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Influence of Terminal Differentiation and PACAP on the Cytokine, Chemokine, and Growth Factor Secretion of Mammary Epithelial Cells

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide with trophic and cytoprotective effects, has been shown to affect cell survival, proliferation, and also differentiation of various cell types. The high PACAP level in the milk and its changes during lactation suggest a possible effect of PACAP on the differentiation of mammary epithelial cells. Mammary cell differentiation is regulated by hormones, growth factors, cytokines/chemokines, and angiogenic proteins. In this study, differentiation was hormonally induced by lactogenic hormones in confluent cultures of HC11 mouse mammary epithelial cells. We investigated the effect of PACAP on mammary cell differentiation as well as release of cytokines, chemokines, and growth factors. Differentiation was assessed by expression analysis of the milk protein β-casein. Differentiation significantly decreased the secretion of interferon gamma-induced protein 10 (IP-10), regulated upon activation normal T cell expressed and presumably secreted (RANTES), and the epidermal growth factor receptor (EGFR) ligands epidermal growth factor (EGF) and amphiregulin. The changes in the levels of IP-10 and RANTES may be relevant for the alterations in homing of T cells and B cells at different stages of mammary gland development, while the changes of the EGFR ligands may facilitate the switch from proliferative to lactating stage. PACAP did not modulate the expression of β-casein or the activity of hormone-induced pathways as determined by the analysis of phosphorylation of Akt, STAT5, and p38 MAPK. However, PACAP decreased the release of EGF and amphiregulin from non-differentiated cells. This may influence the extracellular signal-related transactivation of EGFR in the non-differentiated mammary epithelium and is considered to have an impact on the modulation of oncogenic EGFR signaling in breast cancer.

Keywords Mammary differentiation · PACAP · IP-10 · RANTES · EGF · Amphiregulin

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Abbreviations

ADAM17	ADAM metallopeptidase domain 17
AREG	Amphiregulin
cAMP	Cyclic adenosine monophosphate
CTGF	Connective tissue growth factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
IL-1ra	Interleukin 1 receptor antagonist
IP-10	Interferon gamma-induced protein 10
JAK	Janus kinase
M-CSF	Macrophage colony-stimulating factor
p38 MAPK	p38 Mitogen-activated protein kinases
PACAP	Pituitary adenylate cyclase-activating polypeptide

PDGF	Platelet-derived growth factor
PKA	Protein Kinase A
PRL	Prolactin
PTHLH	Parathyroid hormone-like hormone
RANK-L	Receptor activator of NF-kB ligand
RANTES	Regulated upon activation normal T cell expressed and presumably secreted
STAT	Signal transducer and activator of transcription
TIMP	Tissue inhibitor of metalloproteinase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with diverse effects on cell proliferation and differentiation. The developmental effects of PACAP are best known in the central nervous system, where it exerts trophic factor-like effects (Waschek 2002; Watanabe et al. 2007). PACAP appears very early during the development of the nervous system, where it first stimulates proliferation of the cortical and cerebellar neuroblasts, and at a later stage, it influences differentiation, migration, and neuronal patterning (Watanabe et al. 2007). Similar developmental effects have been found in peripheral nervous structures, for example in dorsal root ganglia (Nielsen et al. 2004). PACAP induces differentiation in human neuroblastoma and mouse embryonic stem cells (Cazillis et al. 2004; Monaghan et al. 2008). PACAP has a biphasic, concentration-dependent effect on neuroblastoma cell lines, i.e., it stimulates cell proliferation at subnanomolar concentrations, while at higher doses, it induces differentiation (Vaudry et al. 2009). Less is known about the effects of PACAP on proliferation and differentiation of nonneural cells. PACAP inhibits osteoblastic and preantral follicle differentiation and is involved in T cell maturation (Delgado et al. 1996; Nagata et al. 2009; Latini et al. 2010). Some experimental data are available on the effects of PACAP on the growth of tumor cells, like pituitary adenoma, schwannoma, prostatic, colon, and lung carcinoma cells (Zia et al. 1995; Oka et al. 1999; Le et al. 2002; Gutierrez-Canas et al. 2003; Castorina et al. 2008).

PACAP is present in certain body fluids, such as human follicular fluid, plasma, and, similarly to vasoactive intestinal peptide (VIP), in the milk (Werner et al. 1985; Borzsei et al. 2009; Brubel et al. 2011; Koppan et al. 2012). PACAP-like immunoreactivity is higher in the milk than in the respective plasma samples and it shows significant changes during lactation (Borzsei et al. 2009; Csanaky et al. 2012). PACAP-immunoreactive nerve fibers and PACAP receptors have been identified in the mammary gland (Skakkebaek et al. 1999; Garcia-Fernandez et al. 2004, 2005). These observations raise the question about a potential role of PACAP in mammary gland development and differentiation.

It is well known that besides primary estrogen, progesterone, and prolactin (PRL), the proliferation and differentiation of mammary cells are influenced by cytokines, growth, and angiogenic factors (Khaled et al. 2007; Watson et al. 2011). PACAP has influence on cytokines, chemokines, and angiogenic factors. The expression of vascular endothelial growth factor (VEGF), a potent angiogenic factor, is increased by binding of PACAP to VPAC1 receptor, and therefore, PACAP is assorted as "nonclassic endogenous regulator of angiogenesis" (Ribatti et al. 2007). Furthermore, PACAP has been shown to be able to modify the cytokine profile by decreasing and increasing the production of pro- and certain anti-inflammatory cytokines, respectively, as well as chemokines and chemokine receptors. PACAP was demonstrated to influence cytokine production not only of immunocompetent cells (macrophages, lymphocytes), but also of other cell types (Nagakawa et al. 2005; Vaudry et al. 2009; Horvath et al. 2010).

In this study, we induced differentiation on HC11 mouse mammary cells, which are responsive to lactogenic hormones and produce β-casein "in vitro" (Ball et al. 1988). We investigated whether PACAP has any effect on this differentiation process. Moreover, PRL- and/or PACAP-induced changes in secreted cytokines, growth, and angiogenic factors were investigated with mouse cytokine and angiogenesis arrays. The observed effects are discussed in light of the current literature on the role of these regulatory factors on growth and differentiation of mammary epithelial cells.

Materials and Methods

Reagents and Antibodies

Bovine insulin, ovine prolactin, and dexamethasone were purchased from Sigma (St. Louis, MO). Recombinant murine epidermal growth factor (EGF) was obtained from Peprotech (Rocky Hill, NJ). Primary antibodies were applied as it follows: antiphospho-Akt (Thr308; Cell Signaling Technology, Beverly, MA) at 1:500; anti Akt-1 (C20; Santa Cruz Biotechnologies) at 1:500; antiphospho-p38 MAP kinase (Thr180/Tyr182; Cell Signaling Technology) at 1:1,000; anti-p38 MAP kinase (Cell Signaling Technology) at 1:1,000; antiphospho-signal transducer and activator of transcription (STAT)-5 (Tyr694, recognizes also Tyr699 of STAT5B; Cell Signaling Technology) at 1:900; anti-STAT5 (Cell Signaling Technology) at 1:900; anti-α-tubulin (Santa Cruz Biotechnologies) at 1:1,000; and anti-β-casein (M-14; Santa Cruz Biotechnologies) at 1:1,000. Corresponding anti

IgG-horseradish peroxidase (HRP) secondary antibodies were purchased from Santa Cruz Biotechnologies. PACAP38 was synthesized using a solid-phase procedure utilizing ^{t}Boc chemistry (Pirger et al. 2010).

Cell Culture and Hormone Induction

HC11 mouse mammary epithelial cells were maintained in growth medium, which contained RPMI-1640, 10 % heat-activated fetal calf serum (FCS), 5 μg/ml insulin (I), and 10 ng/ml EGF supplemented with 50 μg/ml gentamicin and glutamine. Cells were grown in 5 % CO_2 at 37 °C and passaged every 3–4 days. Cells were plated in six-well plates and grown to confluence for 2–3 days. After the cells reached the confluent state, they were washed twice with PBS to remove EGF, and an additional 2-day incubation was carried out in pre-hormone medium (PHM) containing RPMI-1640, 2 % FCS, 5 μg/ml insulin, and 50 μg/ml gentamicin and glutamine. After 2 days, the medium was changed to DIP medium containing 1 μM dexamethasone (D), 5 μg/ml I, and 5 μg/ml prolactin (P) in PHM. The PHM was changed, and D, PRL, and PACAP38 were added to the cell cultures every day (Fig. 1). The cell cultures were co-incubated with 100 nM PACAP38 for 4 days with and without PRL in cell differentiation experiments. In signal transduction experiments, Western blot analysis was performed on confluent cell cultures after 20 min of DIP and 100 nM PACAP38 treatment.

Western Blot Analysis

Cell lysates were prepared by washing cells three times with ice-cold PBS followed by lysis for 30 min at 4 °C in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 25 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 10 % glycerol, 1 % Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1.19 mM Na_3VO_4, and 2.5 mM NaF). Lysates were centrifuged (15,300 rpm) at 4 °C for 10 min to remove insoluble parts. Protein concentration was determined by Bradford, and proteins were separated by SDS-PAGE and blotted on Odyssey membranes. Membranes were blocked with 5 % milk for 30 min at room temperature, incubated overnight at 4 °C with the primary antibodies, and then further incubated for 30 min at room temperature with the appropriate secondary antibodies and the reactions were detected with the ECL Plus Western blotting detection system (GE Healthcare, Little Chalfont, UK).

Mouse Cytokine Array and Mouse Angiogenesis Array

Secreted cytokines and angiogenesis-related proteins were investigated by semiquantitative Mouse Cytokine Array Panel A and Mouse Angiogenesis Array Kit (R&D Systems, Hungary). In these arrays, the investigated proteins bind carefully selected captured antibodies spotted in duplicate on nitrocellulose membranes. The kits contain all buffers, detection antibodies, and membranes necessary for the measurements. The arrays were performed as described by the manufacturer. Briefly, after blocking the array membranes for 1 h, 500 μl medium was added and incubated overnight at 2–8 °C on a rocking platform with detection antibody cocktail. After washing with buffer three times and adding HRP-conjugated streptavidin, the membranes were exposed to chemiluminescent detection reagent. X-ray films were scanned on transmission mode. Factors which showed changes with eye control in each experiment were analyzed by ImageJ software. The positive controls at the reference spots were normalized to non-differentiated cells in order to compare the results from different membranes. Pixel densities were expressed in arbitrary units. The released proteins of stimulated cells were compared to that of the non-differentiated untreated cells. Statistical analysis was performed by one- and two-way ANOVA test.

Results

β-casein was expressed in HC11 cells only after DIP treatment, as described previously (Ball et al. 1988). PACAP co-incubation without PRL did not induce β-casein expression, and it did not modify the DIP-evoked β-casein expression either (Fig. 2). The downstream signaling via phosphorylation

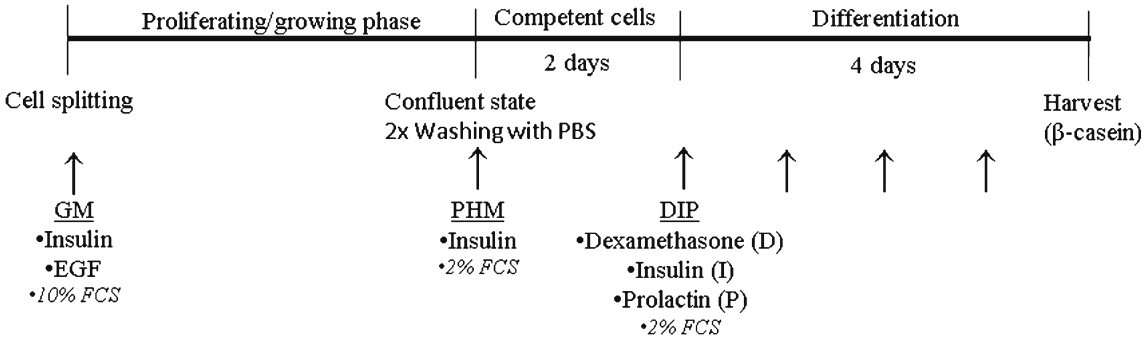
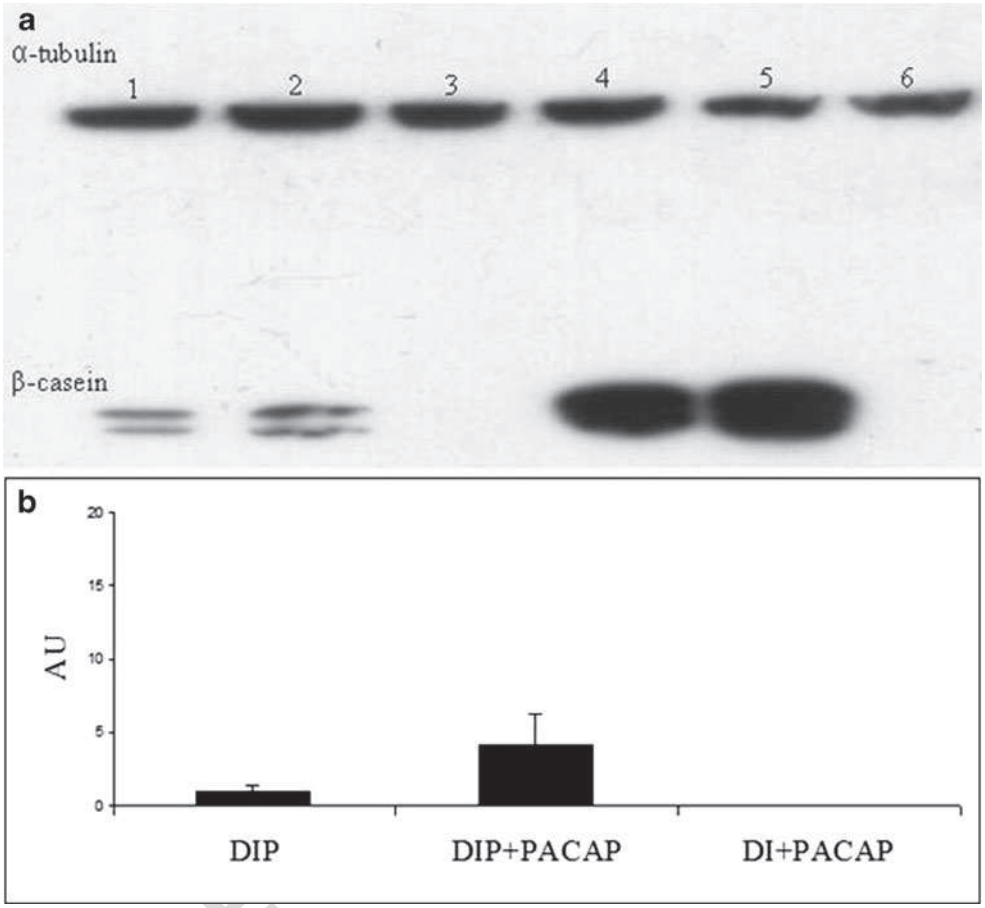


Fig. 1 Timeline of the experiment. *GM* growth medium, *PHM* pre-hormone medium



Q7 **Fig. 2** Western blot analysis of β-casein expression in HC11 cells in the presence and absence of PACAP. Two different HC11 cell clones were investigated. **a** A23 clone (one to three bands): *1* DIP; *2* DIP+PACAP; *3* DI+PACAP; B22 clone (four to six bands): *4* DIP; *5* DIP+PACAP; *6* DI+PACAP. DIP induces β-casein expression (*1* and *4*). The band of β-casein does not appear after PACAP treatment without PRL (*3* and *6*), and PACAP has no modulatory effect on DIP-induced β-casein expression either (*2* and *5*). **b** Densitometric analysis of three independent experiments on B22 clone. Results are shown mean ± SE, and α-tubulin serves as control

of STAT5 was activated by PRL as reported previously (Welte et al. 1994). Akt/p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation, which can be triggered by insulin in these cells (Berlato and Doppler 2009), was observed under all experimental conditions. No modulating effect of PACAP on STAT5, Akt, or p38 MAPK was detectable (Fig. 3).

The mouse cytokine array measurements showed that differentiated HC11 cells secreted significantly lower levels of interferon gamma-induced protein 10 (IP-10) and regulated upon activation normal T cell expressed and presumably secreted (RANTES) compared to the non-differentiated cells. A further decrease was observed in secretion of RANTES after PACAP co-incubation of differentiated cells, but this change did not prove to be statistically significant. In differentiated cells, a consequent increase of released I-309 and interleukin 1 receptor antagonist (IL-1ra) and a decrease of macrophage colony-stimulating factor (M-CSF) were presumed by eye control, but these changes were not significant by densitometric analysis. The interleukin (IL) series showed very weak densities and they were not analyzed further (Figs. 4 and 5).

On the angiogenesis array, the IGFBP10 signal was well detectable, but did not show changes in intensity after DIP or PACAP treatment, while other insulin-like growth factor-binding proteins (IGFBPs), such as IGFBP1, 2, and 9, did not produce signals at all (Fig. 6). The media of differentiated cells showed a significant drop of EGF, amphiregulin (AREG), and IGFBP3 on the angiogenesis array (Fig. 6, compare encircled spots in panels A and C). PACAP co-incubation significantly decreased the expression of EGF and AREG in non-differentiated cells (Fig. 6b), while there were no changes in these factors in differentiated cells (Fig. 6d). Quantification of differentiation and PACAP-induced changes of EGF, AREG, and IGFBP3 is shown in Fig. 7. Neither hepatocyte growth factor (HGF) nor "classic" angiogenic factors, such as VEGF, fibroblast growth factor (FGF)-2, angiopoietin, and thrombospondin, produced signals suitable for densitometric analysis. PDGF-AA did not show changes under eye control after DIP or PACAP co-treatment. Inconsequent or weak signals were seen in connection with other proteins (Fig. 6).

Discussion

Three major stages of mammary gland development can be distinguished, namely ductal elongation/bifurcation in puberty, side branching in estrous cycles, and alveologenesis/

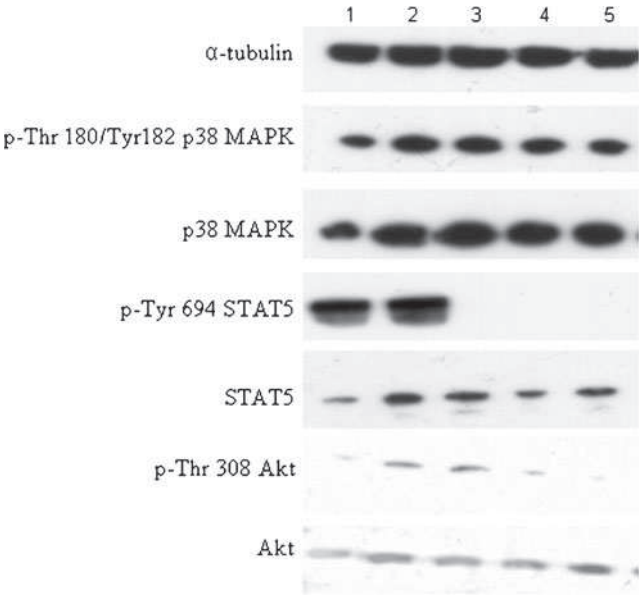


Fig. 3 Western blot analysis of p38 MAPK, STAT5, and Akt in extracts of HC11 cells. *1* DIP; *2* DIP+PACAP; *3* DI+PACAP; *4* I+PACAP; *5* I. The abundance of phosphorylated activated forms of p38 MAPK and Akt remained similar under all experimental conditions investigated, and thus, it did not appear to be significantly influenced by PRL, dexamethasone, and PACAP, while STAT5 was phosphorylated only in the presence of PRL. There was no change in STAT5 activation in case of PACAP co-incubation (similar bands in 1–2). Without PRL, PACAP could not activate STAT5 (lack of pSTAT5 in 3–5)

lactogenic differentiation in pregnancy (Brisken and O'Malley 2010). Lactogenic differentiation of mammary epithelial cells mainly requires hormonal signaling. Binding of PRL to its receptor induces homodimerization resulting in JAK2/STAT5 activation. The STAT5 dimer translocates to the nucleus and promotes transcription of β-casein.

Besides hormones, cytokines/chemokines and growth factors modulate lactogenic differentiation. IL-4/IL13/STAT6 signaling is an important regulator of alveologenesis. This pathway is also associated with differentiation of naive T helper cells (Khaled et al. 2007; Watson et al. 2011). The tumor necrosis factor (TNF) family molecule, RANK-L, and its receptor are also implicated in terminal differentiation (Kim et al. 2002). TNFα stimulates mammary differentiation in vitro, but only in the absence or upon deficiency of EGF (Ip et al. 1992). Furthermore, connective tissue growth factor (CTGF) enhances β-casein transcription, while siRNA-mediated depletion of CTGF blocks differentiation showing that even growth factors intervene with the process of lactogenic differentiation (Morrison et al. 2010).

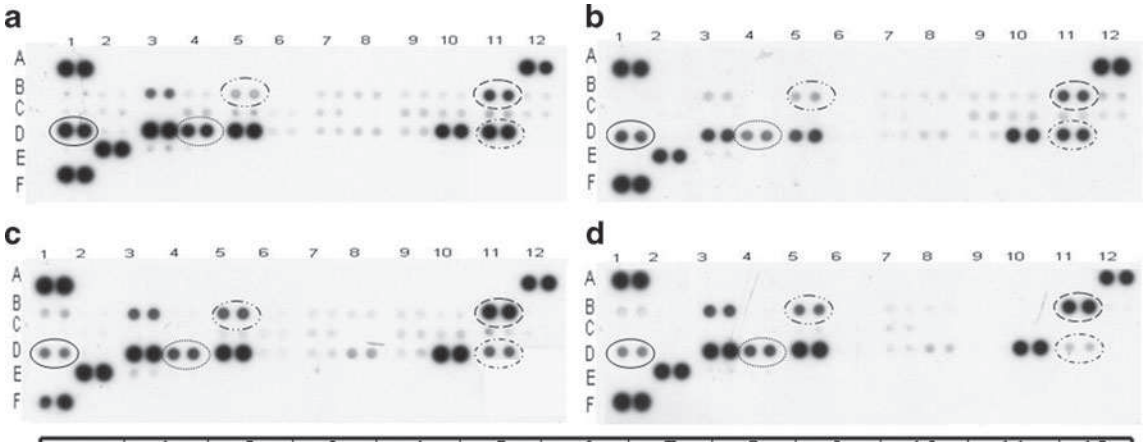
HC11 cells are derived from mid-pregnant BALB/c mouse. This cell line serves as a model to investigate the molecular mechanism of hormones, cytokines, growth, and transcriptional factors involved in differentiation (Ball et al. 1988; Doppler et al. 1989). Confluent HC11 cells are responsive to lactogenic hormones resulting in terminal differentiation and expression of milk proteins as it was proven by the induction of β-casein gene expression in our study. We extended the characterization of lactogenic hormone-induced differentiation by determining secretion of almost 50 chemokines/cytokines, several growth/angiogenesis-related proteins, and some other factors with the mouse cytokine array panel and angiogenesis kits.

A significant decrease of IP-10 and RANTES was measured in the cell culture media of differentiated HC11 cells. IP-10 and RANTES are chemokines, responsible for the recruitment of T lymphocytes and some other leukocytes (Schall et al. 1990; Angiolillo et al. 1995). They are present in the mammary gland and milk, and they are supposed to maintain the balance of lymphocyte homing to the mammary gland at different stages of differentiation (Michie et al. 1998; Takahata et al. 2003). Colonization of mammary gland is dominated by T cells during pregnancy, while Ig-A containing B cells are abundant during lactation (Tanneau et al. 1999). Therefore, decreased release of T cell attractants, such as IP-10 and RANTES in our experiment, may reflect the shift of T to B cells in lactating glands.

Khaled et al. applied a similar cytokine assay on the media of non-differentiated and 8-day differentiated KIM-2 mouse mammary epithelial cells, and they observed that the secretion of IL-4 was higher in the differentiated cells, while the secretion of granulocyte colony-stimulating factor (G-CSF) and IL-6 decreased. Other Th2 cytokines (IL-2, IL-3, IL-5, IL-9, IL-10, and IL-13) did not show changes in their study. Moreover, with the use of qRT-PCR, they observed a Th1/Th2 cytokine switch in the expression profile concomitant with induction of differentiation, i.e., IL-12 and TNFα were downregulated, while IL-4, IL-5, and IL-13 were upregulated (Khaled et al. 2007). We could not detect similar changes in the release of IL-4, IL-6, and G-CSF from differentiated HC11 cells and this may reflect cell line-specific differences as well as the different differentiation protocol used in our study.

In our experiment, IGFBP3 was abundantly present in the media of non-differentiated HC11 cells, while decreased levels of IGFBP3 were measured after DIP treatment. Similarly, Skaar et al. demonstrated decreased IGFBP3 secretion of Comma-1D cells, a progenitor of HC11 cell line after treatment with dexamethasone (Skaar and Baumrucker 1993). IGFBPs are carrier proteins and they modulate the activity of insulin-like growth factors (IGFs). Decreased in vitro IGFBP3 secretion of differentiated HC11 cells is compatible with the physiological decrease of IGFBPs during lactation allowing maximal effect of IGFs, which are recognized as endocrine and paracrine modulators of PRL-induced alveolar differentiation (Allar and Wood 2004).

The decreased AREG and EGF release from differentiated HC11 cells may reflect the switch from proliferative to lactogenic phase. Expression of AREG transcripts has been shown to be regulated by PRL (Ormandy et al. 2003), but, to our knowledge, no data are available about the effect of PRL on



	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref.											Ref.
B	BLC	C5/C5a	G-CSF	GM-CSF	I-309	Ecotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12 p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1								
F	Ref.											PBS (neg)

Fig. 4 Mouse cytokine array panel **a** of non-differentiated (**a**, **b**) and differentiated HC11 cells (**c**, **d**) without PACAP (**a**, **c**) and with PACAP co-incubation (**b**, **d**). Proteins which show obvious changes in expression after DIP and/or PACAP treatment are marked by *circles* and comprise B5 = I-309; B11 = IL-1ra; D1 = IP-10; D4 = M-CSF; and D11 = RANTES

their secretion. Production of AREG is induced by estrogen in peripubertal breasts, and it is downregulated during and after pregnancy. Once expressed, AREG exists as a membrane-associated precursor. AREG released from mammary epithelium binds to epidermal growth factor receptor (EGFR) of stromal cells, and this has been shown to be important for the expression of growth factors (FGF, HGF, IGF1), which are implicated in stimulating the proliferation of other epithelial cells (McBryan et al. 2008). AREG, EGF, and transforming growth factor (TGF)α are structurally related proteins. While AREG is specifically required for ductal morphogenesis, EGF and TGFα are dispensable for this process (Luetteke et al. 1999). EGF blocks functional differentiation (β-casein and WAP production) or results in dedifferentiation (Spitzer et al. 1995).

The regulatory role of neuropeptides outside the nervous and endocrine system is widely accepted, e.g., neuronal peptide galanin not only regulates PRL secretion from the pituitary lactotrophs, but the mammary epithelium is also directly responsive to galanin, as it augments alveolar morphogenesis (Naylor et al. 2003). In our study, PACAP had no influence on

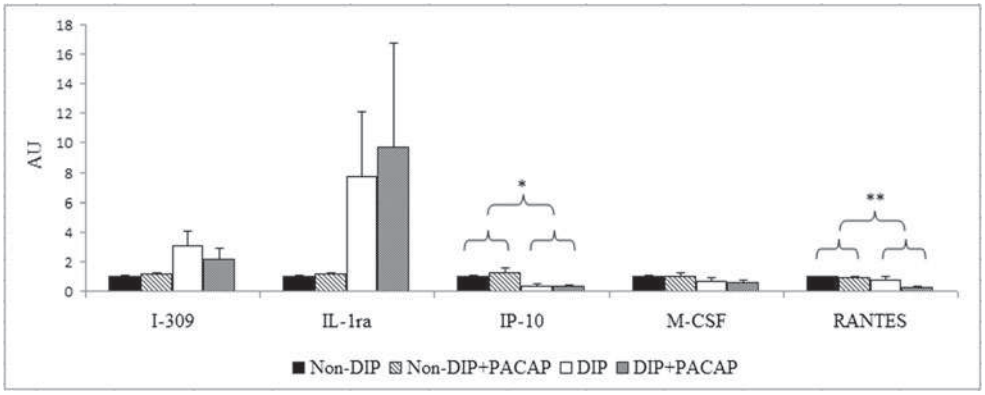
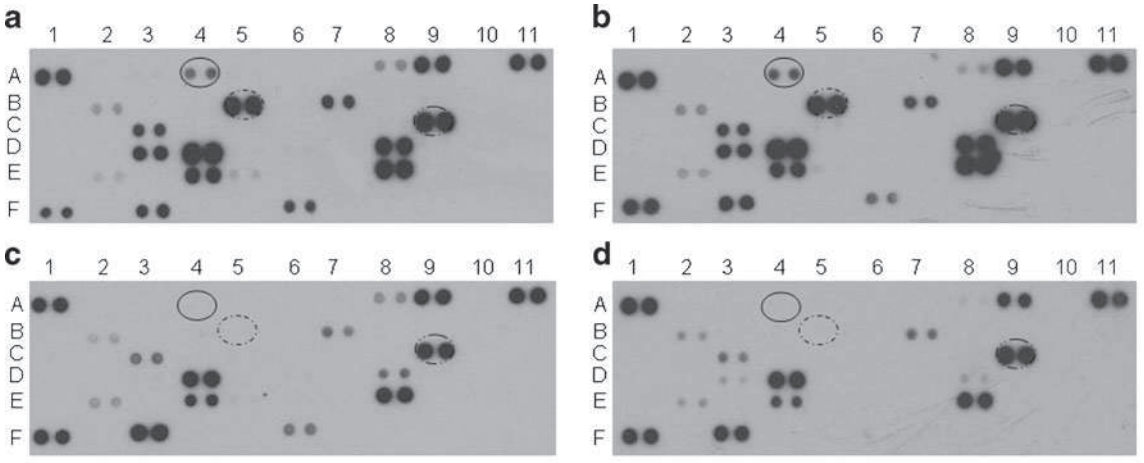


Fig. 5 Image analysis of some secreted cytokines. Secreted IP-10 and RANTES are significantly lower in culture media of differentiated cells compared to non-differentiated ones (*$p < 0.05$; **$p < 0.005$). The different arrays are normalized to the controls of non-differentiated cells, and the *bar charts* show the relative changes in protein expressions based on three independent measurements



	1	2	3	4	5	6	7	8	9	10	11
A	Ref.		ADAMTS1	AREG	ANG	Ang-1	Ang-3	TF	CXCL16		Ref
B		IGFBP10	DLL4	DPPIV	EGF	CD105	Endostatin	ET-1	FGF-1	FGF-2	FGF-7
C		FGF-7	Fractalkine	GM-CSF	HB-EGF	HGF	IGFBP1	IGFBP2	IGFBP3	IL-1α	IL-1β
D		IL-10	IP-10	KC	Leptin	MCP-1	MIP-1α	MMP-3	MMP-8	MMP-9	IGFBP 9
E		OPN	PD-ECGF	PDGF-AA	PDGF-AB/PDGF-BB	PTX3	PF4	PlGF-2	PRL	Proliferin	
F	Ref.	SDF-1	Serpin E1	Serpin F1	TSP-2	TIMP-1	TIMP-4	VEGF	VEGF-B	PBS (neg)	

Fig. 6 Mouse angiogenesis array of non-differentiated (**a**, **b**) and differentiated HC11 cells (**c**, **d**) without PACAP (**a**, **c**) and with PACAP co-incubation (**b**, **d**). Proteins which show obvious changes after DIP and/or PACAP treatment are indicated by *circles* and comprise A4 = AREG, B5 = EGF, and C9 = IGFBP3

the differentiation of HC11 cells either at the level of β-casein production or phosphorylation of proteins involved in lactogenic hormone signaling.

The observed PACAP-induced decrease of secreted AREG and EGF from non-differentiated HC11 cells may be a consequence of (a) decreased ligand shedding and/or (b) decreased expression of these EGFR ligands. A possible mechanism for decreased EGFR ligand shedding could be the reported dependence of TGF-β expression on PACAP, as evident from TGF-β downregulation in PACAP KO mice (Tan et al. 2009), and the role of TGF-β in inhibition of ADAM metallopeptidase domain 17 (ADAM17), a metalloproteinase implicated in shedding of AREG, EGF, TGF-α, and activation of EGFR (Sternlicht et al. 2005). TGF-β downregulates matrix degrading proteinases, including ADAM17, and upregulates their inhibitors, such as TIMP-3 (Leivonen et al. 2013; Wada et al. 2013).

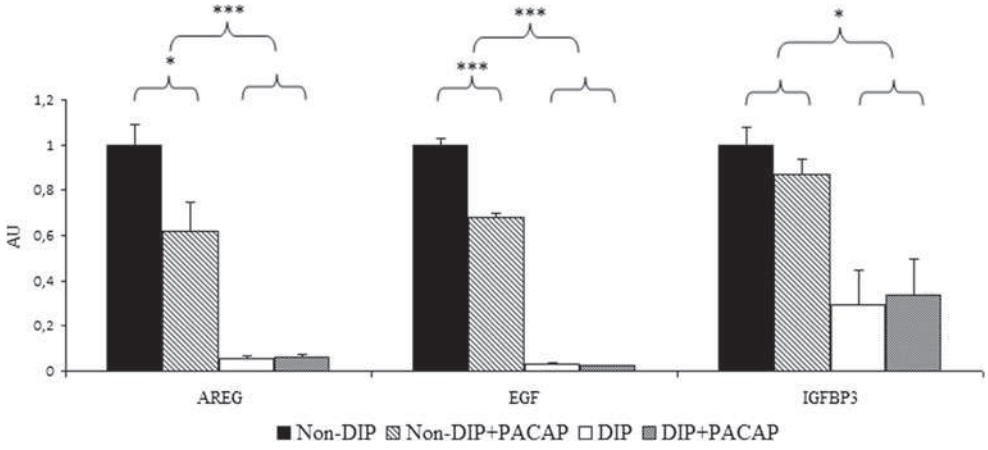


Fig. 7 Image analysis of AREG, EGF, and IGFBP3. Differentiation resulted in significantly decreased levels in all of these growth factors, while PACAP treatment decreased the level of AREG and EGF in non-differentiated cells (*$p<0.05$; ***$p<0.001$). All measurements were repeated three times. The different arrays were normalized to the controls of non-differentiated cells

Whether TGF-β is expressed in HC11 cells and modified by PACAP remains to be shown in further studies.

In contrast to our findings with non-differentiated mammary epithelial cells, where PACAP inhibited expression of growth factors, PACAP has a growth factor-like activity on neural cells (Grumolato et al. 2003; Somogyvári-Vigh and Reglodi 2004; Vaudry et al. 2009). PACAP/PAC1-R interaction via cAMP/PKA signaling-stimulated Src-ADAM17 increased TGF-α release and transactivated EGFR on lung carcinoma cells. Administration of anti-AREG did not reverse the PACAP-induced transactivation of EGFR (Moody et al. 2012). Likewise, VIP transactivated EGFR and induced VEGF release on mammary carcinoma cells (Valdehita et al. 2008).

Our results on the PACAP-mediated downregulation of AREG and EGF may also have significance in light of some experimental oncology data. AREG is a crucial growth factor influencing the proliferation of mammary epithelial tumor cells, and EGFR transactivation-dependent breast cancers utilize ADAM-mediated EGFR ligand shedding. Therefore, AREG is a promising target for drug intervention (Moody et al. 2012). Interestingly, AREG is supposed to contribute even to bone metastasis by stimulation of bone resorption via autocrine AREG-EGFR signaling to promote PTHLH production (Gilmore et al. 2008).

In summary, we demonstrated that PACAP had no direct effect on the lactogenic hormone-induced terminal differentiation of HC11 mouse mammary epithelial cells. A significant decrease in the release of IP-10/RANTES was detected during differentiation which might be relevant for influencing the altered recruitment of lymphocytes in the terminal differentiated gland as it is observed under "in vivo" conditions. The decreased secretion of AREG/EGF is considered to contribute to the proliferative to lactogenic phase switch in terminal differentiated gland. Interestingly, PACAP co-incubation significantly decreased the levels of AREG and EGF secreted from non-differentiated mammary cells, which may have physiological implications. Furthermore, in the light of the prominent role of EGFR signaling in breast cancer, this inhibitory effect of PACAP could be relevant in influencing the development and progression of this disease.

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