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Declaration of interest

Conflicts of interest: none

Abstract

In the adult female, within the estrous cycle, the mammary gland undergoes multiple rounds of growth, with increased cellular proliferation, and involution, with increased apoptosis. The increase in proliferation is elicited by endocrine (Estrogen, Progesterone), as well as locally produced (Epidermal growth factor, Insulin-like growth factor, etc) growth factors. Among the genes that are modulated during cellular proliferation, immediate early genes play a fundamental role, being rapidly upregulated and then downregulated within the G0/G1 phase of the cell cycle, allowing the progression to the subsequent phases. Egrs (1 to 4) are immediate early genes that encode for trascription factors that promote, within different cell types and depending on the strength and duration of the stimuli, several different responses like mitogenesis, differentiation, apoptosis or even anti-apoptosis. In this work we have studied the mechanisms of modulation of the Egr family, in mammary epithelial cells of different origin (bovine, canine, feline, murine). Following stimulation with growth medium, Egr mRNA expression showed a strong upregulation reaching a peak at 45-60 minutes, that rapidly declined. Among several cytokines, particularly important for mammary morphogenesis, that we have tested (EGF, IGF-I, insulin, estrogen, progesterone), only EGF upregulated Egrs to levels close to those elicited by growth medium. In order to understand how the Egr trascription factors were regulated, we have inhibited Erk 1/2 and PI3K, molecules that drive two major intracellular signaling pathways. Inhibition of the Erk 1/2 pathway totally abolished Egr upregulation mediated by growth medium or EGF. On the other hand, the PI3K-Akt pathway played a minor role on Egr levels, with a strong inhibitory effect on cat GH2 cells only, that could be ascribed to reduced Erk phosphorylation following PI3K inhibition. Finally we showed that addition of growth medium also upregulated that the mammary luminal marker cytokeratin 18, but only in the murine NMuMG cell line. This is the first manuscript describing how the Egr trascription factors are expressed in mammary epithelial cells of domestic animals and which growth factors and signaling pathways modulate their expression.

Keywords

Egr; mammary gland; immediate early gene; proliferation; epidermal growth factor

1. Introduction

The mammary gland is one of few organs that develops almost exclusively postnatally. Before puberty this organ grows isometrically with the animal, by developing rudimentary branches that are originated by multipotent mammary epithelial cells that progressively invade the surrounding stroma, mainly composed of adipose tissue and fibroblasts. In this phase, in mice, estrogen provides the endocrine signal that induces the release of locally produced amphiregulin, one on the seven ligands able to activate the epidermal growth factor receptor (EGFR). This endocrine-paracrine system is fundamental for prepubertal growth of the mammary gland: estrogen receptor alpha (Mallepell, S. et al. 2006) or amphiregulin knock-out mice are unable to develop this organ (Ciarloni, L. et al. 2007). Once adulthood is reached, the estrous cycle provides the endocrine milieu that stimulates cycles of mammary growth and involution. Estrogen and progesterone (Tanos, T. et al. 2012), acting through locally released growth factors, drive mammary cellular proliferation that form newly developed lateral branches. The major locally produced factors that act during this phases are Wnt-4 (Brisken, C. et al. 2000) and RANKL (Fernandez-Valdivia, R. et al. 2009). Once the estrous cycle ends, lateral branches disappear, and are recreated the successive cycle. Within this system, control of cell proliferation and death, is a major requisite.

Immediate early genes (IEGs) are a class of genes that encode for a variety of proteins involved in many cellular functions, among which proliferation and differentiation. IEGs are expressed within minutes of stimulation and thus, do not require de novo protein synthesis (Herschman, H.R. 1991). Within proliferation, IEGs are normally induced in interphasic cells following stimulation by growth factors (e.g. insulin-like growth factor I, epidermal growth factor, etc) as well as many mitogenic signals (Fowler, T. et al. 2011). Following stimulation, IEGs mRNAs and proteins are rapidly expressed, but being unstable, rapidly degraded as well (Healy, S. et al. 2013). Thus, the peak in expression lasts for a very short period of time, usually less than an hour. Even if brief, this exposure drives the cell to turn on a set of secondary response genes that allow the progression to the subsequent phases of the cycle.

A subclass of IEGs is represented by trascription factors like Egr (1, 2, 3 and 4), Myc, Fos, and Jun, key players in cellular proliferation and well known proto-oncogenes, often found deregulated in cancers (Huang, H. et al. 2014; Verde, P. et al. 2007; Zwang, Y. et al. 2012). Egrs are zinc finger trascription factors with

nuclear localization, that bind preferentially to GC-rich sequences (Thiel, G. and Cibelli, G. 2002). Upon DNA-binding they promote many biological functions that range from proliferation to differentiation to apoptosis and carcinogenesis, depending on the cell type, duration of the growth factor stimuli and intracellular context (Adamson, E.D. and Mercola, D. 2002; Fang, F. et al. 2013; Mayer, S.I. et al. 2009; Seiler, M.P. et al. 2012).

No detailed analysis has been made on Egrs in cells obtained by domestic animals of different species and no data is available on the timing of Egr modulation, which growth factors and which signaling pathways mediate their expression. Therefore we decided to use mammary epithelial cells from bovine, mice and cat (non tumor-derived) as well as dog (tumor-derived) in order to understand the basic mechanisms of modulation of Egrs in these species. We focused our study on different aspects of Egr biology: a) basal expression of Egrs, b) modulation of expression following growth factor addition, c) study of extrinsic stimuli able to modulate Egrs in mammary cells, d) examination of intracellular pathways able to modulate Egrs.

2. Materials and methods

2.1 Materials and cell lines

All reagents, unless specified, were from Sigma–Aldrich (St. Louis, MO, USA); recombinant Epidermal Growth Factor (EGF), Insulin-Like Growth Factor I (IGF-I) were from Immunotools (Friesoythe, Germany) and were resuspended in water + 0,1 % BSA; UO126, Selumetinib, Wortmannin and phorbol 12-myristate 13-acetate (PMA) were from LC Laboratories (Woburn, MA, USA) and were resuspended in DMSO; DC Protein Assay was from Bio-Rad Laboratories (Hercules, CA, USA); Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane was from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK); Clarity Western ECL Substrate was from Bio-Rad Laboratories (Hercules, CA, USA); CL-XPosure Film was from Thermo Scientific (Waltham, MA, USA).

Antibodies. Anti-Egr-1 (1:1000; Sc-189), anti-Erk 1 (1:1000; Sc-94) and anti-IGF-IR alpha (1:1000; Sc-712) rabbit policlonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-α-tubulin

(1:5000) and anti-phospho-Erk 1/2 (1:5000; M 8159) mouse monoclonal antibodies were from Sigma–Aldrich; anti-phospho-Akt mouse monoclonal (1:1000; #4051; ser-473), anti-Akt (1:1000; #9272) and anti-EGFR (1:1000; #2232) rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA); rabbit policlonal anti-Keratin 14 (1:500; Poly19053) was from BioLegend (Dedham, MA, USA); mouse monoclonal anti-Keratin 18 (1:200; RGE 53) was from Progen Biotechnik (Heidelberg, Germany).

NMuMG murine mammary epithelial cell line (ATCC number CRL-1636) was kindly provided by R. Montesano (University of Geneva Medical School, Switzerland) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). BME-UV bovine mammary epithelial cell line kindly provided by I. Politis (Agricultural University of Athens, Athens, Greece; (Zavizion, B. et al. 1996)). BME-UV cells were grown in DMEM supplemented with 10 % FBS. *Felis catus* mammary cells (GH2) were obtained by an healthy mammary gland following mastectomy in a cat with a mammary tumor (the tumor was in the inguinal mammary gland while mammary cells were obtained from the thoracic mammary gland). Consensus for the surgery was given by the owner of the animal and approved by the Ethical and Welfare Committee of the Department of Veterinary Sciences. Fresh mammary tissue was dissected, minced, added to a 1:1 v/v mixture of DMEM/F12 medium supplemented with 2 % w/v bovine serum albumin (BSA, Fraction V), 300 U/ml collagenase, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 µg/ml streptomycin and placed in a shaking incubator at 37 °C for three h. Epithelial cell aggregates obtained by centrifugation at 80 g for 30 seconds were washed in fresh DMEM/F12 medium for 3 times. Aggregates were incubated with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA followed by vigorous pipetting for 4 minutes and subsequent washing in Hank's balanced salt solution (HBSS) supplemented with 2 % FBS. Cells were then treated for 3 min with 5 mg/ml dispase and 100 µg/ml DNAseI and passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA, USA) to remove remaining cell aggregates. GH2 cells were then cultured in collagen coated wells with DMEM/F-12 media supplemented with cholera toxin (100 ng/ml), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), EGF (20 ng/ml) and 5 % horse serum. GH2, by immunostaining, were mostly keratin 14 positive, a unique marker for cells belonging to the basal compartment of mammary gland, with a small proportion of keratin 18 positive cells (luminal compartment; Supplementary Fig. 3). CF33 cells, a canine breast cancer cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). CF33 cells were grown in DMEM supplemented with 10 % heat-inactivated FBS. All cell lines

were maintained in a 5 % CO2–water-saturated atmosphere and routinely passaged when confluent by washing with PBS followed by trypsinization.

2.2 RNA extraction and quantitative RT-PCR

Total RNA was isolated from primary mammary cell subpopulations using Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories) following the manufacturer's instructions. The purification procedure included a DNase I digest step to produce a DNA-free total RNA. One microgram of total RNA was reverse transcribed with iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories) using a blend of oligo(dT) and random primers following the manufacturer's instructions. Real-time PCR was used to measure the quantity of the genes of interest relative to the quantity of HPRT-1 (hypoxanthine phosphoribosyl-transferase 1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. HPRT-1 and GAPDH were used to correct for sample concentration and reverse transcription efficiency because their expression is stable in these cells under our experimental conditions. Efficiencies for the different genes were calculated by standard dilution curves and used to correct for amplification. The specificity of each amplification was confirmed by initial sequencing of the reaction product and by subsequent verification of the dissociation curve consisting of a single peak. Diluted cDNAs (1:20, 30 ng per reaction) were used for real-time PCR amplification (CFX Connect real-time PCR system; Bio-Rad Laboratories) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). Real-time PCR parameters were: cycle 1, 95 °C for 30 s; cycle 2, 95 °C 10 s, 60 °C 30 s for 40 cycles. Quantitative analisys and correction for efficency was calculated by the CFX Manager Software version 3.1 (Bio-Rad Laboratories) using the Δ(ΔCT) method by Livak and Schmittgen (Livak, K.J. and Schmittgen, T.D. 2001) and compared to a comparator sample (CTRL=1). Primers were used at a final concentration of 250 nM (MWG-Biotech; Ebersberg, Germany). Primers, accession number, amplicon size, melting temperature and efficiency (%) are indicated in Supplementary Table 1. N/D = Not Determined, due to very low expression of the target mRNA (> than 30 Cq).

2.3 Analysis of cell cytodieresis through time-lapse video microscopy

Cells were plated and cultivated in their respective growth medium (20-30 % confluence) then either left in their growth medium (GM) or starved in serum and growth factors deprived medium (ST) and photographed

155 at 4 min intervals for 24 h (360 frames) with a Leica AF6000 LX (Leica Microsystems, Wetzlar, Germany)
156 inverted microscope equipped with a Leica DFC350FX digital camera and a motorized stage controlled by
157 the LAS AF software (Leica Microsystems, Wetzlar, Germany). All cells within a 10x field were followed
158 from time 0 to time 24 h and the frames in which the cytodieresis event occurred were recorded. Finally, the
159 total amount of divisions occurring within every hour was calculated and divided by the mean number of
160 cells present at the same time interval (number of divisions / h / number of cells).
161
162 *2.4 Cell cycle analysis*
163 For cell cycle analysis cells were seeded in 6 cm plates in their respective growth medium then either left in
164 proliferation medium or starved in serum/growth factors deprived medium for 16 h. Cells were then
165 trypsinized, washed with PBS, fixed for 1 h in 50 % ethanol (in PBS), then centrifuged, resuspended in PBS
166 and stained with DAPI (1 µg/ml final concentration) for 2 h at 37 °C. Samples were run on a Attune
167 Acoustic Focusing Cytometer (Applied Biosystems) and the cell cycle distribution in G0/G1 and G2/M
168 phases was calculated using the Attune Cytmetric Software (Applied Biosystems). Each experiment was
169 repeated four times independently, and within each experiment, each treatment was performed with two
170 replicate culture wells.
171
172 *2.5 Western-blot analysis*
173 For western-blot, all cell lines were seeded in six-well plates and allowed to grow to 50 % confluence. If
174 needed, cells were starved for 16 h in serum deprived medium. The indicated cytokines were added for 1 h.
175 UO126 (20 µM), Selumetinib (1 µM) and Wortmannin (100 nM) were resuspended in dimethyl sulfoxide
176 (DMSO) at 1000x concentration and added 1h before lysis or the indicated treatments. Control samples were
177 added with an equivalent amount of DMSO. Cells were washed with ice-cold PBS, lysed, and scraped in
178 lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X 100, 1mM h
179 glycerolphosphate) with Protease Inhibitor Cocktail (1:100) and 1 mM sodium orthovanadate. Protein lysates
180 (10 mg) were cleared of cellular debris by centrifugation at 4 °C for 10 min at 12 000 g, quantified using DC
181 Protein Assay, resolved in 10 % SDS-PAGE gels, and transferred to Hybond-C Extra nitrocellulose
182 membranes. After the transfer of proteins, the membranes were blocked at room temperature for 2 h with

Tris-buffered saline (TBS, 10 mM Tris and 150 mM NaCl, pH 7.4) containing 10 % BSA and then incubated overnight at 4 °C with the appropriate primary antibodies. The membranes were washed six times for 5 min each in TBS–Tween and then incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. The membranes were again washed six times in TBS–Tween and incubated for 5 min at room temperature with Clarity Western ECL Substrate. The proteins were visualized by briefly exposing the membrane to an autoradiographic CL-XPosure Film. Densitometric analysis for keratin-18 and p-Erk 1/2 levels was performed with ImageJ 1.50 (National Institutes of Health, USA; https://imagej.nih.gov/ij/) with total Tubulin and Erk levels used as internal normalization controls (for Krt18 and p-Erk respectively).

2.6 Statistics

Experimental data are presented as mean ± sem. Statistical differences between treatments were calculated with one-way ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by an unpaired Student's t-test. For all tests, the level of statistical significance was set at $p < 0.05$.

3. Results

3.1 Egr-1, 2, 3 and 4 levels in mammary epithelial cells.

In order to study the Egr behavior, we first analyzed its mRNA levels in mammary epithelial cells of different origin under two different conditions: cells cultivated in their respective growth medium (GM, see Materials and methods) or cultivated under starving conditions for 16 h (medium without any supplement: ST). Basal expression rates, as indirectly monitored by Cq levels (in 30 ng of retrotranscribed RNA), were higher for Egr-1 and progressively lower for Egr-2, 3 and 4 (Fig. 1A). Even though these transcription factors are tightly connected to the cell cycle Egrs levels in GM and ST cells were similar. To understand this result we used time lapse video microscopy (Supplementary Video 1) and counted all cytodieresis events occurring simultaneously, e.g. every h (Fig. 1B). Under the GM condition cells showed a 3,2±0,8% (NMuMG), 3,6±1,3% (CF33), 4,4±1,2% (BME-UV) and 5,8±0,8% (GH2) cells undergoing cytodieresis

every h. Under the ST condition (after 14-18 h) cells showed a mild reduction of cytodieresis events occurring every h: 1,4±0,5% (NMuMG), 1,9±0,5% (CF33), 2,4±0,9% (BME-UV) and 3,4±1,0% (GH2). These data indicate that under both the GM or ST condition a very low proportion of cells are within the exact same phase of the cycle with more than 94% of the cells being unsynchronized. This effect produces a very high "background noise" that masks the behavior of Egrs. We thus decided to increase per percentage of cells within the same phase of the cycle in order to better study Egr modulation. For this purpose, cells were serum starved. Analysis by flow cytometry confirmed that, to the exception of BME-UV cells, starving increased the percentage of mammary cells in G0/G1 (Fig. 1C, D).

3.2 Egr-1, 2 and 3 modulation in mammary epithelial cells

Following starvation for 16 h cells were stimulated with their respective growth medium (see materials and methods) and RNA was extracted at multiple time points (from 15 to 120 min). Egr-1, 2 and 3 levels were measured by real-time PCR with time = 0 min being set arbitrarily to 1 (Fig. 2; CTRL). All cell lines reacted similarly reaching a peak in Egr levels at 45 to 60 min after growth medium addition (Fig. 2). Egr-1 and 2 returned to almost basal levels after 120 min of stimulation, while Egr-3 had a slightly delayed curve of increase and decrease in expression, in particular in CF-33 cells reaching a peak at 75-90 min. Egr-4 (data not shown) showed a similar, but much lower, trend of regulation with levels of expression that remained always very low (> 28 Cq). For this reason Egr-4 was not analyzed any further.

3.3 Growth factors that affect Egr modulation in mammary epithelial cells

We then verified the ability of multiple cytokines, important for mammary growth, to modulate Egr levels. Following 16 h of serum starvation several growth factors (EGF, IGF-I, insulin, 17β-estradiol, progesterone) were added for 1 h and Egr mRNAs were analyzed by real-time PCR. Among all the growth factors that we tested, epidermal growth factor (EGF) was unique in eliciting an Egr-1, -2 and -3 increase to levels close to growth medium (Fig. 3). In mouse NMuMG cells, insulin promoted some increase on Egr expression. Other cytokines (FGFβ, PDGFaa, VEGF, TNFα), with limited effect in mammary morphogenesis, were tested and found to have no effect on Egr expression (data not shown).

We thus assayed whether the cell lines expressed the receptors for the cytokines that we had tested (Epidermal Growth Factor, Insulin, IGF-I, Estrogen or Progesterone receptors). EGFR and Erb-B2, activated by EGF as homo- (EGFR/EGFR) or hetero- (EGFR/Erb-B2) dimers, and IGF-I receptors (activated by IGF-I and insulin at the high concentrations that we used) were present in all cell lines (Fig. 3 A and B). Insulin, Estrogen and Progesterone receptors were not expressed or expressed at very low levels.

3.4 Identification of pathways that modulate expression of Egr-1, 2 and 3

We analyzed whether two major intracellular pathways, the Erk-1/2 and the PI3K-Akt pathways, could modulate Egr expression in mammary epithelial cells. To this end, starved mammary cells (16 h) were pretreated for 1 h with UO126 (Erk-1/2 inhibitor, 20 μM) or wortmannin (PI3K inhibitor, 100nM) and stimulated with GM or EGF for 1 h in order to increase Egr-1, -2 and -3 expression. Inhibiting the Erk-1/2 pathway abolished the Egr mRNA increase downstream GM or EGF in all cell lines (Fig. 4A, GM vs GM+UO and EGF vs EGF+UO). Inhibiting the PI3K pathway had no effect on bovine BME-UV and canine CF33 cells, a mild effect on mouse NMuMG cells treated with GM (Fig. 4A, GM vs GM+W) and a strong reducing effect on GH2 cells (Fig. 4A, GM vs GM+W and EGF vs EGF+W). Egr-1 protein levels, visualized by western-blot in GH2 and NMuMG cells, reflected the results obtained by mRNA quantification (Fig. 4B). We were unable to visualize Egr-1 in BME-UV and CF33 cells by western-blot, possibly because the antibodies did not work in these species (2 different antibodies were tested). p-Erk and p-Akt levels, as shown by western-blot, confirmed that UO126 and wortmannin inhibited their targets (Fig. 4B, p-Erk and p-Akt) but showed also a reduction in p-Erk levels in GH2 cells treated with wortmannin. UO126 specificity was confirmed with another more recently developed Erk inhibitor, Selumetinib (Zhou, Y. et al. 2016). Used at 1 μM, Selumetinib prevented Erk phosphorylation (supplementary Fig. 2A), and inhibited Egr-1, -2 and -3 upregulation following GM or EGF addition (supplementary Fig. 2B) in all cell lines.

To better clarify whether the strong inhibitory effect observed on GH2 cells treated with wortmannin could depend on reduced of Erk rather than Akt activation, we performed time-course western-blot analysis of p-Erk 1/2 and p-Akt following addiction of GM, EGF or phorbol 12-myristate 13-acetate (PMA, a strong activator of protein kinase C). PMA was included in this assay because it is a strong activator of Erk 1/2 with different modulating activities on Akt phosphorylation (Han, S., and Meier, K.E. 2009; Liu, H. et al. 2006;

Moelling, K. et al. 2002). Western-blot analysis showed that wortmannin had a strong inhibitory effect on Erk activation at 10 and 30 min following GM and EGF, but not PMA addition (Fig. 5A, 10' and 20'). At 40 and 60 min p-Erk was inhibited downstream all growth factors (Fig. 5A, 40' and 60'). p-Akt levels were increased following GM or EGF treatment. On the contrary, PMA treatment reduced p-Akt below starved (ST) levels. Egr-1, -2 and -3 levels under PMA stimulation, were minimally reduced by wortmannin (Fig. 5B: PMA+W), confirming that in GH2 Erk, but not Akt phosphorylation is the major inducer of these IEGs.

3.5 Regulation of cytokeratin 18 following growth medium stimulation

Recent evidence has shown that Egr-1 binds to the cytokeratin 18 promoter and modulates the expression its mRNA and protein (Zhang, H. et al. 2014). In mammary cells, cytokeratin 18 (Krt18) is a marker of the luminal lineage. Since Egr-1 is strongly upregulated by growth medium, we tested whether growth medium could alter Krt18 expression in mammary cells that express this marker. We first identified Krt18 expressing cells by immunohistochemistry (supplementary Fig. 3). BME-UV, GH2 and NMuMG cells express Krt18, while CF33 do not. Real-time PCR confirmed this data (Cq levels: BME-UV 18,0±1,2, GH2 22,3±1,0, NMuMG 17,5±1,3). To analyze whether Krt18 was modulated in these cell lines, serum starved BME-UV, GH2 and NMuMG where stimulated with GM and Krt18 mRNA was quantified. Only NMuMG showed a stable Krt18 mRNA upregulation following GM addition (Fig. 6A), that was confirmed by western-blot (Fig. 6B). Cytokeratin 14, expressed at high levels by GH2 cells only, did not show any modulation by GM (data not shown).

4. Discussion

Egr-1 was first identified by screening cells for genes that are upregulated by the addition of serum (Sukhatme, V.P. et al. 1987). The discovery of Egr-2, -3 and -4 followed briefly after (Chavrier, P. et al. 1988; Muller, H.J. et al. 1991; Patwardhan, S. et al. 1991). Egrs are zinc finger trascription factors that regulate trascription, unusually, as a monomers. They have found many roles in cellular physiology, most importantly by regulating genes that allow the cell to proceed through the different phases of its cycle, but also, in particular cell types and under well defined stimulatory conditions, promoting differentiation, motility or even apoptosis (Oliveira, F.M. and Tourtellotte, W.G. 2015; Seiler, M.P. et al. 2012; Tao, X. et al.

2015; Tourtellotte, W.G. et al. 1999; Zwang, Y. et al. 2011). In pathology Egrs may act paradoxically, usually as a tumor suppressors by maintaining normal growth regulation, but also as a tumor progression agents, like in prostate cancer (Baron, V. et al. 2006; Baron, V.T. et al. 2015).

In this work we describe the biological behavior of Egr-1, -2 and -3 in three non tumorigenic mammary epithelial cell lines isolated from different species (bovine, mouse, cat) and in one tumorigenic mammary cell line isolated from the dog specie (Saito, T. et al. 2014). We first analyzed the levels of expression of the four Egrs under proliferating (GM) and serum starved (ST) culture conditions. We were expecting lower Egr levels under serum-starved conditions, but unexpectedly all cell lines showed similar levels (Fig. 1A). Since Egrs are Immediate Early Genes tightly connected to the cell cycle, we counted the number of cytodieresis events occurring every hour (a direct indicator of cells that are at the exact same point of the cell cycle). We found out that the maximum number of cytodieresis events occurring simultaneously in any 1 h in GM cells is about 5% (GH2 cells) that decreases in ST cells to about 3%. All other cells lines behave similarly with a small decrease in the percentage of cells undergoing cytodieresis when passing from the GM to the ST condition. This helped us to understand that, within a large population, only a minimal fraction of cells is at the exact same time point of the cell cycle. Since IEGs are highly upregulated in a narrow time interval that lasts less than one hour, having more than 95% of the cells unsynchronized during normal growth, explains why GM and ST treated cells have similar Egr levels.

Synchronization by serum starvation was a prerequisite that allowed us to analyze Egr modulation downstream growth factor stimulation. This treatment increased the percentage of cells in G0/G1 in three cell lines. BME-UV showed an opposite response probably due to the fact that, following serum starvation for 16 h, 30-40% of these cells undergo cell death and are visible as sub-G0 events (supplementary Fig. 1). After stimulation with growth medium all cell lines had a similar trend of Egr modulation, reaching a maximum level after 45-60 minutes that rapidly declined to near basal level after 120 min. Since starvation could not synchronize all cells we hypothesize that the real increase is much higher than the one we have observed. Egr-3 levels were maintained for up to 75-90 minutes before rapidly declining. This different mechanism of modulation has not been described before.

To the exception of CF33 cells (dog), which have been obtained from a mammary cancerous tissue, all other cell lines come from healthy mammary glands. Grossly, the mammary epithelium is organized in two layers of cells: luminal cells that face the lumen of the mammary ducts and express cytokeratin 18 and basal cells that form the outer layer of the ducts and express cytokeratin 14. The cells that we have used in our work express differentially markers of both compartments (data by real-time PCR, not shown, and immunostaining, Supplementary Fig. 2): NMuMG cells are almost all Krt18 positive; GH2 are Krt14 positive with a small percentage of Krt18 positive cells, distributed in islands; BME-UV cells are mostly Krt18 positive. CF33 canine tumoral cells are negative for both markers, therefore are probably of stromal rather than epithelial origin, or have lost the keratin markers during selection. Our results show that the behavior of Egrs is similar in all the cell lines that we examined, indicating that the cell of origin (basal or luminal) or the cancerous state do not seem to modify the modulation of these trascription factors.

Our observation that only EGF can increase Egr expression close to the levels obtained by GM stimulation is intriguing. In fact, we were expecting that other mammary "specific" growth factors or hormones (IGF-I, estrogen, progesterone) could also modulate Egr expression. This result is probably a consequence of the selection operated during the immortalization process that has stabilized these cell lines, in which the majority of the multiple cellular lineages present within the mammary gland disappear. For example, expression of estrogen or progesterone receptors is limited to a small percentage of terminally differentiated cell population that is probably difficult to retain in culture and these receptor were absent in all the cell lines examined. Even though all the cell lines express IGF-IR, and a low quantity of Insulin R, there was no Egr modulation following treatment with IGF-I or insulin (that at the concentration used can activate the IGF-IR). This effect can be explained by the low capacity of the IGF-IR to activate the Erk rather than Akt (Moelling, K. et al. 2002), providing another confirmation that Erk is the major upregulator o Egrs.

EGF is a strong growth-promoting agent and, within the mammary gland, its receptor has long ago found an important biological role (Fowler, K.J. et al. 1995). In depth analyses have then elucidated that among the seven known EGF receptor ligands, only amphiregulin has a fundamental role during the pubertal mammary development (Ciarloni, L. et al. 2007). Interestingly, this role is tied to the presence of the EGF receptor in the stromal, but not the epithelial compartment of the mammary gland (Wiesen, J.F. et al. 1999). Our data show that EGF, acting through its receptor, is the only growth factor able to elicit an increase in Egr

trascription factors in all the cell lines that we have tested. Therefore, a biological role for this growth factor and its receptor, mediated through Egrs, is possible even in the epithelial compartment. The NMuMG line depends on EGFR for its proliferation (Accornero, P. et al. 2012), therefore in this cell line Egr modulation downstream EGF might drive proliferation. Another possible function of Egrs could be to modulate the expression of some differentiation markers, like Krt14 or Krt18. A recent report has outlined a connection between Egr-1 levels and Krt18 (Zhang, H. et al. 2014). Krt18 expression is directly regulated by an Egr-1 binding site situated within its promoter. Among the cell lines examined, only NMuMG showed a mild upregulation of Krt18 expression following GM addiction. We think unlikely that the basal expression of this keratin is maintained by Egr levels for two reasons: a) BME-UV and GH2 Krt18 levels were unaffected following GM addition and, b) MCF-7, T47D and ZR-75D human mammary tumor cells that have high Krt18 basal expression (Cq levels around 16-18, data not shown) have basal Egr-1 levels lower than the cells that we have tested (Cq levels of 27-29; data not shown). Therefore in our models, Egr modulation downstream EGF or GM, most likely promotes a proliferative, antiapoptotic or migratory stimuli (Baron, V. et al. 2006; Tarcic, G. et al. 2012; Yu, J. et al. 2009). By using lipofectamine transfected siRNA (MISSIOIN esiRNA; Sigma-Aldrich) we have tried to downregulate Egr expression but, unfortunately, we were unable to reduce their expression. For this reason, we cannot confirm what is the exact role of these transcription factors in these cell lines.

We have also analyzed which signaling pathway modulates Egr-1, -2 and -3 expression downstream GM or EGF. Our data show clearly that Erk 1/2 activation has a preeminent role in Egr modulation. All cell lines had all Egrs lowered to levels close or lower than the CTRL unstimulated samples, when the Erk 1/2 pathway was inhibited by UO126 or Selumetinib. Our data is in line with literature (Murphy, L.O. et al. 2002; O'Donnell, A. et al. 2012; Tarcic, G. et al. 2012; Whyte, J. et al. 2009), and extends the its significance to mammary cell lines of multiple origin. Inhibiting the PI3K pathway determined a strong reduction of Egrs in GH2 cells only. A possible explanation to this reduction is that inhibition on the PI3K-Akt pathway determined by wortmannin also induces a reduction on the Erk1/2 pathway. This action is apparent when observing the reduction in p-Erk in western-blot. This event could be due either by an off target effect of wortmannin (even at the low concentration used, 100nM, and only in this cell line) or by an Akt-Erk1/2 cross talk active exclusively in this cell line. We addressed this issue by examining p-Erk and p-Akt levels

downstream GM, EGF and PMA at multiple time points. We observed that wortmannin elicit a strong reduction in p-Erk levels, but not under PMA treatment. The inability of wortmannin to reduce p-Erk levels downstream PMA at 10' and 20' min was coupled with a minimal reduction of Egrs in PMA+wortmannin treated samples. This data together with the observation that PMA reduces p-Akt levels in this cell line, confirm that Egrs are modulated in an Erk-dependent but not Akt-dependent manner. Other authors have observed that, in hemopoietic cells, Erk activation requires a PI3K generated signal (Sutor, S.L., et al. 1999). In our assays we show, for the first time in mammary cells, that the PI3K pathway is not insulated from the Erk signaling cascade, following GM or EGF treatment, thus extending previous observations to a different cell type and stimulus. Future studies will address at what level in the signaling cascade PI3-K is required for Erk activation.

4.1 Conclusions

In conclusion, in this study we have evaluated the mechanisms of modulation of Egr-1, -2 and -3 trascription factors that belong to the immediate early genes group, in mammary epithelial cells of different species. We have observed that transcription of Egr-1 and Egr-2 reach a maximum 45 to 60 min after a stimulatory event, then rapidly decline. Egr-3 show a slightly delayed response. We have found that, among different growth factors and hormones that we tested, only EGF is able to increase the Egrs to levels similar to those elicited by growth medium. We have also identified the Erk 1/2 pathway as the major regulator of all three Egrs. Finally, we have shown that NMuMG, but not BME-UV and GH2 cells, exhibit a Krt18 increase following the growth medium acute stimulus.

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514 Figure Captions

515

(A)

Cell line	Cq (mean ± SEM)			
	Egr-1	Egr-2	Egr-3	Egr-4
BME-UV GM	23,1 ± 1,4	29,6 ± 1,7	34,5 ± 1,2	36,4 ± 1,3
BME-UV ST	23,6 ± 1,2	29,4 ± 1,5	31,2 ± 2,0	35,8 ± 1,4
CF33 GM	26,5 ± 0,7	29,8 ± 1,4	31,8 ± 1,4	33,0 ± 1,6
CF33 ST	25,4 ± 1,2	28,5 ± 1,3	32,3 ± 1,3	32,0 ± 1,7
GH2 GM	24,6 ± 1,2	28,0 ± 1,6	27,2 ± 0,9	30,7 ± 1,4
GH2 ST	24,8 ± 1,1	27,5 ± 1,9	27,6 ± 1,0	30,9 ± 1,3
NMuMG GM	25,4 ± 0,8	32,0 ± 0,9	31,7 ± 0,9	36,8 ± 1,8
NMuMG ST	24,0 ± 0,9	30,2 ± 1,0	29,8 ± 1,2	36,2 ± 1,3

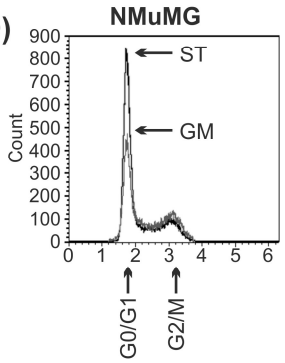
(B)

Cytodieresis events/h (%)			
BME-UV GM	4,4 ± 1,2	GH2 GM	5,8 ± 0,8
BME-UV ST	2,4 ± 0,9	GH2 ST	3,4 ± 1,0
CF33 GM	3,6 ± 1,3	NMuMG GM	3,2 ± 0,8
CF33 ST	1,9 ± 0,5	NMuMG ST	1,4 ± 0,5

(C)

	% G0/G1	% G2/M
BME-UV GM	61,4±6,0	12,3±3,7
BME-UV ST	49,5±9,0	9,9±1,4
CF33 GM	50,2±4,5	20,0±3,2
CF33 ST	64,9±4,7	14,2±3,1
GH2 GM	32,6±4,2	26,7±5,4
GH2 ST	46,3±6,8	20,5±7,2
NMuMG GM	42,0±3,6	17,7±1,9
NMuMG ST	57,8±5,6	15,8±2,0

(D)



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517 **Fig. 1.** Basal **e**xpression of Egr-1, 2 and 3 in mammary epithelial cells of different origin. **A:** Cq levels of
518 Egr-1, Egr-2, Egr-3 and Egr-4 in mammary epithelial cells cultivated in their respective growth medium
519 (GM) or under serum starved (ST) condition for 16 h. Each number represents the mean ± standard error of
520 the mean of the four replicate experiments. **B:** Mean ± standard error of the number of cytodieresis events
521 per h every 100 cells in mammary epithelial cells cultivated under GM or ST conditions (from the 14th to the
522 18th h if under serum starving condition). Time lapse video microscopy of multiple fields (n=4) were
523 acquired and cytodieresis events occurring every h were manually counted (see supplementary Video 1) and
524 divided by the number of cells in the field. **C:** Mean ± standard error of the percentage of cells in G0/G1 and
525 G2/M in mammary cells cultivated under GM or ST conditions for 16 h (n = 4). **D:** Representative
526 cytofluorimetric plot of the NMuMG population (50.000 events), showing the variation in G0/G1 when cells
527 are cultivated for 16 h in ST condition.

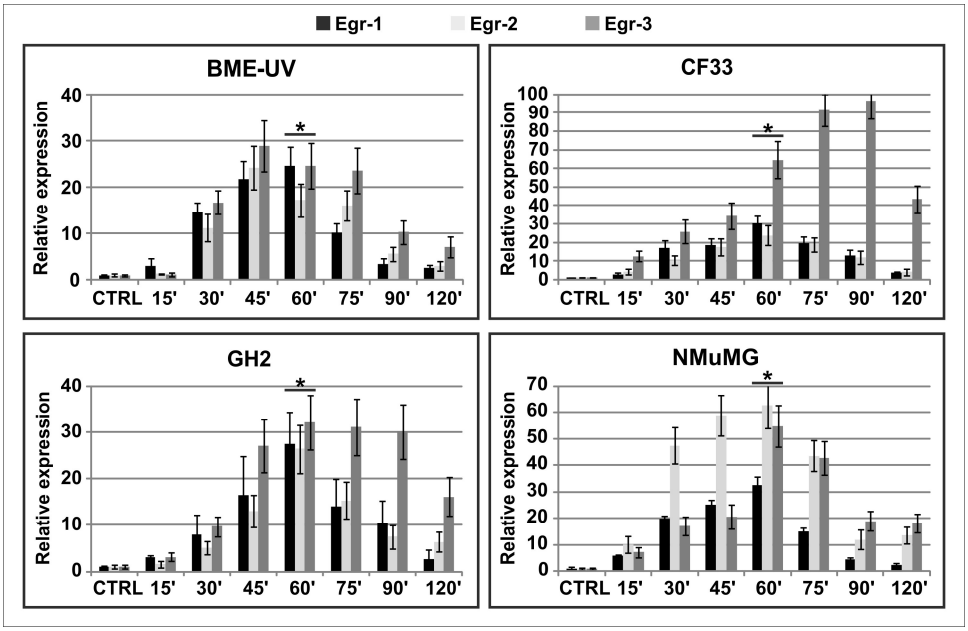
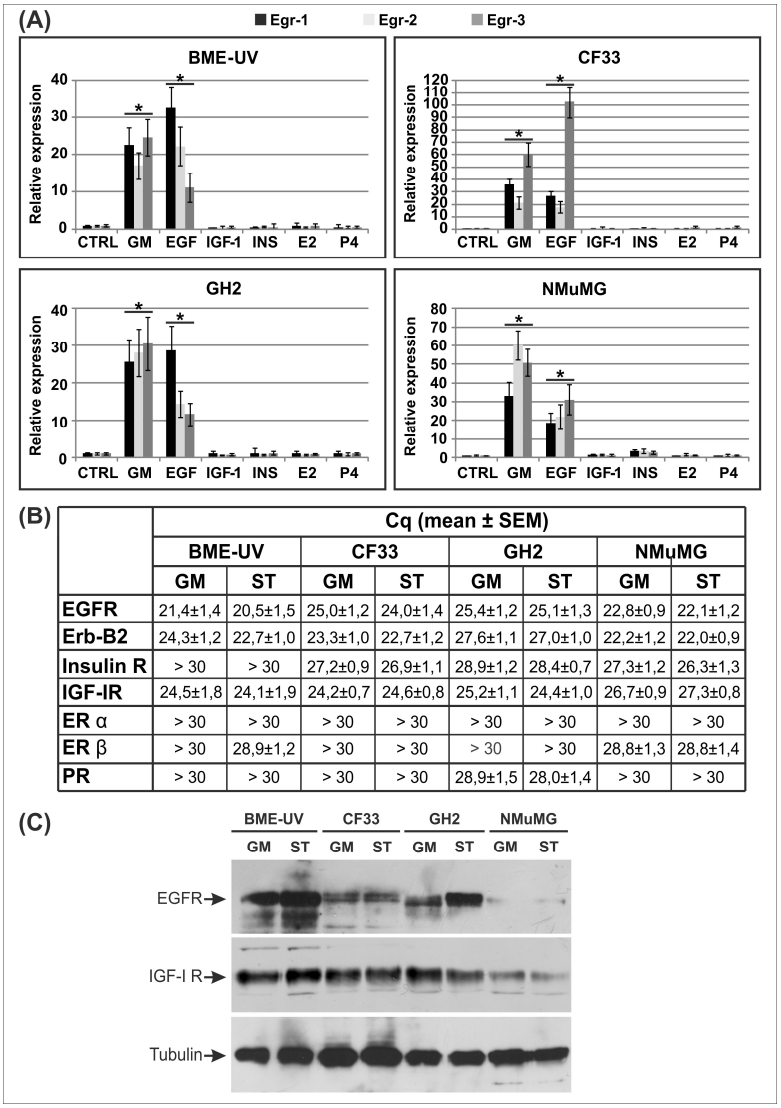


Fig. 2. Time course of Egr-1, Egr-2 and Egr-3 expression in mammary epithelial cell lines following stimulation with growth medium. Cells were synchronized by serum-starvation for 16 h then treated with their respective GM. RNA was extracted at the indicated time points and Egr expression was measured by real-time PCR and normalized with HPRT-1 and GAPDH. Egr expression in cells at the zero time point was set to 1. Each point represents the mean ± standard error of the mean of the four replicate experiments. *P<0,001 versus CTRL.



(B)

	Cq (mean ± SEM)							
	BME-UV		CF33		GH2		NMuMG	
	GM	ST	GM	ST	GM	ST	GM	ST
EGFR	21,4±1,4	20,5±1,5	25,0±1,2	24,0±1,4	25,4±1,2	25,1±1,3	22,8±0,9	22,1±1,2
Erb-B2	24,3±1,2	22,7±1,0	23,3±1,0	22,7±1,2	27,6±1,1	27,0±1,0	22,2±1,2	22,0±0,9
Insulin R	> 30	> 30	27,2±0,9	26,9±1,1	28,9±1,2	28,4±0,7	27,3±1,2	26,3±1,3
IGF-IR	24,5±1,8	24,1±1,9	24,2±0,7	24,6±0,8	25,2±1,1	24,4±1,0	26,7±0,9	27,3±0,8
ER α	> 30	> 30	> 30	> 30	> 30	> 30	> 30	> 30
ER β	> 30	28,9±1,2	> 30	> 30	> 30	> 30	28,8±1,3	28,8±1,4
PR	> 30	> 30	> 30	> 30	28,9±1,5	28,0±1,4	> 30	> 30

Fig. 3. A: Egr modulation in mammary epithelial cell lines treated with different growth factors or hormones. Cells were serum-starved for 16 h then treated with the indicated growths factors/hormones/growth medium (EGF 20 ng/ml; IGF-I 10 ng/ml; Insulin 10µg/ml, β-estradiol 20 nM, 17α-hydroxyprogesterone 1 µM). RNA was extracted after 1 h of treatment. Egrs expression was evaluated by real-time PCR and normalized with HPRT-1 and GAPDH. The serum starved untreated control (CTRL) was set to 1. Each point represents the mean ± standard error of the mean of the four replicate experiments. **P*<0,001 versus CTRL. **B:** Cq levels of EGFR, Erb-B2, Insulin R, IGF-IR, ER α, ER β and PR in mammary epithelial cells cultivated in their respective growth medium (GM) or under serum starved (ST) condition for 16 h. Each number represents the mean ± standard error of the mean of the four replicate experiments. **C:** GM or serum-starved (16 h) cultivated cells were analyzed by western-blot for EGFR and IGF-IR. Tubulin was used as an internal control.

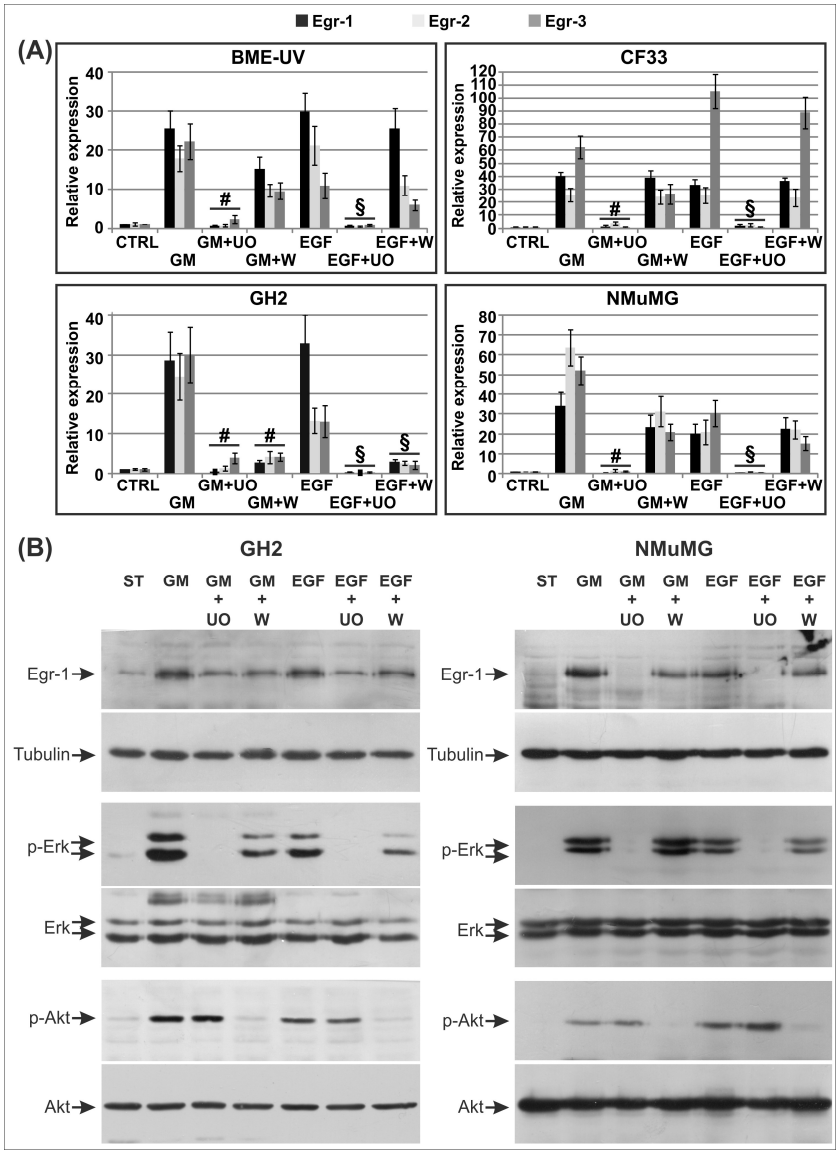


Fig. 4. A: Egr-1, 2 and 3 mRNA and protein analysis in cells treated with growth medium or EGF in the presence of an Erk1/2 or PI3K inhibitor. Cells were serum-starved for 16 h then pretreated for 1 h with UO126 (20 μM) or wortmannin (100 nM) and added with GM or EGF (10 ng/ml) always in presence of the inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set to 1. Each point represents the mean ± standard error of the mean of the four replicate experiments. #P<0,001 versus GM. §P<0,001 versus EGF. **B:** Western-blot analysis of the Egr-1 and tubulin normalization control in GH2 and NMuMG cells treated like in A. Phospho-Erk 1/2 and phospho-Akt were used as internal controls to evaluate the efficacy of the inhibitors (in this case proteins were extracted 10 min after the treatment with GM or EGF). Total Erk 1/2 and total Akt are internal controls for phospho-Erk 1/2 and phospho-Akt, respectively.

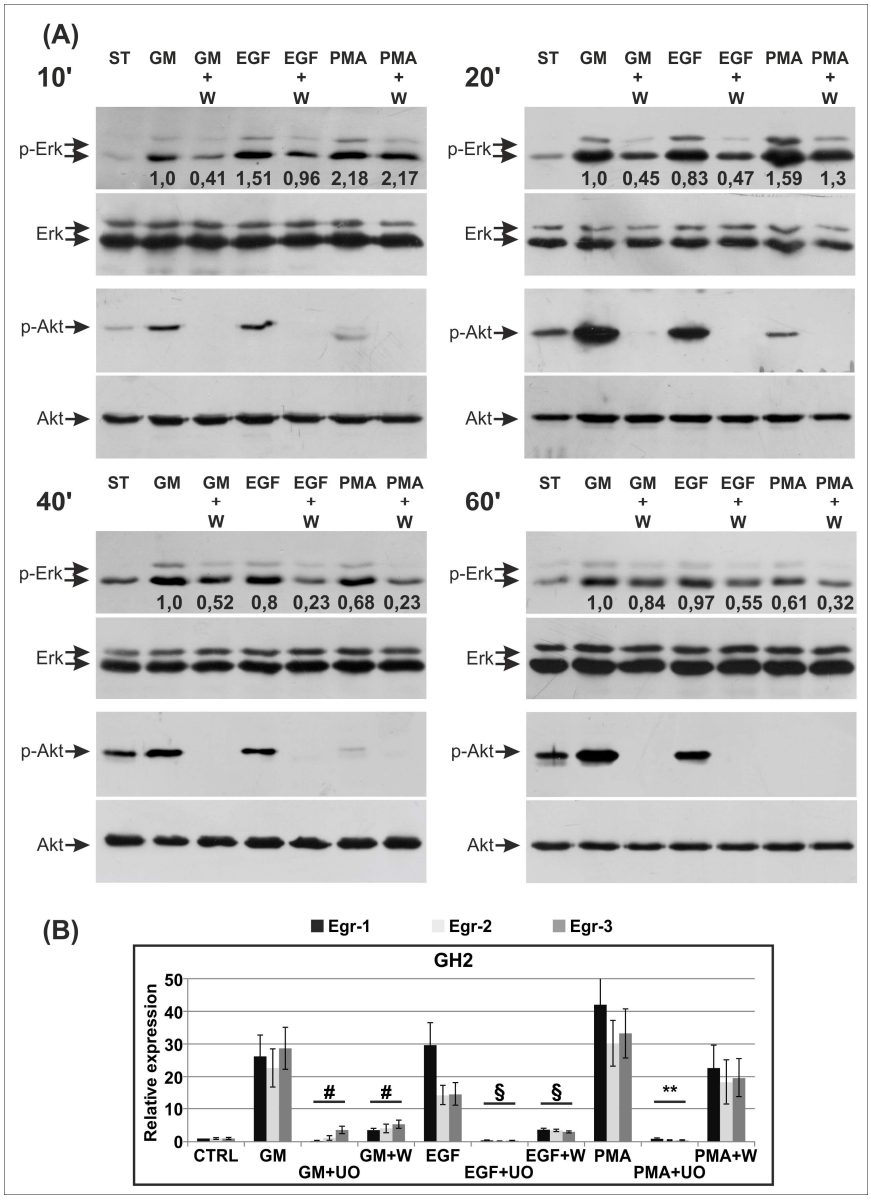


Fig. 5. A: Egr-1, 2 and 3 mRNA and protein analysis in cells treated with growth medium, EGF or PMA in the presence of an Erk 1/2 or PI3K inhibitor. Cells were serum-starved for 16 h then pretreated for 1 h with UO126 (20 μM) or wortmannin (100 nM) and added with GM, EGF or PMA (10 ng/ml) always in presence of the inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set to 1. Each point represents the mean ± standard error of the mean of the four replicate experiments. #P<0,001 versus GM. §P<0,001 versus EGF. **P<0,001 versus PMA. **B:** Time-course western-blot analysis of phospho-Erk 1/2 (with densitometric quantification relative to Erk levels) and phospho-Akt in GH2 cells treated with GM, EGF or PMA (10 ng/ml) in the absence or presence of Wortmannin (100 nM). Total Erk1/2 and total Akt are internal controls for phospho-Erk 1/2 and phospho-Akt, respectively.

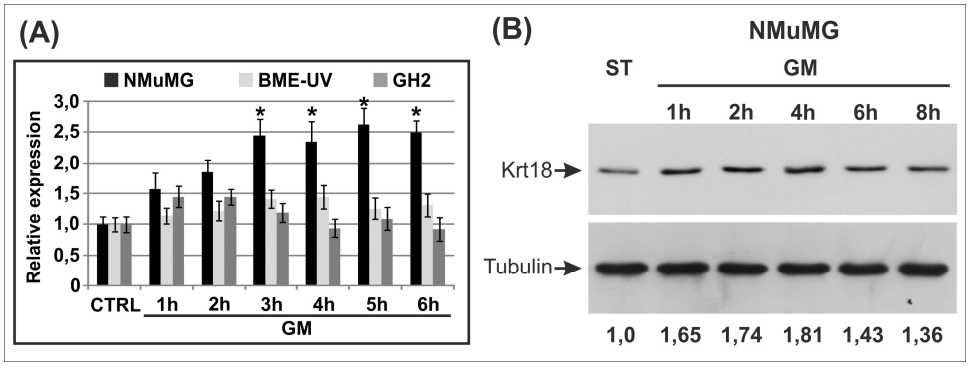
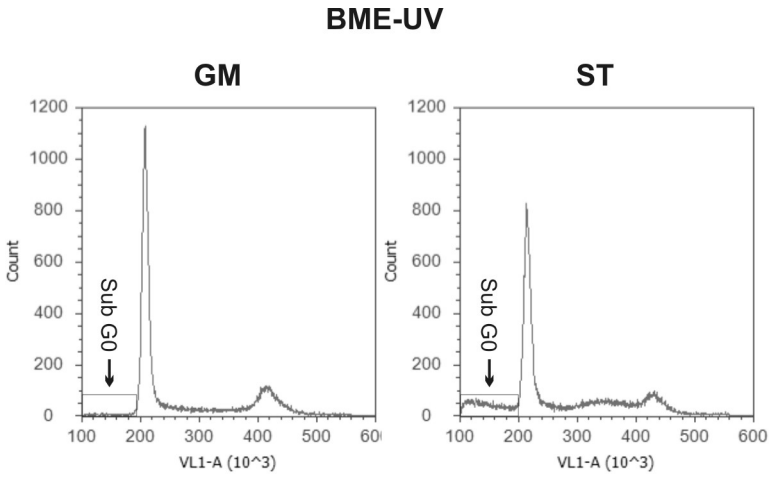
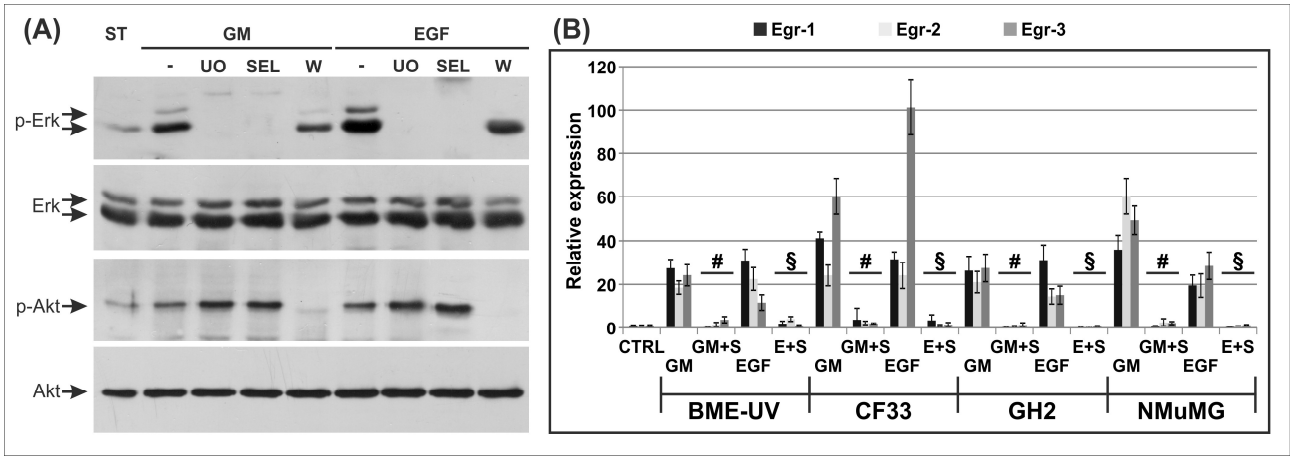


Fig. 6. Cytokeratin 18 mRNA and protein levels following treatment with GM. **A:** Cells were serum-starved for 16 h then treated with their respective GM. RNA was extracted at the indicated time points and cytokeratin 18 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH. **P*<0,05 versus CTRL. **B:** Serum-starved NMuMG cells (16 h) were treated with GM and protein were extracted at the indicated time points and analyzed by western-blot. Tubulin was used as an internal control for the densitometric analysis.



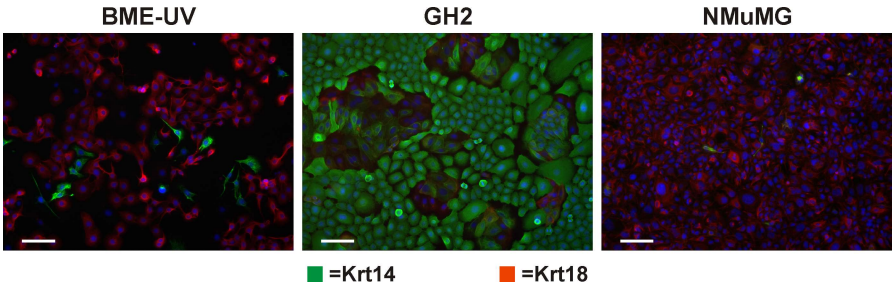
Supplementary Fig. 1.

Cytofluorimetric plot of BME-UV cells cultivated in growth medium or under serum-starving conditions for 16 h. Following serum-starvation for 16 h BME-UV cells show a decrease in the G0/G1 population and the appearance of a sub-G0 population that probably indicates cells undergoing apoptosis.



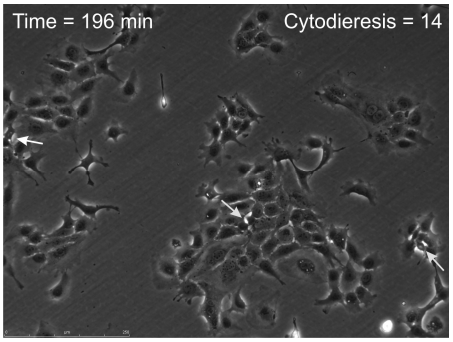
Supplementary Fig. 2.

A: Western-blot analysis of phospho-Erk 1/2 and phospho-Akt levels in GH2 cells treated for 10 min with GM or EGF and UO126 (20 µM), Selumetinib (1 µM) or Wortmannin (100 nM) showing the efficacy of the inhibitors. Total Erk 1/2 and total Akt are internal controls for phospho-Erk 1/2 and phospho-Akt, respectively. **B:** Egr-1, 2 and 3 mRNA levels in cells treated with growth medium or EGF in the presence of the Erk1/2 inhibitor Selumetinib. Cells were serum-starved for 16 h then pretreated for 1 h with Selumetinib (1 µM) and added with GM or EGF (10 ng/ml) always in presence of the inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set to 1. Each point represents the mean ± standard error of the mean of the four replicate experiments. #P<0,001 versus GM. §P<0,001 versus EGF.



Supplementary Fig. 3.

Immunohistochemistry analysis of non tumorigenic mammary epithelial cells for cytokeratin markers. BME-UV (bovine) and NMuMG (murine) cells are mainly Krt18 positive while GH2 (feline) cells are largely Krt14 positive with a small percentage of Krt18 positive cells that tend to group in islands. Bars = 100 µm.



Supplementary Video 1.

Example of the technique used for cytodieresis quantitative analysis. Time lapse video (NMuMG cell line) was acquired, with every frame corresponding to a 4 min interval, then manually analyzed to identify the cytodieresis events (yellow arrow). All the events occurring every h were divided by the number of cells present in the frame at that same time interval to obtain cytodieresis events per h %.

Supplementary Table 1.

Gene	Specie	Accesion number	Primer Forward	Primer Reverse	Amplicon size	Melting temp. °C	Efficiency
Egr-1	Bos t.	NM_001045875.1	AGCCCTACGAGCACCTGAC	GGTTTGGCTGGGGTAACTC	92	82,5	102,8
	Canis f.	XM_846145.3	ACCTGACCGCAGAGTCTTTT	GTGGTTTGGCTGGGGTAACT	82	81,5	105,6
	Felis c.	XM_003980809.3	same as Canis f.	same as Canis f.	82	80,5	100,4
	Mus m.	NM_007913.5	AACCCTATGAGCACCTGAC	CGTTTGGCTGGGATAACTC	92	82,0	96,1
Egr-2	Bos t.	XM_003585156.4	TTTTCCCCAATGCCGAACTG	GCCTGCACTCACAATGTTGA	245	86,5	103,1
	Canis f.	XM_536361.5	TCTTCCCCAATGCCGAACTG	TGTTGATCATGCCATCTCCG	75	83,5	104,9
	Felis c.	XM_003993969.3	same as Canis f.	same as Canis f.	72	83,0	103,3
	Mus m.	NM_010118.3	CTCGTCGGTGACCATCTTCC	TTGATCATGCCATCTCCCGC	84	84,0	104,5
Egr-3	Bos t.	NM_001289818.1	CGCGCTCAACCTCTTTTCTG	TCCATCACATTCTCTGTAGCCA	75	81,5	104,3
	Canis f.	XM_543247.5	TGGCTACAGAGAATGTGATGGAC	CCCGAATAAGAGAGTTCCGGG	70	81,5	104,3
	Felis c.	XM_003984699.3	same as Canis f.	same as Canis f.	70	81,5	100,9
	Mus m.	NM_018781.3	ACAATCTGTACCCCGAGGAGA	TCACATTCTCTGTAGCCATCTGA	99	85,5	101,2
Egr-4	Bos t.	NM_001040497.1	CACCTTAGCGAGTTTTCCGG	GAGGCAGGAGTCGGTTAAGT	195	89,5	N/D
	Canis f.	XM_540228.5	ATCCAAGCAGTACCCGAACA	ATCCGGGGAGTAAAGGTCCG	183	90	N/D
	Felis c.	XM_006930186.2	same as Canis f.	CCAGATCCGGGGAGTAAAGG	187	90	N/D
	Mus m.	NM_020596.2	CTTCTTCATCCAGGCGGTTC	CTTCTTCATCCAGGCGGTTC	192	84,0	N/D
Krt-14	Bos t.	NM_001166575.1	TTGCAAAACCTGGAGATCGA	CACGCTGCTGATCAGCTCCT	129	N/D	N/D
	Canis f.	NM_001253741.1	GTGCAGAACCTGGAGATCGA	CACGTTGCCAATCAGGTCCT	129	N/D	N/D
	Felis c.	XM_003996860.3	same as Canis f.	CACGTTGCCGATCAGGTCCT	129	87	102,3
	Mus m.	NM_016958.2	ATGCAGAACCTGGAGATCGA	CACACTGCCGATCATCTCCT	129	N/D	N/D
Krt-18	Bos t.	NM_001192095.1	AAGGGTCTACAAAACCAGATTG	CTGCGAGGTGACCACTGTGG	192	87	104,8
	Canis f.	XM_534794.5	AAGGGTCTACAAAACCAAATCG	CTGTGTGGTGACCACTGTGG	192	86	104,4
	Felis c.	XM_003988746.2	AAGGGTCTACAAAACCAAATTG	CTGGGAGGTGATCACTGTGG	192	86,5	100,7
	Mus m.	NM_010664.2	AAGGTCTGGAAGCCCAGATT	CTTGGTGGTGACAACTGTGG	191	86,5	99,8
IGF-IR	Bos t.	NM_001244612.1	AGCTGATGTGTACGTTCCTGAC	CTCATCCTTGACCACGCCC	127	88,5	96,4
	Canis f.	XM_545828.5	GGACGCAGTATGCGGTTTAT	AGAGAGGGTGGGTTCCACTT	187	85	95,4
	Felis c.	XM_003986871.2	GACTAAAGCCTTGGACGCAGT	TGGAAGGAACTGAAGCATTGG	129	84	103,4
	Mus m.	NM_010513.2	CTTTCCGAGGATCGTCGCCT	CGGGCCCACAGATTTCTCC	247	88	108
Insulin R	Bos t.	XM_015464089.1	ACGCTAGGCCTTCGAGGAAA	CACGGCTGCTGTCACATTC	62	84	N/D
	Canis f.	XM_005633222.1	GATCTAGCAGCCCGAAACTG	AGTGGTAAAGACCCCGTCCT	171	85,5	104,8
	Felis c.	XM_011288834.1	TCGGTTTCGAATTCCTCCTCT	GGAAGGCAGCTTCAACCCTTT	159	85,5	N/D
	Mus m.	NM_010568.3	TCAGCCAGTCTTCGAGAACG	AAGAAGGCGGACCACATGAT	87	83	105,6
EGFR	Bos t.	XM_002696890.4	CTTCGGCTGCCTGCTGGACTA	CCAGGTAATTCATGCCCTTTGC	104	86,5	104,3

	Canis f.	XM_014120756.1	GGACTATGTCCGCGAGCACAA	CCAGGTAGTTCATGCCCTTTGC	89	84,5	96,7
	Felis c.	XM_006929086.2	same as Canis f.	CCAGGTAGTTCATGCCCTTCGC	89	83,5	92,4
	Mus m.	NM_207655.2	GGACTACGTCCGAGAACACAA	CCAGGTAGTTCATGCCCTTTGC	89	82,5	98,3
Erb-B2	Bos t.	NM_001303629.1	CTGAACTGGTGTGTGCAGATTGC	CATGGTTGGGACTCTTGACCA	177	87	N/D
	Canis f.	NM_001003217.2	CTGAACTGGTGTGTGCAGATTG	CATGGTTGGGACTCTTGACCA	109	86	103,4
	Felis c.	NM_001048163.1	same as Bos t.	CGTGGTTGGGACTCTTGACCA	109	86	N/D
	Mus m.	NM_001003817.1	CTCAACTGGTGTGTTCAGATTGC	CGTGGTTGGGACTCTTGACTA	109	84,5	95,7
ERalfa	Bos t.	NM_001001443.1	AGGGAAGCTCCTATTTGCTCC	CGGTGGATGTGGTCCTTCTCT	234	N/D	N/D
	Canis f.	NM_001286958.1	AGGGAAGCTCTTCTTTGCTCC	CGGTGGATATGGTCCTTCTCT	234	N/D	N/D
	Felis c.	NM_001024231.1	same as Bos t.	CGATGGATATGGTCCTTCTCT	234	N/D	N/D
	Mus m.	NM_007956.5	GGGGAAGCTCCTGTTTGCTCC	same as Bos t.	234	N/D	N/D
ERbeta	Bos t.	NM_174051.3	GCTTCGTGGAGCTCAGCCTG	AGGATCATGGCCTTGACACAGA	262	87,5	N/D
	Canis f.	XM_005623489.2	GCTTTGTGGAGCTCAGCCTC	AGGACCATGGCCTTGACACAGA	262	N/D	N/D
	Felis c.	XM_006932907.2	GCTTTGTGGAGCTCAGCCTG	same as Canis f.	262	N/D	N/D
	Mus m.	NM_207707.1	same as Felis c.	AGAATCATGGCCTTCACACACA	316	85	N/D
PR	Bos t.	NM_001205356.1	GAGAGCTCATCAAGGCAATTGG	CACCATCCCTGCCAATATCTT	227	N/D	N/D
	Canis f.	NM_001003074.1	GAGAGCTCATCAAAGCAATTGG	CACCATCCCTGCCAAGATCTT	227	N/D	N/D
	Felis c.	XM_011286504.1	same as Bos t.	same as Canis f.	227	82,5	N/D
	Mus m.	NM_008829.2	GCGAATTGATCAAGGCAATTGG	CACCATGCCCGCCAGGATCTT	227	N/D	N/D
HPRT-1	Bos t.	NM_001034035.2	CACTGGGAAGACAATGCAGA	GGTCCTTTTCATCAGCAAGCT	92	81,5	99,4
	Canis f.	NM_001003357.2	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTTATAGCCAACA	123	82,5	99,9
	Felis c.	XM_006944016.2	TGACACTGGCAAAACAATGCA	GGTCCTCTTCACCAGCAAGCT	94	82,5	97,7
	Mus m.	NM_013556.2	TGACACTGGtAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	94	8,5	101,3
GAPDH	Bos t.	NM_001034034.2	ACTTATGACCACTGTCCACGC	GATATTCTGGGCAGCCCCTC	100	87,5	100,8
	Canis f.	NM_001003142.2	TGAACGGATTTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	90	84,0	103,2
	Felis c.	NM_001009307.1	same as Canis f.	same as Canis f.	93	84,0	101,6
	Mus m.	NM_001289726.1	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87	84,0	95,5

Note:N/D = not determined due to too low abundance of target gene.