Molecular and Cellular Endocrinology 419 (2016) 259–267

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Role of 3′-5′-cyclic adenosine monophosphate on the epidermal growth factor dependent survival in mammary epithelial cells

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ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form
28 September 2015
Accepted 26 October 2015
Available online 30 October 2015

Keywords:
Mammary epithelial cells
bcl-X
Apoptosis
EGF
cAMP
CREB

ABSTRACT

Epidermal growth factor (EGF) has been suggested to play a key role in the maintenance of epithelial cell survival during lactation. Previously, we demonstrated that EGF dependent activation of PI3K pathway prevents apoptosis in confluent murine HC11 cells cultured under low nutrient conditions. The EGF protective effect is associated with increased levels of the antiapoptotic protein Bcl-XL. Here, we identify the EGF-dependent mechanism involved in cell survival that converges in the regulation of *bcl-X* expression by activated CREB. EGF induces Bcl-XL expression through activation of a unique *bcl-X* promoter, the P1; being not only the PI3K/AKT signaling pathway but also the increase in cAMP levels and the concomitant PKA/CREB activation necessary for both bcl-XL upregulation and apoptosis avoidance. Results presented in this work suggest the existence of a novel connection between the EGF receptor and the adenylate cyclase that would have an impact in preventing apoptosis under low nutrient conditions.

1. Introduction

In vivo proliferation, differentiation and apoptosis of mammary epithelial cells are carefully regulated by systemic and local factors acting in highly controlled, multi-step processes that take place during all stages of mammary gland development. In particular, the ErbB receptor/ligand system represents one of the major signaling networks that control these processes in a tightly regulated way.

All the ErbB receptors and numerous epidermal growth factor (EGF)-related peptide growth factors are co-expressed together in dynamic patterns along murine mammary gland development. Particularly, the ErbB1 specific ligands transforming growth factor (TGF)-α and amphiregulin (AR); the dual ErbB1 and ErbB4 ligands betacellulin (BTC), heparin-binding EGF (HB-EGF), epiregulin (EPR) and neuregulin 1 (NRG-1) that binds both ErbB3 and ErbB4 are broadly expressed in mature virgin and mid-pregnant glands and

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPAC, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; Grb2, growth factor receptor-bound protein 2; GSK3β, glycogen synthase kinase 3 beta; IBMX, 3-isobutyl-1-methylxanthine; IkB, inhibitor of nuclear factor kB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Mcl-1, induced myeloid leukemia cell differentiation protein Mcl-1; MEK, MAP kinase kinase; mTOR, mammalian target of rapamycin; NSCLC, non−small cell lung cancer; PI3K, phosphoinositide 3-kinase; Shc, Src homology and collagen homology; RTK, receptor tyrosine kinase.

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http://dx.doi.org/10.1016/j.mce.2015.10.026
0303-7207/

drop during late pregnancy and lactation (Schroeder and Lee, 1998). In contrast, EGF, whose expression levels are low in the virgin and early pregnant glands, is the sole factor whose expression dramatically increases towards the end of pregnancy and peak during lactation. Even more, high levels of EGF molecules were observed in the mouse milk (Beardmore and Richards, 1983; Grueters et al., 1985). EGF binds to and activates the ErbB1 receptor (EGFR) and this may lead to further stimulation of other members of the ErbB receptor family like ErbB2 (Beerli et al., 1994) and ErbB3 (Kim et al., 1994). Although the role of EGFR signaling during alveolar morphogenesis and lactation is controversial (Troyer and Lee, 2001), it has been shown that EGF acts as survival factor of mammary epithelial cells (Rosfjord and Dickson, 1999). Together, these observations suggest a key role for this growth factor in the inhibition of the mammary epithelium cell death and/or in the maintenance of epithelial cell survival during lactation.

Activated EGFR undergoes autophosphorylation of tyrosine residues in their cytoplasmic tail. As a consequence, phosphotyrosine binding proteins are recruited, activating multiple signal transduction pathways that include the MAPK signaling cascade (mediated by the adapters Grb2 and Shc) and the phosphoinositide-3-kinase (PI3K) pathway. The latter acting through the recruitment of the kinase AKT to the plasma membrane followed by the phosphorylation of diverse substrates, such as the nuclear transcription factor κB inhibitor (IκB), the pro-apoptotic protein Bad, mTOR, and GSK3β among others (Lill and Sever, 2012). The consequence of triggering ErbB receptor signaling pathways is to promote cell survival and/or the inhibition of apoptosis in mammary epithelial cells (Danielsen and Maihle, 2002), generally via the up- and down-regulation of Bcl-2 family member proteins, including Bcl-XL/-2/Mcl-1 and Bax, respectively, which mediate the protective effect of EGF (Nass et al., 1996; Milani et al., 2007; Galbaugh et al., 2006; Strange et al., 2001; Shiozaki et al., 2009; Schulze-Bergkamen et al., 2008).

We have previously demonstrated that EGF prevents apoptosis in the confluent non-tumoral murine HC11 cell line cultured under low nutrient conditions (Romorini et al., 2009). This cell line is a broadly used model to investigate the molecular mechanisms of hormones and cytokines, as well as growth and transcription factors implicated in differentiation and cell survival (Ball et al., 1988; Doppler et al., 1989). Importantly, we found that the EGF dependent activation of PI3K signaling pathway is associated with an increase in bcl-XL mRNA levels, the most abundant member of the Bcl-2 family in mammary epithelial cells. On the other hand, c-Jun N-terminal kinase (JNK) activity participates in the control of apoptosis by increasing the levels of the Bcl-XL protein (Romorini et al., 2009; Schorr and Furth, 2000; Heermeier et al., 1996). The effects of EGF stimulation on the promotion of cell survival disappeared in *bcl-X* depleted cells using siRNA (Romorini et al., 2009), suggesting that the EGF dependent cell response requires Bcl-XL. *In vivo*, the mammary epithelial specific knockout of *bcl-X* was shown to amplify cell death and accelerate tissue remodeling during the first stage of mammary gland involution without compromising lactation performance (Walton et al., 2001). Taken together, these results highlight Bcl-XL as a key factor for maintaining cell survival *in vitro* as well as during lactation in the mouse.

The bcl-XL isoform is generated by alternative splicing of a single gene (bcl2l1; Ch2qH1). Mouse *bcl-X* promoter region has a complex structure containing at least five different alternative transcriptional start sites in its promoter (P1−P5), which are regulated in a tissue-specific and cell-context dependent manner (Pecci et al., 2001). As a result, a variety of transcripts are generated, each differing in the 5′-leading exon dependent upon alternative promoter usage. External signals, such as growth factors, directly influence the patterning of splice variants through different signaling pathways to regulate promoter selection.

In the present study, we examine the EGF-dependent mechanisms regulating cell survival and *bcl-X* expression regulation in serum depleted confluent mammary epithelial HC11 cells. We found that the addition of EGF induces bcl-XL expression throughout the activation of the *bcl-X* promoter 1 (P1) mediated in part by the PI3K/AKT signaling pathway. To our surprise, the increase in the cellular cAMP levels with concomitant PKA/CREB activation, is also responsible for the EGF mediated P1 activity. Moreover, both signals are necessary for the promotion of cell survival driven by the growth factor. These data provide evidence for the existence of a novel connection between the EGF receptor and the adenylate cyclase activating machinery that would have an impact in preventing cell death under low nutrient conditions.

2. Materials and methods

2.1. Reagents

Recombinant Human EGF (Cat. No. PHG0311), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Cat. No. M6494), RPMI 1640 and RPMI 1640 free of phenol red culture media were purchased from Invitrogen (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) was acquired from Internegocios S.A. (Mercedes, BA, ARG). Wortmannin, LY294002, H89 and Forskolin were purchased from Calbiochem (San Diego, CA, USA) and A6730 (AKT inhibitor VIII), 3-isobutyl-1-methylxanthine (IBMX), and AG1478 from Sigma Aldrich (St. Louis, MO, USA). For *in vitro* assays, 1000X stock solutions of kinase inhibitors were prepared in dimethyl sulfoxide.

2.2. Cell cultures and transfections

HC11 cells, derived from BALB/c mouse normal mammary glands (gently provided by Dr. Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland) were grown in RPMI 1640 growth medium (GM) supplemented with 10% FBS containing insulin (5 μg/ml) (Sigma Aldrich, St. Louis, MO, USA), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA). Serum-free medium (SFM) was prepared with RPMI 1640 medium supplemented only with penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For transient transfections, 5×10^5 cells were seeded in 6 well plates and 3 μg of a Promoter 1 fragment containing luciferase reporter vector (pP1-Luc) was co-transfected with 3 μg of pAKT-Myr or pCREB and/or pDN-CREB, when correspond, by using the Lipofectamine 2000 (Gibco, Inc.) method according to manufacturer's instructions. pCMV-LacZ (1 μg) was used as transfection efficiency marker and an pCMV empty vector was transfected to balance DNA amounts. Briefly, plasmids were diluted in 100 μl medium and added drop wise to an equal volume of medium containing 2 μl of Lipofectamine 2000. After 20 min, the transfection mixture was added drop wise to the cells and incubated overnight at 37 °C in 5% CO_2 atmosphere. Then, the serum-free medium was replaced by regular medium supplemented with 10% charcoal-dextran delipidated FCS as previously described (Lippman et al., 1976). Cells were incubated under the corresponding conditions during 48 h. Luciferase activity was measured with the Luciferase Assay System according to the manufacturer's protocol (Promega Inc. cat #E1501). β-galactosidase activity was measured as previously described (Truss et al., 1995). For stable transfections, P1*bclX*-luciferase stable HC11 cells were obtained by transfecting HC11 cells with 10 μg of purified P1*bclX* vector (Viegas et al., 2004). Stable clones were selected with 2.5 μg/ml of puromycin (Invitrogen) and analyzed by determining the luciferase

expression in response to EGF.

2.3. Mutagenesis

In order to eliminate the CRE-like consensus sequence (TGACTTCA) found within P1bcl-X promoter region, pP1-Luc construct was used to generated two different CREB binding site mutants: Mut1: TGgtTTCA (Castro-Caldas et al., 2003) and Mut2: a 4 bp deletion (TG—CA) (Travnickova-Bendova et al., 2002). In both cases, the mutations were generated by using the QuikChange II XL Site Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Primers are available upon request.

2.4. Cell treatments by serum withdrawal

Cells were trypsinized and plated in 6 well plates in the conditions described above. When cultures reached confluence, the medium was removed; the cells were washed twice with PBS and maintained in SFM during 1–2 h for starvation in the presence or absence of the different kinase inhibitors; LY294002 (10 μM); Wortmannin (100 nM); H89 (20 μM); A6730/AktVIII (1 μM and 10 μM) and AG1478 (100 nM). After this period, SFM containing EGF (100 ng/ml) either with or without the kinase inhibitors was added. The cells were then incubated for the time course described on each experiment.

2.5. RNA isolation and RT-real time PCR

RNA was isolated using TRIzol reagent (Invitrogen), purified and treated with DNase I (Invitrogen, cat #18068-015), according to the manufacturer's instructions. cDNA was synthesized using 1 μg of RNA with a MMLV enzyme (Promega), and real-time PCR was performed in a DNA Engine Opticon instrument (MJ Research Inc., Waltham, MA, USA). All reactions were conducted in a volume of 25 μl containing 4 mM $MgCl_2$ (Invitrogen), 0.25 mM dNTPs (Invitrogen), 1.25 U of Taq polymerase (Invitrogen), 1/30000 Sybr Green (Roche, Indianapolis, IN, USA) and specific oligonucleotides (Supplementary Table 1). The amplification of unique products in each reaction was verified by melting curve and agarose gel electrophoresis. Each sample was run in triplicate. The expression level was normalized to *actin* or *hspcb* expression levels using standard curve method. Means and standard errors from at least 3 independent samples were calculated and shown as fold changes respect to vehicle group.

2.6. Protein analysis

Total proteins were extracted from HC11 cells in RIPA protein extraction buffer (50 mM Tris—HCl, 150 mM NaCl, 1% triton, 0.25% sodium deoxycholate, 1 mM EDTA pH 7.4) supplemented with protease (Protease inhibitor cocktail set I, Calbiochem) and phosphatase inhibitors (25 mM NaF, 1 mM Na_2VO_4). The lysates were centrifuged at 14,300 g and 4 °C for 10 min, and the pellets discarded. Cleared lysates were combined with SDS sample buffer (50 mM Tris pH 6.8, 1% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). For western blot samples were boiled for 5 min and electrophoresed for 3 h at 100 V in 15% or a 12% SDS-polyacrylamide gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine, 0.025 M Tris-base (pH 8.3) at 300 mA for 1.5 h at 0 °C. Blots were blocked 1 h at room temperature in TBS (20 mM Tris-Cl, pH 7.5, 500 mM NaCl) containing low-fat powered milk (5%) and Tween 20 (0.1%). The incubations with primary antibodies were performed overnight at 4 °C in blocking buffer (3% skim milk, 0.1% Tween, in Tris-buffered saline). The membranes were then incubated with the corresponding counter-antibody and the proteins evidenced by ECL Plus System enhanced chemiluminescence detection (GE Health Care, Little Chalfont, Buckinghamshire, UK). α-AKT (sc-1618); α-CREB (sc-186) and α-actin (sc-1616-R) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-P-CREB (#9198) and α-P-AKT (Cell Signaling Technology, Beverly, MA, USA) primary antibodies were used. As secondary antibodies, a peroxidase-labeled α-rabbit IgG and α-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

2.7. cAMP measurements

Confluent and 1 h starved HC11 cells incubated with different kinase inhibitors were pretreated for 3 min in SFM in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM) as described elsewhere (Copsel et al., 2011) and then stimulated for different times (0, 2, 5, 10, 15, 30 and 60 min) with EGF (100 ng/ml) or 10 min with Forskolin (20 μM). Incubations were stopped by rapid homogenization in ice-cold absolute ethanol and then centrifuged for 15 min at 1200 g. The supernatant was dried, and the residue was resuspended to determine the cAMP content by competitive protein binding assay (Gilman, 1970), modified according to Davio et al. (Davio et al., 1995). Results are expressed as fold change versus control.

2.8. Cell viability assay

Cell viability was determined by a 3,4,5-dimethylthiazol-2,5-biphenyl tetrazolium bromide assay (Mosmann, 1983). Briefly, HC11 cells where grown to confluence on 96-well plates and then starved with SFM free of phenol red in the presence or absence of the different kinase inhibitors. Afterwards, cells were treated with EGF (100 ng/ml) and incubated for 72 h at 37 °C in 5% CO_2. For labeling, the medium was removed and replaced with 100 μl of fresh culture medium to which 10 μl of MTT (12 mM, 5 mg/ml in PBS) stock solution were added. Finally, 75 μl of medium was removed and 50 μl of dimethyl sulfoxide were added in order to dissolve the formazan crystals. The absorbance was monitored using a spectrophotometer with a microplate reader at a wavelength of 540 nm (Bio-Rad, Hercules, CA) and a 650 nm reference wavelength. Results are expressed as fold change versus control.

2.9. Statistical analysis

Results were expressed as means ± standard error of at least 3 independent experiments. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc.) and consisted of T-test analysis (for *bcl-X* promoter studies) and one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons tests as indicates in the figure legends. Before statistical analysis, Q—Q plot and Shapiro Wilks test were performed for normality. Homocedasticity was assessed with Levene's test. Different letters or asterisk means significant differences ($p < 0.05$) between means or versus Control, respectively.

3. Results

3.1. EGF induces bcl-XL expression via activation of the P1 promoter

Previous work indicated that EGF prevents apoptosis through the induction of an antiapoptotic isoform Bcl-XL in confluent HC11 cells (Romorini et al., 2009). The contribution of each *bcl-X* promoter region (Fig. 1A; P1—P5) necessary for EGF induction was identified (Fig. 1B). Cells were treated with EGF in serum free media

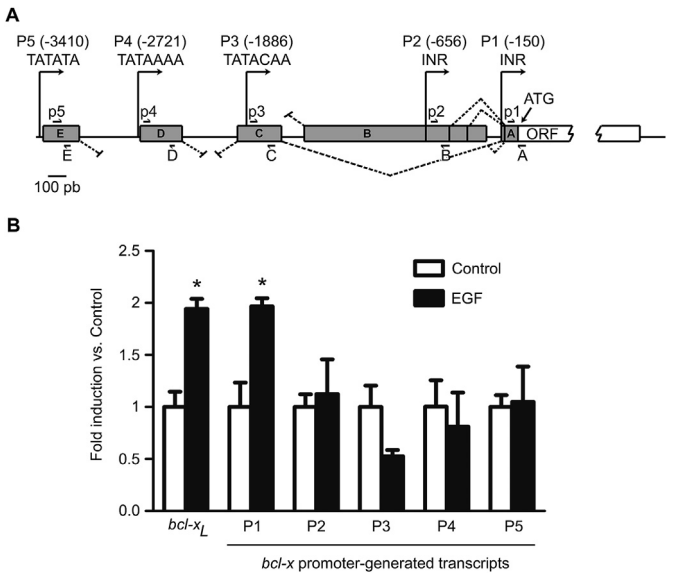

Fig. 1. EGF dependent increase of *bcl-x* P1 mRNA **(A)** The 5′-UTR region of the mouse *bcl-x* gene promoter is indicated in grey and shows the five alternative gene promoters. The numbers between brackets correspond to the upstream distance respect to the translation initiation site and letters A to E to different exons. The specific oligonucleotides designed for determining each promoter derived transcripts are shown. **(B)** HC11 cells were grown up to confluence and then treated with EGF for 5 h as indicated in Materials and Methods. qPCR using specific oligonucleotides for total bcl-XL, and for transcripts generated by each individual promoter (P1 to P5) was performed. Values were normalized to β-actin mRNA levels. Bars indicate mean ± S.E of three independent experiments. (*) denotes significant differences versus its respective control for each pair of samples ($p < 0.05$).

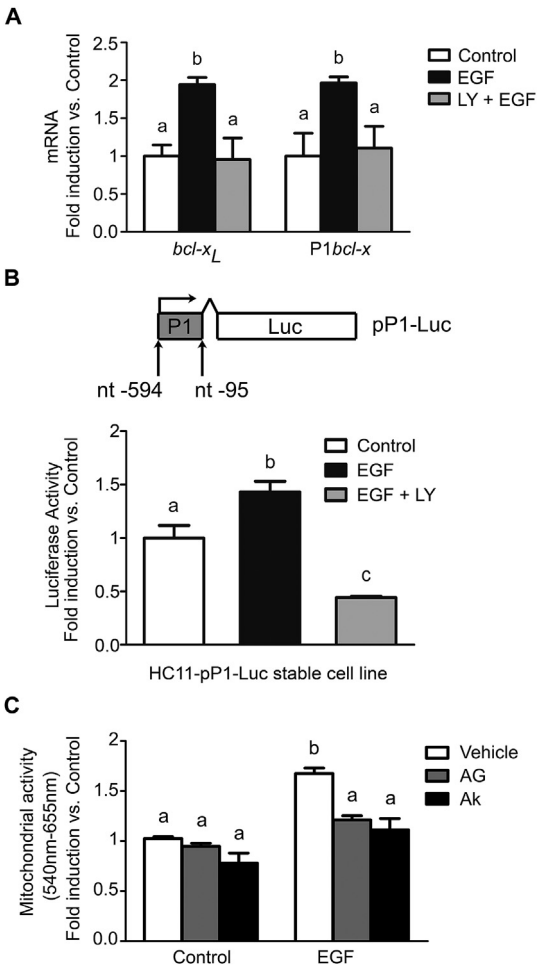

Fig. 2. PI3K/AKT signaling pathway mediates EGF survival effect trough the activation of P1-*bclX*. **(A)** Confluent HC11 cells pre-treated for 1 h with SFM in the presence of the PI3K inhibitor LY (10 μM) were stimulated with EGF (100 ng/ml) alone or EGF plus LY (10 μM) for 3 h. qPCR using specific oligonucleotides for total bcl-XL, *bcl-x* promoter 1 (P1*bcl-X*) derived transcripts and β-actin was performed. Values were normalized to β-actin and bars indicate mean of fold induction versus control ± S.E from three independent experiments. **(B)** Confluent and stably transfected HC11 cells with pP1-luc were incubated in SFM with EGF (100 ng/ml) with or without LY (10 μM) for 36 h. Luciferase activity was measured and normalized with total protein levels. Bars indicate mean of fold induction versus control ± S.E from three independent experiments. Values for each sample were significantly different to the other ones ($p < 0.05$). **(C)** Cells were seeded into 96-well plates. After reaching confluence, cells were starved and treated with the EGFR inhibitor AG1478 (AG) 100 nM or with AKTVIII (Ak) 10 μM for 72 h in the presence or absence of EGF (100 ng/ml). Then, MTT assays were performed as described in Materials and Methods. Experiments were repeated five times, data are presented as means ± SD (vertical bars). AG. AG1478; Ak: AktVIII.

(SFM) for 5 h, and the transcriptional patterning of the promoter regions was evaluated using real time quantitative PCR (qPCR) with primers designed to discriminate and amplify individual transcripts containing the P1–P5 *bcl-X* promoter regions following EGF activation (see scheme in Fig. 1A) (Viegas et al., 2004). Transcripts generated from all promoters were detected; however, bcl-X transcripts originating from promoter P1 were the most abundant in this cell type (data not shown). EGF treatment led to a slight but insignificant decrease in the expression levels of transcripts generated by promoter P3; no regulation was observed for transcripts generated by promoters P2, P4 and P5. However, the levels of those mRNA generated by promoter P1 significantly increased upon EGF treatment, which was consistent with the observed increase of bcl-XL mRNA levels measured by primers targeted to an internal region of the transcript in response to the growth factor (Fig. 1B).

Previously, we demonstrated that EGF increases bcl-XL expression levels through the activation of PI3K (Romorini et al., 2009). In an effort to extend those observations, we evaluated the role of the PI3K signal transduction cascade in the EGF dependent expression of *bcl-X* P1 promoter driven transcripts. HC11 cells were treated with EGF in the presence or absence of PI3K kinase inhibitor LY294002 (LY), and bcl-XL and P1 transcripts were measured by qPCR. Treatment of HC11 cells with LY blocked the increase in levels of P1-dependent *bcl-X* transcripts when cells were stimulated with EGF (Fig. 2A). Similarly to the P1 specific mRNA, the PI3K inhibitor completely impaired the EGF mediated induction of bcl-XL expression measured by qPCR using primers that anneal to the bcl-XL mRNA transcribed by all promoters (Fig. 2A and (Romorini et al., 2009)). To further establish the contribution of P1 promoter region involved in this effect, a luciferase reporter construct containing the P1 promoter region from −594 to −95 nt upstream the bcl-XL translation start site (Viegas et al., 2004) was created. The luciferase reporter construct corresponding to the P1 promoter of *bcl-X* was stably transfected in HC11 cells (Fig. 2C; HC11–P1luc). When confluent, the HC11–P1luc cells were treated with EGF in the presence or absence of LY (10 μM).

Stimulation of HC11–P1luc cells with EGF significantly increased luciferase activity, and P1 driven luciferase expression induced by the growth factor was abrogated in the presence of LY (Fig. 2B). In a different set of experiments, P1 driven luciferase activity was significantly increased 1.43 ± 0.08 fold ($p < 0.05$) when HC11 cells were co-transfected with an expression vector encoding a constitutive active AKT isoform (pAKTMyr) together with the P1*bcl-X* reporter construct (data not shown). Taken together, these observations suggest that the 500 bp fragment from the P1 region

contains response elements activated by downstream factors of the PI3K/AKT signaling pathway involved in transcription via the P1*bcl-X* promoter. Nevertheless, considering that the expression induction of the endogenous bcl-x P1 generated transcripts is higher compared to HC11−P1luc in response to EGF (compare Fig. 2A and B), we propose that other regions in addition to the 500 bp would contribute to modulate the expression induction of P1 driven transcripts in response to the growth factor.

Cells were then stimulated with EGF in the presence of AG1478 (100 nM), the EGF receptor inhibitor, or with AktVIII (10 μM), a specific AKT inhibitor that binds the pleckstrin homology (PH) domain of AKT1 and AKT2 (Bain et al., 2007) (Fig. 2C). Inhibition of the EGF receptor (AG1468) and AKT inhibition (AktVIII) suppressed the EGF mediated cell survival assessed by MTT (Fig. 2C), indicating that the inhibition of cell death and the bcl-XL induction via the P1 promoter region by EGF stimulation requires both PI3K and AKT kinase activities.

3.2. Role of CREB in the EGF mediated cell survival

In silico analysis of potential response elements present along the P1 promoter (Cartharius et al., 2005) revealed the presence of a full cAMP responsive element (CRE)-like site located between −265 and −257 bp upstream *bcl-X* translation start site. This CRE-like site (TGACTTCA) contains a single mismatch compared to the human sequence. Since CREB has been extensively reported to be induced by growth factors signaling cascades we tested if it is activated by EGF stimulation in this cellular model. A time course of EGF dependent CREB activation was evaluated by measuring the phosphorylation levels of CREB (Ser133) and AKT (Ser473). EGF administration increased the phosphorylation levels of both AKT (p-AKT) and CREB (p-CREB) with different kinetics, suggesting that AKT activation precedes CREB phosphorylation (Fig. 3A). The highest activation levels were detected after 5 min of EGF treatment for p-AKT, and between 10 and 15 min for p-CREB that was sustained for up to 30 min. HC11 cells also showed impaired CREB phosphorylation induction following stimulation with EGF when incubated with a transphosphorylation inhibitor of the ErbB1 receptor AG1478 (Fig. 3B). In order to validate that AKT activation due to EGF addition results in the phosphorylation of CREB, confluent HC11 cells were incubated in the presence of AKT inhibitor (AktVIII; 1 μM or 10 μM). The activation of AKT by EGF stimulation was completely suppressed by treatment of 10 μM AktVIII and markedly reduced at the 1 μM dose (Fig. 3C). Unexpectedly, the AKT inhibitor had no effect on Ser133 phosphorylation of CREB, suggesting that the activation of CREB driven by EGF is not directly mediated by AKT signal transduction.

The EGF phosphorylation of CREB was evaluated in cells treated with the PKA inhibitor H89 (20 μM). In the presence of H89, CREB phosphorylation of Ser133 was lower relative to that observed in cells treated with EGF alone (Fig. 3D), indicating that PKA is necessary in the activation of CREB triggered by EGF signal transduction. Consistent with these observations, HC11 viability was significantly diminished when cells were treated with EGF in the presence of the PKA inhibitor (Fig. 4A), suggesting a predominant role for the PKA pathway in EGF dependent cell survival. The induction of bcl-XL and P1 generated transcripts observed when confluent HC11 cells were treated with EGF was attenuated in the presence of the PKA inhibitor (Fig. 4B), connecting activation of CREB transcription via PKA signal transduction with HC11 cell survival.

To deepen our understanding of bcl-XL expression regulation, we assessed the ability of CREB to activate the *bcl-X* P1 promoter. HC11 cells were transiently co-transfected with expression vectors containing CREB1 and the P1*bcl-X* reporter construct (Fig. 5A). Transient overexpression of CREB1 significantly increased luciferase activity compared to control treatment. Consistently, co-transfection with a dominant negative CREB mutant expression vector (pDN-CREB) completely inhibited P1*bcl-X* luciferase activity. This result indicates that CREB1 transcription factor is able to induce the expression of bcl-X mRNAs driven by promoter P1. In alignment with these observations, treatment of confluent HC11 cells with Forskolin, a known adenylate cyclase activator that leads to the activation of cAMP/PKA/CREB signaling pathway, significantly increased P1 dependent bcl-XL transcripts by 1.4 ± 0.2 fold ($p < 0.05$) relative to the control (data not shown).

In order to evaluate CREB binding to the single CRE-like site present in the promoter P1, we used two P1-Luc mutants that differ from the wild type vector only in their CRE-like sequence. As shown in Fig. 5B, both mutants were unable to prevent the increased luciferase activity triggered by CREB1 overexpression indicating that CREB activates *bcl-x* P1 through a different site or by an indirect manner. Additionally, there is no induction of luciferase activity when CREB is coexpressed with a P2 to P5-Luc vector, highlighting the importance and specificity of the P1 response to CREB. Therefore, together this results support the idea that P1-bcl-x promoter is the unique promoter activated by CREB protein by a mechanism that does not involve the CRE-like specific site.

3.3. Activation of cAMP/PKA pathway by EGF

The preceding results suggested a key role for cAMP/PKA pathway in the EGF dependent HC11 cell survival. In order to further address this observation, cAMP levels were quantified by performing a time course radio binding protein assay in cell extracts from confluent HC11 cells treated with EGF (Fig. 5A). Surprisingly, cAMP levels were significantly increased after 2 min following EGF addition, and high levels were maintained over the course of the hour due to the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM). Although lower than the increase produced by the strong adenylate cyclase activator Forskolin, the EGF dependent increase of cAMP was significant (Fig. 6A, right panel). The increase in cAMP levels stimulated by EGF was blocked by the EGF receptor inhibitor AG1478 but not by the PI3K inhibitor wortmannin (Fig. 6B), despite suppression of phosphorylated AKT under both conditions (Fig. 6C). Cumulatively, these results suggest a direct link between EGF receptor stimulation and the activation of adenylate cyclase to control HC11 cell survival that is independent of PI3K/AKT pathway activation by EGF.

4. Discussion

During postnatal development of the mouse mammary gland, the epidermal growth factor ligands and the ErbB receptors have multiple and recurring functions being not only essential for proliferation, invasion and differentiation of mammary epithelial tissue but also implicated in tumorigenic progression (Hardy et al., 2010; Sebastian et al., 1998). Given that many of these ligands and receptors are highly expressed in breast cancer, it is crucial to identify potential activation mechanisms of the survival pathways involved in mammary tissue. Here, we report the CREB mediated induction of the bcl-XL pro-survival transcript induced specifically from the P1 promoter region, working independently of EGF mediated PI3K/AKT activation.

Several lines of evidence strongly suggest that EGF plays a key role as a survival factor of mammary epithelial cells (Merlo et al., 1995; Nass et al., 1996; Amundadottir et al., 1996). For example, addition of EGF prevents apoptosis of confluent HC11 cells cultured in serum-free medium (Merlo et al., 1996). We previously

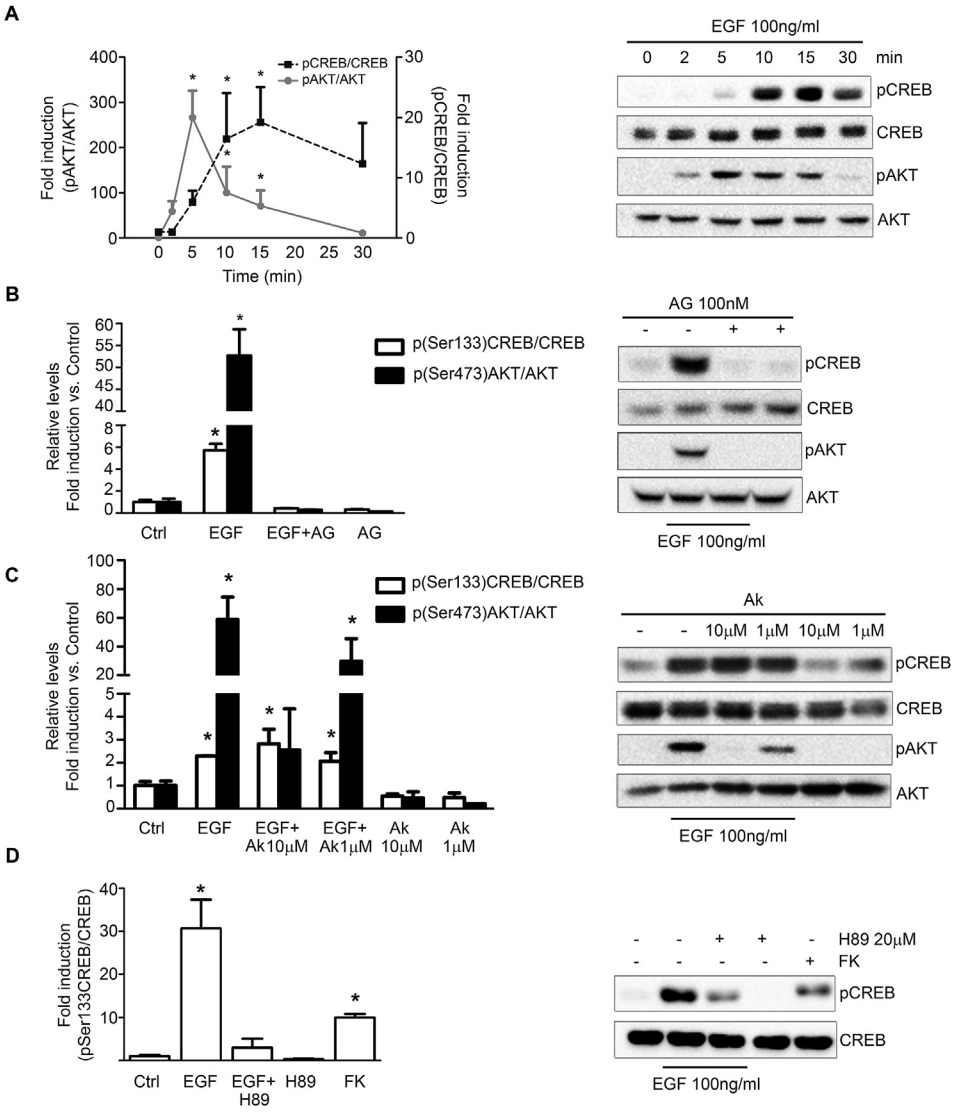

Fig. 3. EGF induces CREB activation independently from the PI3K/AKT pathway. Confluent HC11 cells were starved for 1 h and treated with EGF (100 ng/ml) over a time course series **(A)** or incubated for an hour with AG1478 (100 nM) **(B)**, AktVIII (1, 10 μM) **(C)** or H89 (20 μM) **(D)** and then treated with EGF (100 ng/ml) for 15 min with the corresponding inhibitor **(B–C)**. Forskolin (10 μM) was used as a positive control **(D)**. Then, harvested cells lysates were subjected to Western blotting and probed for p(Ser473)-AKT and AKT **(A–C)** and/or p(Ser-133)-CREB, CREB **(A–D). (A–D)**: In the right panels representative gels are shown of three independent experiments. In the left panels, the relative protein value of phosphoproteins is presented as the ratio between the phosphoprotein signal and total protein for either CREB or AKT. Quantification of three independent experiments as mean ± S.E is shown. Densitometric analysis of protein levels was performed with ImageJ 1.46r software (Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/). (*) denotes significant differences versus control (no treatment) ($p < 0.05$). AG. AG1478; Ak: AktVIII; FK, Forskolin.

demonstrated that, although EGF leads to the activation of PI3K/AKT and MAPK, cell viability and the mRNA induction of the anti-apoptotic protein Bcl-XL primarily relies on activation of PI3K signaling (Romorini et al., 2009). Herein, we report an additional pathway triggered by EGF, independent of PI3K/AKT pathway activity and involving cAMP/PKA/CREB. These two mechanisms converge in the activation of only one *bcl-X* promoter in response to EGF treatment (P1) spanning from −594 to −95 nts upstream the transcriptional start site. Loss of activation of either PI3K/AKT or cAMP/PKA/CREB signal transduction pathways results in a complete inhibition of pro-survival bcl-XL induction mediated by EGF, favoring intrinsic cell death pathways.

We have focused on Bcl-XL since it is the most abundant member of the Bcl-2 family in mammary epithelial cells, plays a key role in the inhibition of cell death in mammary gland and its overexpression in several breast tumors is associated with increased resistance to chemotherapy (Olopade et al., 1997; Liu et al., 1999). Conversely, downregulation of Bcl-XL leads to apoptosis and ameliorates the pro-survival influences of EGF (Romorini et al., 2009). In this sense, identifying the relationship between EGF mediated avoidance of apoptosis and the induction of the anti-apoptotic Bcl-XL protein could have profound implications for the treatment of solid tumors including cancers of the breast.

PI3K/AKT involvement in the up-regulation of Bcl-XL and suppression of apoptosis upon EGFR activation has been observed in several cell types including mammary epithelial cells (Shiozaki et al., 2009; Kim et al., 2015; Schulze-Bergkamen et al., 2008; Ramljak et al., 2003). However, the participation of CREB as an activator of bcl-XL expression in response to EGF in mammary epithelial cells is described here for the first time. In non−small cell lung cancer (NSCLC), CREB dependent up-regulation of Bcl-XL was reported (Aggarwal et al., 2008) wherein its induction was effectively suppressed either by knockdown of CREB expression or by overexpressing a dominant negative CREB, promoting apoptosis of

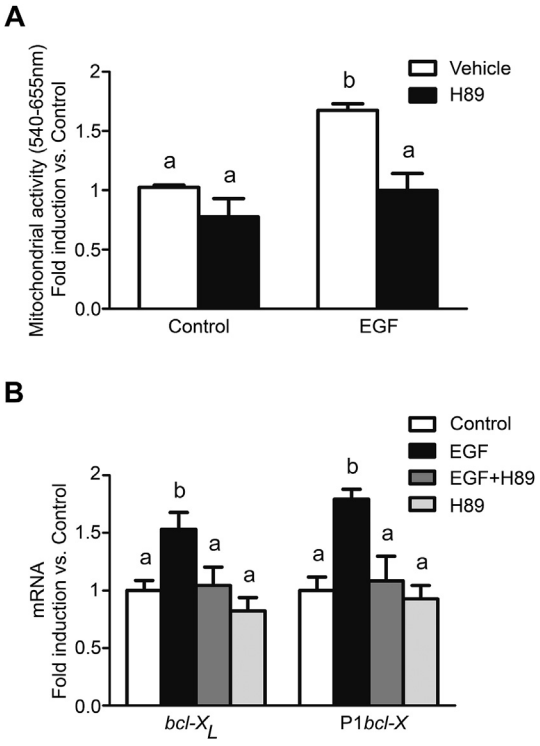

Fig. 4. PKA is necessary for the EGF mediated survival effect. **(A)** MTT assay was performed as indicated in Materials and Methods to examine the involvement of PKA in the EGF mediated survival effect in HC11 cells. Briefly, after reaching confluence, cells were starved and treated with the PKA inhibitor H89 (20 μM) for 72 h in the presence or absence of EGF (100 ng/ml). Data are presented as means of fold induction ± SD of three independent experiments. **(B)** Confluent cells pre-treated for 1 h with SFM in the presence or absence of H89 (20 μM) were stimulated with EGF (100 ng/ml) for 6 h. qPCR using specific oligonucleotides for bcl-XL, *bcl-x* promoter P1 (P1*bcl-X*) derived transcripts and β-actin was performed. Values were normalized to hspcb and bars indicate mean of fold induction versus control ± S.E from three independent experiments.

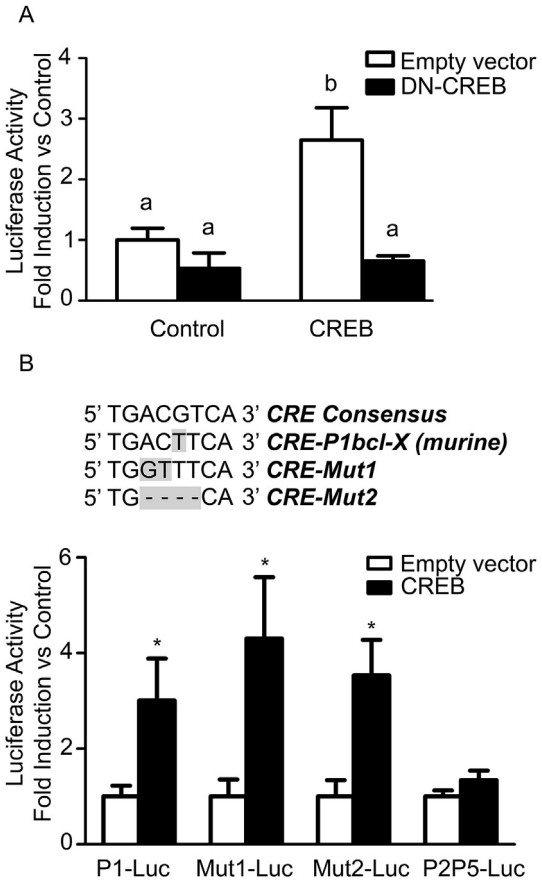

Fig. 5. CREB specifically activates promoter P1. **(A)** HC11 cells were transiently co-transfected with the reporter construct pP1-Luc and expression vectors that over-express CREB1 protein and/or a dominant negative version of CREB (pDN-CREB). **(B)** pP1-Luc, Mut1-Luc, Mut2-Luc or pP2P5-Luc reporter vectors were transiently co-transfected with CREB1 in HC11 cells. An empty vector was used as Control. pCMV-LacZ was added as a transfection control. Luciferase activity was measured 24 h post transfection and normalized to β-galactosidase activity. Bars indicate mean of fold induction versus Control (empty vector) ± S.E from three independent experiments. Bars with different letters in **(A)** are significantly different. (*) denotes significant differences versus control (no treatment) ($p < 0.05$).

these NSCLC. Coincidently, we observe that overexpression of the dominant-negative form of CREB mutant prevents *bcl-X* P1 activation.

PI3K/AKT-dependent activation of CREB in breast cancer cells was previously demonstrated (Phuong et al., 2014); however, our findings indicate that PI3K/AKT pathway is not necessary for Ser133-CREB phosphorylation as, using the AktVIII inhibitor in EGF stimulated HC11 cells, levels of p-CREB were unchanged. Instead, PKA is primarily responsible since the increase in p-CREB levels in cells treated with EGF was blocked by the addition of the EGFR-tyrosine kinase inhibitor AG1478 and the PKA inhibitor H89. EGF led to significantly increased cAMP levels, indicating that activation of CREB would be mostly coordinated through cAMP/PKA. Importantly, inhibition of PKA activity using H89 impaired EGF mediated induction of bcl-XL mRNA via the P1 promoter, reducing HC11 cell viability. It is relevant to note that many other regulatory stimuli have been reported to promote Ser133-CREB phosphorylation in addition to PKA (Yamamoto et al., 1988; Johannessen et al., 2004; Johannessen and Moens, 2007). Given that H89 did not completely attenuate Ser133 p-CREB levels, we cannot yet rule out the involvement of alternative kinases during EGF dependent CREB activation.

EGF activation of PKA was in fact described in murine metastasic fibrosarcoma cells where EGFR catalytic domain is necessary for adenylate cyclase dependent PKA induction (Fishman et al., 1997). An increasing body of evidence supports the idea that receptor tyrosine kinases (RTK) can utilize some G-coupled protein receptor signal transducer molecules (Chaturvedi et al., 2006). EGF augments cAMP accumulation in cardiomyocytes, stimulating adenylate cyclase via activation of Gαs (Nair et al., 1990). *In vitro* studies also demonstrated that the EGFR is able to activate the adenylate cyclase by phosphorylating a Gs protein (Poppleton et al., 1996). How the active EGFR modulates cAMP accumulation in mammary epithelial cells is a matter of further mechanistic studies. Nonetheless, we show that the increased cAMP levels were only affected by EGFR and not PI3K inhibition, suggesting a direct crosstalk between the activated RTK and adenylate cyclase to modulate the balance between cell survival and intrinsic cell death pathways.

cAMP can either stimulate or inhibit programmed cell death. In fact, several studies identified that the anti-apoptotic response to cAMP may involve the cAMP-regulated effector protein Epac (Insel et al., 2012). Here, we report that the accumulation of cAMP initiated by EGF stimulation was associated with the induction of bcl-XL expression and, consequently, the prevention of apoptosis triggered by the activation of the PKA/CREB signaling pathway. Supporting these results, a survival role for PKA/CREB pathway was also observed in several cell types including alveolar type II cells (Barlow et al., 2008), mouse photoreceptor cells (O'Driscoll et al., 2007), and in ischemic primary hippocampal neurons, among others (Yu et al., 2014).

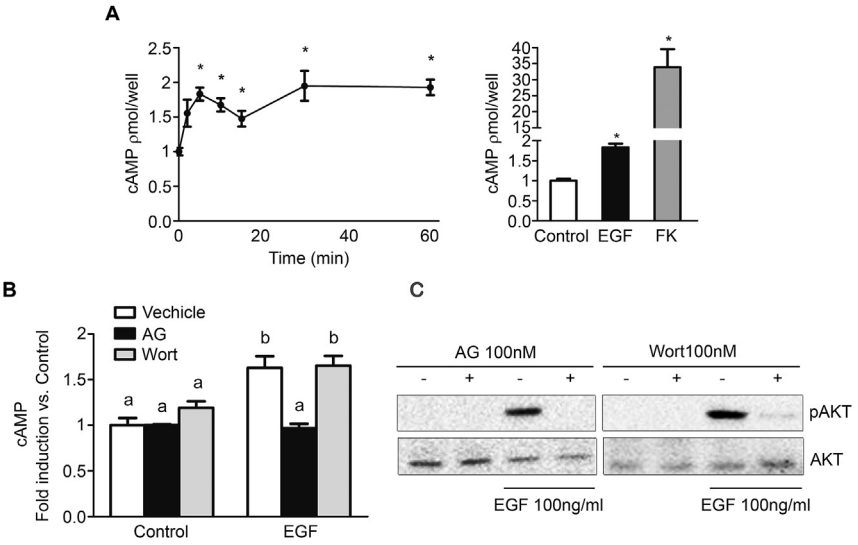

Fig. 6. EGF stimulation increases cAMP levels. **(A)** cAMP levels were measured in confluent and 1 h starved HC11 cells treated with EGF (100 ng/ml) in the presence of IBMX (1 mM) at increasing times (left) or at 10 min in cells treated with either EGF or Forskolin (FK, 10 μM) (Right). Results are expressed as mean ± S.E of two wells in three independent experiments. (*) denotes significant differences versus control (EGF 0 min) ($p < 0.05$). **(B–C)** Confluent cells were incubated in SFM with the EGFR inhibitor AG1478 (100 nM) or the PI3K inhibitor Wortmannin (100 nM) and then stimulated with EGF (100 ng/ml) for 10 min **(B)** cAMP content was measured and results are expressed as mean of fold induction versus control (no treatment) ± S.E from three independent experiments. **(C)** The phosphorylation levels of AKT protein from whole-lysates were measured to assess the integrity of the kinase inhibitors used in cAMP assay and a representative gel from three repetitions of each experiment is shown. Wort: Wortmannin.

The results presented here, in combination with our previous studies support the idea that at least two signaling pathways working in parallel ensure EGF directed cell survival: one through PI3K/AKT and the other via cAMP/PKA/CREB. In this case, the CREB transcription factor is responsible for promoter P1 activation and subsequent bcl-XL increase in response to cAMP accumulation triggered by EGF. The CREB arm of the pathway is independent of bcl-XL upregulation through PI3K/AKT signaling. We propose that other transcription factor/s could be activated by AKT and combine with CREB in regulating bcl-X gene expression induction. The fact that two different P1 promoter mutants with their single CRE-like site impaired respond similarly to the wild type, suggests that CREB links indirectly to another site located within the proximal 500 bp region upstream the transcription start site to regulate the expression of P1 driven transcripts. Evidence supporting this possibility was identified in T cells, where Bcl-XL upregulation requires co-activation of CREB and NFκB (Mori et al., 2001). Further studies are still needed to understand the complex mechanisms involved in EGF dependent *bcl-X* P1 activation and increased cell viability.

5. Conclusions

To our knowledge, this is the first study that provides details regarding the molecular mechanism involved in the regulation of *bcl-X* expression in mammary epithelial cells necessary to avoid apoptosis and increase cell viability. The description of a novel cross-talk between the EGF receptor and the adenylate cyclase activating pathways that prevent intrinsic cell death highlights the complexity of specific mechanisms, signaling pathways, and regulation of target genes that control cell survival in mammary epithelial cells.

Acknowledgments

The authors wish to thank Dr. Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland for kindly providing HC11 cells. We thank Dr. Michael Rudolph for critical reading of the manuscript and Yanina Sica for her support. We also thank Dr. Edith C. Kordon for the fruitful discussion of experiments. The authors are members of the National Council for Scientific Research.

Author contribution

DY Grinman; L Romorini; L Rocha-Viegas; C Davio and Adali Pecci conceived and designed the experiments. DY Grinman; L Romorini and DM Presman performed the experiments. DY Grinman; L Romorini; OA Coso; C Davio and Adali Pecci analyzed the data and wrote the paper.

Funding

This work was supported by grants from the University of Buenos Aires [20020130100363BA] and the National Agency of Scientific Promotion of Argentina [BID-2011 PICT 1321].

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.10.026.

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