence
458 supported our main interest in Erk 1/2. EGFR is a strong activator of Ras/Erk pathway, by means of multiple
459 binding sites for Grb2 and SHC. Moreover, the ductal branching triggered *in vitro* by EGF-like factors
460 depends on p Erk, in the murine species (Camacho Leal et al., 2012; Fata et al., 2007; Kariagina et al., 2010).
461 The analysis of the status of Erk 1/2 phosphorylation in our cells deprived of any external growth factor, the
462 starving condition, showed that phosphor-Erk was still elevated (to the exception of MCF-10A) and that
463 AG1478 abolished this signaling almost completely. This trend was substantially aligned with the
464 phosphorylation (i.e activation) of EGFR and indicates that cells retain active EGFR signaling when all
465 external growth factors have been withdrawn, by producing their own EGFR-ligands. The activation of p Erk
466 in AG1478-treated cells is further abolished when external factors are withdrawn (compare the G+A with
467 S+A lanes in HC11 and MCF-10A cells, Fig. 2B). We can therefore conclude that the Erk activity is, to some
468 extent, independent of EGFR signaling which is consistent with the multiple signaling pathways relying on
469 this kinase. EGFR is also a potential activator of PI3K/Akt pathway, and our data confirms that link,
470 showing that Akt phosphorylation is partially abolished in the presence of AG1478, in all the lines except
471 NMuMG. On the other hand, differences between S and S+A were barely visible. It is possible that this
472 pathway is activated in the context of a hypothetical autocrine, EGFR-mediated signaling in our cell lines,
473 but p Akt plays a minor role compared to p Erk, consistently with the mild effects upon wortmannin
474 treatment. It is also possible that these two pathways play complementary roles in mammary morphogenesis,
475 as previously demonstrated for MCF-10A cells (Tang et al., 2014; Tarcic et al., 2012). According to our
476 results, there are no links between p EGFR and the activation of p STAT3 (apparent in HC11 and MCF-10A)
477 or p STAT5 (evidenced in none of the lines) in the lines here investigated.

478 When we determined the expression of all seven EGFR-ligands we found that AREG mRNA was present at
479 high levels in all the cell lines, with other factors (to the exception of EGF) often reaching considerable
480 levels of expression. We also observed a strong downregulation of some ligands (AREG, EREG, HBEGF

481 and BTC) when medium derived factors were removed or EGFR signaling was inhibited. These data
482 indicated that the gene expression of several EGFR ligands is regulated in a fashion consistent with cell
483 proliferation and survival, suggesting that these factors could be the mediators of the autocrine activity we
484 demonstrated, in a similar way to what was described in breast cancer by (Zhou et al., 2014). The ligands for
485 the EGFR receptor have been described as immediate early genes with fast regulation kinetics that depend on
486 the protein kinase C and/or extracellular-signal-regulated kinase (Barnard et al., 1994; Berasain & Avila,
487 2014; Kerpedjieva et al., 2012; Shirakata et al., 2010; Taylor et al., 2014). It is therefore likely that, in our
488 cellular system, EGFR signaling maintain these ligands upregulated, by promoting Erk 1/2 phosphorylation.
489 A detailed study of the signaling regulating these ligands in the mammary compartment is underway.
490 Surprisingly, AREG protein levels seemed to be unaffected either by starving condition or EGFR-inhibition.
491 Although we cannot rationally explain this evidence, it could be speculated that only the release of active
492 AREG (not measured in the present study) is impaired by EGFR-inhibition. Another possibility is that only
493 AREG gene expression is regulated, and other EGF-like effectors are involved downstream of EGFR, or that
494 the half-life of AREG is longer than 48 h in the lines here examined (current experiments are underway). On
495 the other side, EGFR and Erb-B2 showed that their protein, but not their mRNA was upregulated under the
496 G+A, S or S+A treatments. This finding was not surprising, since it was proved and extensively reviewed
497 that tyrosine kinase receptors become downregulated when activated by their own ligands (Darcy et al.,
498 1999; Edery et al., 1989; Waterman & Yarden, 2001).

499 According the results published herein, proliferation and activation of Erk 1/2, in MCF-10A cells, almost
500 exclusively relied on EGF included in the growth medium. These data were in marked contrast with other
501 lines, that self-sustained in starving condition, by activating EGFR-Erk 1/2 signaling pathway. We therefore
502 investigated which characteristics could make MCF-10A unique over the other lines. HC11 are all Krt14
503 positive, BME-UV and NMuMG are all Krt18 positive, while MCF-10A express markers of both the basal
504 and luminal compartments (Qu et al., 2015). MCF-10A cells treated with AG1478, UO126 or starved
505 exhibited a strong reduction of the Krt14 negative / Krt18 positive cell population, which was restored upon
506 EGF treatment almost back to the growth condition. Our data are in line with previous observations that a
507 basal cell fate might be supported in the absence of EGF (Deugnier et al., 1999). In MCF10A cells AREG
508 promotes an epithelial cell fate, by activating EGFR-ERK signaling even if less strongly than EGF. In the

509 same study, Krt14 gene was differentially expressed in AREG- or EGF-stimulated cells (Fukuda et al.,
510 2016). Those previous results might also explain why MCF-10A cells, that express high levels of AREG
511 mRNA and protein, did not maintain proliferation or Erk 1/2 phosphorylation in our experiments, when EGF
512 was removed from the medium. All in all, the results about cell proliferation, cell death and Krt14/18
513 expression, together with previous findings, suggest that EGFR signaling is important for the viability of
514 Krt18 cells (NMuMG and BME-UV) negative for Krt14 (MCF-10A). The decrease of such a population
515 might explain why an EGFR-dependent autocrine signaling was not apparent in the MCF-10A here
516 examined.

517 Although our data were obtained from four cell lines of different source, we are aware that our
518 demonstration of a potential autocrine signaling is “indirect”, being focused on the potential of cells to
519 survive without external growth factors. This first, significant evidence encourages a more direct
520 demonstration. We also acknowledge that the observed effect may depend on the immortalization process
521 that has selected “EGFR dependent” cells and that immortalized lines are not a realistic model of the real
522 mammary epithelium. We are currently addressing these issues by using primary, animal-isolated mammary
523 cells from different sources. In this regard, the first data obtained in the swine and bovine are promising and
524 may prove that our assessment is also correct in an *ex vivo* system.

525 In conclusion, we identified a gap in the scientific background, whereby stromal EGFR certainly plays a
526 major role in the pubertal development of the mammary gland, but the contribution of its epithelial
527 counterpart is not elucidated. We have shown that EGFR is expressed by several epithelial cell lines, here
528 used as *in vitro* model of the mammary epithelium, and that p EGFR (activated receptor) triggers a signaling
529 that promotes the proliferation and survival of cells, even in the absence of external stimuli (i.e factors
530 included in the medium), mainly by activating Erk 1/2. This proves the existence of an autocrine signal
531 among mammary epithelial cells *in vitro* and lays the foundations for further studies *ex vivo*.

532

533 **Conflict of interest:** The authors declare no conflict of interest.

534

535 Akita, R. W., & Sliwkowski, M. X. (2003). Preclinical studies with erlotinib (Tarceva). *Seminars in*
536 *Oncology*, 30(3 SUPPL. 7), 15–24. [https://doi.org/10.1016/S0093-7754\(03\)70011-6](https://doi.org/10.1016/S0093-7754(03)70011-6)

537 Andrechek, E. R., White, D., & Muller, W. J. (2005). Targeted disruption of ErbB2/Neu in the mammary
538 epithelium results in impaired ductal outgrowth. *Oncogene*, *24*(5), 932–937.
539 <https://doi.org/10.1038/sj.onc.1208230>

540 Barnard, J. A., Graves-Deal, R., Pittelkowll, M. R., Duboisn, R., Cookllll, P., Ramseys, G. W., Bishop, P. R.,
541 Damstrupn, L., & Coffeefl, R. J. (1994). Auto-and Cross-induction within the Mammalian Epidermal
542 Growth Factor-related Peptide Family. *Journal of Biological Chemistry*, *269*(36), 22817–22822.

543 Baselga, J., & Averbuch, S. D. (2000). ZD1839 ('Iressa') 1,2 as an Anticancer Agent. *Drugs*, *60*(SUPPL. 1),
544 33–40.

545 Berasain, C., & Avila, M. A. (2014). Amphiregulin. *Seminars in Cell and Developmental Biology*, *28*, 31–
546 41. <https://doi.org/10.1016/j.semcdb.2014.01.005>

547 Camacho Leal, M. del P., Pincini, A., Tornillo, G., Fiorito, E., Bisaro, B., Di Luca, E., Turco, E., Defilippi,
548 P., & Cabodi, S. (2012). p130Cas Over-Expression Impairs Mammary Branching Morphogenesis in
549 Response to Estrogen and EGF. *PLoS ONE*, *7*(12), e49817.
550 <https://doi.org/10.1371/journal.pone.0049817>

551 Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adélaïde, J., Cervera, N., Fekairi, S., Xerri, L.,
552 Jacquemier, J., Birnbaum, D., & Bertucci, F. (2006). Gene expression profiling of breast cell lines
553 identifies potential new basal markers. *Oncogene*, *25*(15), 2273–2284.
554 <https://doi.org/10.1038/sj.onc.1209254>

555 Ciarloni, L., Mallepell, S., & Briskin, C. (2007). Amphiregulin is an essential mediator of estrogen receptor
556 alpha function in mammary gland development. *Proceedings of the National Academy of Sciences of
557 the United States of America*, *104*(13), 5455–5460. <https://doi.org/10.1073/pnas.0611647104>

558 Coleman, S., Silberstein, G. B., & Daniel, C. W. (1988). Ductal morphogenesis in the mouse mammary
559 gland: Evidence supporting a role for epidermal growth factor. *Developmental Biology*, *127*(2), 304–
560 315. [https://doi.org/10.1016/0012-1606\(88\)90317-X](https://doi.org/10.1016/0012-1606(88)90317-X)

561 D'Cruz, C. M., Moody, S. E., Master, S. R., Hartman, J. L., Keiper, E. A., Imielinski, M. B., Cox, J. D.,
562 Wang, J. Y., Ha, S. I., Keister, B. A., & Chodosh, L. A. (2002). Persistent Parity-Induced Changes in
563 Growth Factors, TGF- β 3, and Differentiation in the Rodent Mammary Gland. *Molecular
564 Endocrinology*, *16*(9), 2034–2051. <https://doi.org/10.1210/me.2002-0073>

565 Darcy, K. M., Wohlhueter, A. L., Zangani, D., Vaughan, M. M., Russell, J. A., Masso-Welch, P. A., Varela,
566 L. M., Shoemaker, S. F., Horn, E., Lee, P.-P. H., Huang, R.-Y., & Ip, M. M. (1999). Selective changes
567 in EGF receptor expression and function during the proliferation, differentiation and apoptosis of
568 mammary epithelial cells. *European Journal of Cell Biology*, *78*(7), 511–523.
569 [https://doi.org/10.1016/S0171-9335\(99\)80077-6](https://doi.org/10.1016/S0171-9335(99)80077-6)

570 Darcy, K. M., Zangani, D., Wohlhueter, A. L., Huang, R.-Y., Vaughan, M. M., Russell, J. A., & Ip, M. M.
571 (2000). Changes in ErbB2 (her-2/neu), ErbB3, and ErbB4 during Growth, Differentiation, and
572 Apoptosis of Normal Rat Mammary Epithelial Cells. *Journal of Histochemistry & Cytochemistry*,
573 *48*(1), 63–80. <https://doi.org/10.1177/002215540004800107>

574 Deugnier, M. A., Faraldo, M. M., Rouselle, P., Thiery, J. P., & Glukhova, M. A. (1999). Cell-extracellular
575 matrix interactions and EGF are important regulators of the basal mammary epithelial cell phenotype.
576 *Journal of Cell Science*, *112*(7), 1035 LP – 1044. <http://jcs.biologists.org/content/112/7/1035.abstract>

577 DiAugustine, R. P., Richards, R. G., & Sebastian, J. (1997). EGF-Related Peptides and Their Receptors in
578 Mammary Gland Development. *Journal of Mammary Gland Biology and Neoplasia*, *2*(2), 109–117.
579 <https://doi.org/10.1023/A:1026395513038>

580 Edery, M., Pang, K., Larson, L., & Nandi, S. (1989). Turnover of epidermal growth factor binding sites in
581 mouse mammary epithelial cells. *Biomedicine & Pharmacotherapy*, *43*(5), 361–368.
582 [https://doi.org/10.1016/0753-3322\(89\)90062-0](https://doi.org/10.1016/0753-3322(89)90062-0)

583 Fata, J. E., Mori, H., Ewald, A. J., Zhang, H., Yao, E., Werb, Z., & Bissell, M. J. (2007). The MAPK ERK-
584 1,2 pathway integrates distinct and antagonistic signals from TGF α and FGF7 in morphogenesis of
585 mouse mammary epithelium. *Developmental Biology*, *306*(1), 193–207.
586 <https://doi.org/10.1016/j.ydbio.2007.03.013>

587 Fry, D. W., Kraker, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., &
588 Bridges, A. J. (1994). A Specific Inhibitor of the Epidermal Growth Factor Receptor Tyrosine Kinase.
589 *Science*, *265*(5175), 1093–1095. <https://doi.org/10.1126/science.8066447>

590 Fukuda, S., Nishida-Fukuda, H., Nanba, D., Nakashiro, K. I., Nakayama, H., Kubota, H., & Higashiyama, S.
591 (2016). Reversible interconversion and maintenance of mammary epithelial cell characteristics by the
592 ligand-regulated EGFR system. *Scientific Reports*, *6*(1), 1–16. <https://doi.org/10.1038/srep20209>

593 Harris, R. C., Chung, E., & Coffey, R. J. (2003). EGF receptor ligands. *Experimental Cell Research*, 284(1),
594 2–13. [https://doi.org/10.1016/S0014-4827\(02\)00105-2](https://doi.org/10.1016/S0014-4827(02)00105-2)

595 Hedengran Faulds, M., Olsen, H., Helguero, L. A., Gustafsson, J.-åke, & Haldosé, L. N. (2004). Estrogen
596 Receptor Functional Activity Changes during Differentiation of Mammary Epithelial Cells. *Molecular*
597 *Endocrinology*, 18(2), 412–421. <https://doi.org/10.1210/me.2003-0290>

598 Hevir, N., Trošt, N., Debeljak, N., & Rižner, T. L. (2011). Expression of estrogen and progesterone receptors
599 and estrogen metabolizing enzymes in different breast cancer cell lines. *Chemico-Biological*
600 *Interactions*, 191, 206–216. <https://doi.org/10.1016/j.cbi.2010.12.013>

601 Jackson-Fisher, A. J., Bellinger, G., Ramabhadran, R., Morris, J. K., Lee, K.-F., & Stern, D. F. (2004).
602 ErbB2 is required for ductal morphogenesis of the mammary gland. *Proceedings of the National*
603 *Academy of Sciences*, 101(49), 17138–17143. <https://doi.org/10.1073/pnas.0407057101>

604 Jenkins, E. C., Debnath, S., Gundry, S., Gundry, S., Uyar, U., & Fata, J. E. (2012). Intracellular pH
605 regulation by Na⁺/H⁺ exchanger-1 (NHE1) is required for growth factor-induced mammary branching
606 morphogenesis. *Developmental Biology*, 365(1), 71–81. <https://doi.org/10.1016/j.ydbio.2012.02.010>

607 Kariagina, A., Xie, J., Leipprandt, J. R., & Haslam, S. Z. (2010). Amphiregulin Mediates Estrogen,
608 Progesterone, and EGFR Signaling in the Normal Rat Mammary Gland and in Hormone-Dependent
609 Rat Mammary Cancers. *Hormones and Cancer*, 1(5), 229–244. [https://doi.org/10.1007/s12672-010-](https://doi.org/10.1007/s12672-010-0048-0)
610 0048-0

611 Kerpedjieva, S. S., Kim, D. S., Barbeau, D. J., & Tamama, K. (2012). EGFR ligands drive multipotential
612 stromal cells to produce multiple growth factors and cytokines via early growth response-1. *Stem Cells*
613 *and Development*, 21(13), 2541–2551. <https://doi.org/10.1089/scd.2011.0711>

614 Levitzki, A., & Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. *Science*,
615 267(5205), 1782–1788. <https://doi.org/10.1126/science.7892601>

616 Luetkeke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A., & Lee, D. C. (1999).
617 Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor
618 ligands in mouse mammary gland development. *Development (Cambridge, England)*, 126(12), 2739–
619 2750. <http://www.ncbi.nlm.nih.gov/pubmed/10331984>

620 Macias, H., & Hinck, L. (2012). Mammary gland development. *Wiley Interdisciplinary Reviews*.

621 *Developmental Biology*, 1(4), 533–557. <https://doi.org/10.1002/wdev.35>

622 Moran, T. J., Gray, S., Mikosz, C. A., & Conzen, S. D. (2000). The Glucocorticoid Receptor Mediates a
623 Survival Signal in Human Mammary Epithelial Cells 1. *Cancer Research*, 60(4), 867–872.

624 Qu, Y., Han, B., Yu, Y., Yao, W., Bose, S., Karlan, B. Y., Giuliano, A. E., & Cui, X. (2015). Evaluation of
625 MCF10A as a Reliable Model for Normal Human Mammary Epithelial Cells. *PLOS ONE*, 10(7),
626 e0131285. <https://doi.org/10.1371/journal.pone.0131285>

627 Schroeder, J. A., & Lee, D. C. (1998). Dynamic expression and activation of ERBB receptors in the
628 developing mouse mammary gland. *Cell Growth & Differentiation : The Molecular Biology Journal of*
629 *the American Association for Cancer Research*, 9(6), 451–464.
630 <http://www.ncbi.nlm.nih.gov/pubmed/9663464>

631 Shirakata, Y., Tokumaru, S., Sayama, K., & Hashimoto, K. (2010). Auto- and cross-induction by betacellulin
632 in epidermal keratinocytes. In *Journal of Dermatological Science* (Vol. 58, Issue 2, pp. 162–164).
633 Elsevier. <https://doi.org/10.1016/j.jdermsci.2010.03.016>

634 Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A., & Bissell, M. J. (2001). The interplay of matrix
635 metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial
636 cells. *Development*, 128(16), 3117–3131. <http://www.ncbi.nlm.nih.gov/pubmed/11688561>

637 Sisto, M., Lorusso, L., Ingravallo, G., & Lisi, S. (2017). Exocrine Gland Morphogenesis: Insights into the
638 Role of Amphiregulin from Development to Disease. *Archivum Immunologiae et Therapiae*
639 *Experimentalis*, 65(6), 477–499. <https://doi.org/10.1007/s00005-017-0478-2>

640 Sobolewska, A., Motyl, T., & Gajewska, M. (2011). Role and regulation of autophagy in the development of
641 acinar structures formed by bovine BME-UV1 mammary epithelial cells. *European Journal of Cell*
642 *Biology*, 90, 854–864. <https://doi.org/10.1016/j.ejcb.2011.06.007>

643 Sornapudi, T. R., Nayak, R., Guthikonda, P. K., Pasupulati, A. K., Kethavath, S., Uppada, V., Mondal, S.,
644 Yellaboina, S., & Kurukuti, S. (2018). Comprehensive profiling of transcriptional networks specific for
645 lactogenic differentiation of HC11 mammary epithelial stem-like cells. *Scientific Reports*, 8(11777).
646 <https://doi.org/10.1038/s41598-018-30122-4>

647 Spivak-Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J., & Lax, I. (1992).
648 Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits

649 stimulatory or inhibitory responses. *The Journal of Biological Chemistry*, 267(12), 8056–8063.
650 <http://www.ncbi.nlm.nih.gov/pubmed/1349015>

651 Sternlicht, M. D., Sunnarborg, S. W., Kouros-Mehr, H., Yu, Y., Lee, D. C., & Werb, Z. (2005). Mammary
652 ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding
653 of epithelial amphiregulin. *Development*, 132(17), 3923–3933. <https://doi.org/10.1242/dev.01966>

654 Sumbal, J., & Koledova, Z. (2019). FGF signaling in mammary gland fibroblasts regulates multiple
655 fibroblast functions and mammary epithelial morphogenesis. *Development*, 146(23), dev185306.
656 <https://doi.org/10.1242/dev.185306>

657 Tang, W. Y. Y., Beckett, A. J., Prior, I. A., Coulson, J. M., Urbé, S., & Clague, M. J. (2014). Plasticity of
658 mammary cell boundaries governed by EGF and actin remodeling. *Cell Reports*, 8(6), 1722–1730.
659 <https://doi.org/10.1016/j.celrep.2014.08.026>

660 Tarcic, G., Avraham, R., Pines, G., Amit, I., Shay, T., Lu, Y., Zwang, Y., Katz, M., Ben- Chetrit, N.,
661 Jacob- Hirsch, J., Virgilio, L., Rechavi, G., Mavrothalassitis, G., Mills, G. B., Domany, E., & Yarden,
662 Y. (2012). EGR1 and the ERK- ERF axis drive mammary cell migration in response to EGF. *The*
663 *FASEB Journal*, 26(4), 1582–1592. <https://doi.org/10.1096/fj.11-194654>

664 Taylor, S. R., Markesbery, M. G., & Harding, P. A. (2014). Heparin-binding epidermal growth factor-like
665 growth factor (HB-EGF) and proteolytic processing by a disintegrin and metalloproteinases (ADAM):
666 A regulator of several pathways. *Seminars in Cell and Developmental Biology*, 28, 22–30.
667 <https://doi.org/10.1016/j.semcdb.2014.03.004>

668 Thiel, G., & Cibelli, G. (2002). Regulation of life and death by the zinc finger transcription factor Egr-1.
669 *Journal of Cellular Physiology*, 193(3), 287–292. <https://doi.org/10.1002/jcp.10178>

670 Wang, Q. A., & Scherer, P. E. (2019). Remodeling of Murine Mammary Adipose Tissue during Pregnancy,
671 Lactation, and Involution. *Journal of Mammary Gland Biology and Neoplasia*, 24(3), 207–212.
672 <https://doi.org/10.1007/s10911-019-09434-2>

673 Waterman, H., & Yarden, Y. (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB
674 receptor tyrosine kinases. *FEBS Letters*, 490(3), 142–152. [https://doi.org/10.1016/S0014-](https://doi.org/10.1016/S0014-5793(01)02117-2)
675 [5793\(01\)02117-2](https://doi.org/10.1016/S0014-5793(01)02117-2)

676 Wiesen, J. F., Young, P., Werb, Z., Cunha, G. R., Mandel, R., Krajewski, S., Reed, J. C., & Rosen, J. M.

677 (1999). Signaling through the stromal epidermal growth factor receptor is necessary for mammary
678 ductal development. *Development*, *126*(2), 335–344. <http://www.ncbi.nlm.nih.gov/pubmed/9847247>

679 Williams, C., Helguero, L., Edvardsson, K., Haldosén, L. A., & Gustafsson, J. Å. (2009). Gene expression in
680 murine mammary epithelial stem cell-like cells shows similarities to human breast cancer gene
681 expression. *Breast Cancer Research*, *11*(3), R26. <https://doi.org/10.1186/bcr2256>

682 Zhang, X., Martinez, D., Koledova, Z., Qiao, G., Streuli, C. H., & Lu, P. (2014). FGF ligands of the
683 postnatal mammary stroma regulate distinct aspects of epithelial morphogenesis. *Development*,
684 *141*(17), 3352–3362. <https://doi.org/10.1242/dev.106732>

685 Zhou, Z. N., Sharma, V. P., Beaty, B. T., Roh-Johnson, M., Peterson, E. A., Van Rooijen, N., Kenny, P. A.,
686 Wiley, H. S., Condeelis, J. S., & Segall, J. E. (2014). Autocrine HBEGF expression promotes breast
687 cancer intravasation, metastasis and macrophage-independent invasion in vivo. *Oncogene*, *33*(29),
688 3784–3793. <https://doi.org/10.1038/onc.2013.363>

689