

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  $\beta$ -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  $\beta$ -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

Abbreviations

ADAM17	ADAM metalloproteinase domain 17	50
AREG	Amphiregulin	53
cAMP	Cyclic adenosine monophosphate	56
CTGF	Connective tissue growth factor	59
EGF	Epidermal growth factor	60
EGFR	Epidermal growth factor receptor	63
FGF	Fibroblast growth factor	63
G-CSF	Granulocyte colony-stimulating factor	66
HGF	Hepatocyte growth factor	69
IGF	Insulin-like growth factor	70
IGFBP	Insulin-like growth factor-binding protein	73
IL	Interleukin	73
IL-1ra	Interleukin 1 receptor antagonist	76
IP-10	Interferon gamma-induced protein 10	79
JAK	Janus kinase	80
M-CSF	Macrophage colony-stimulating factor	83
p38	p38 Mitogen-activated protein kinases	84
MAPK		85
PACAP	Pituitary adenylate cyclase-activating polypeptide	88

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90	PDGF	Platelet-derived growth factor
93	PKA	Protein Kinase A
93	PRL	Prolactin
96	PTH LH	Parathyroid hormone-like hormone
Q3 98	RANK-L	Receptor activator of NF- $\kappa$ B ligand
100	RANTES	Regulated upon activation normal T cell
102		expressed and presumably secreted
103	STAT	Signal transducer and activator of transcription
106	TIMP	Tissue inhibitor of metalloproteinase
108	TGF	Transforming growth factor
100	TNF	Tumor necrosis factor
112	VEGF	Vascular endothelial growth factor
113	VIP	Vasoactive intestinal peptide
115		

## 116 Introduction

117 Pituitary adenylate cyclase-activating polypeptide (PACAP) is  
 118 a neuropeptide with diverse effects on cell proliferation and  
 119 differentiation. The developmental effects of PACAP are best  
 120 known in the central nervous system, where it exerts trophic  
 121 factor-like effects (Waschek 2002; Watanabe et al. 2007).  
 122 PACAP appears very early during the development of the  
 123 nervous system, where it first stimulates proliferation of the  
 124 cortical and cerebellar neuroblasts, and at a later stage, it  
 125 influences differentiation, migration, and neuronal patterning  
 126 (Watanabe et al. 2007). Similar developmental effects have  
 127 been found in peripheral nervous structures, for example in  
 128 dorsal root ganglia (Nielsen et al. 2004). PACAP induces  
 129 differentiation in human neuroblastoma and mouse embryonic  
 130 stem cells (Cazillis et al. 2004; Monaghan et al. 2008).  
 131 PACAP has a biphasic, concentration-dependent effect on  
 132 neuroblastoma cell lines, i.e., it stimulates cell proliferation  
 133 at subnanomolar concentrations, while at higher doses, it  
 134 induces differentiation (Vaudry et al. 2009). Less is known  
 135 about the effects of PACAP on proliferation and differentia-  
 136 tion of nonneural cells. PACAP inhibits osteoblastic and pre-  
 137 antral follicle differentiation and is involved in T cell maturation  
 138 (Delgado et al. 1996; Nagata et al. 2009; Latini et al.  
 139 2010). Some experimental data are available on the effects of  
 140 PACAP on the growth of tumor cells, like pituitary adenoma,  
 141 schwannoma, prostatic, colon, and lung carcinoma cells (Zia  
 142 et al. 1995; Oka et al. 1999; Le et al. 2002; Gutierrez-Canas  
 143 et al. 2003; Castorina et al. 2008).

144 PACAP is present in certain body fluids, such as human  
 145 follicular fluid, plasma, and, similarly to vasoactive intestinal  
 146 peptide (VIP), in the milk (Werner et al. 1985; Borzsei et al.  
 147 2009; Brubel et al. 2011; Koppan et al. 2012). PACAP-like  
 148 immunoreactivity is higher in the milk than in the respective  
 149 plasma samples and it shows significant changes during lac-  
 150 tation (Borzsei et al. 2009; Csanaky et al. 2012). PACAP-  
 151 immunoreactive nerve fibers and PACAP receptors have been

identified in the mammary gland (Skakkebaek et al. 1999; 152  
 Garcia-Fernandez et al. 2004, 2005). These observations raise 153  
 the question about a potential role of PACAP in mammary 154  
 gland development and differentiation. 155

It is well known that besides primary estrogen, progester- 156  
 one, and prolactin (PRL), the proliferation and differentiation 157  
 of mammary cells are influenced by cytokines, growth, and 158  
 angiogenic factors (Khaled et al. 2007; Watson et al. 2011). 159  
 PACAP has influence on cytokines, chemokines, and angio- 160  
 genic factors. The expression of vascular endothelial growth 161  
 factor (VEGF), a potent angiogenic factor, is increased by 162  
 binding of PACAP to VPAC1 receptor, and therefore, 163  
 PACAP is assorted as “nonclassic endogenous regulator of 164  
 angiogenesis” (Ribatti et al. 2007). Furthermore, PACAP has 165  
 been shown to be able to modify the cytokine profile by 166  
 decreasing and increasing the production of pro- and certain 167  
 anti-inflammatory cytokines, respectively, as well as 168  
 chemokines and chemokine receptors. PACAP was demon- 169  
 strated to influence cytokine production not only of immuno- 170  
 competent cells (macrophages, lymphocytes), but also of 171  
 other cell types (Nagakawa et al. 2005; Vaudry et al. 2009; 172  
 Horvath et al. 2010). 173

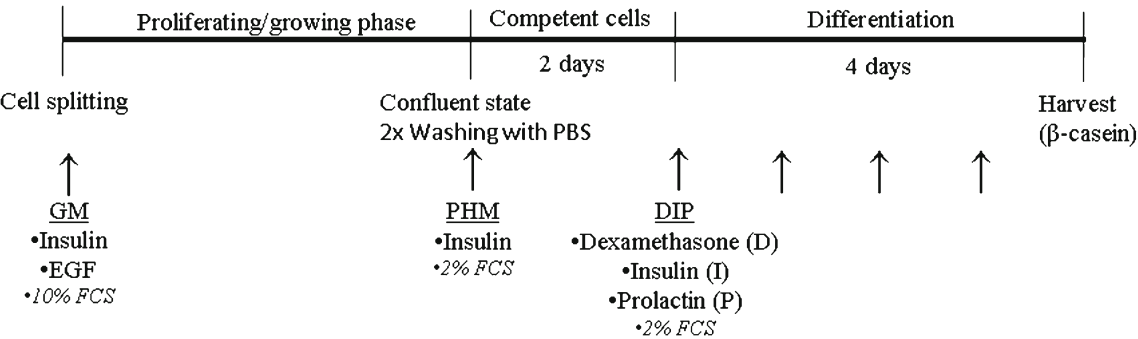
In this study, we induced differentiation on HC11 mouse 174  
 mammary cells, which are responsive to lactogenic hormones 175  
 and produce  $\beta$ -casein “in vitro” (Ball et al. 1988). We inves- 176  
 tigated whether PACAP has any effect on this differentiation 177  
 process. Moreover, PRL- and/or PACAP-induced changes in 178  
 secreted cytokines, growth, and angiogenic factors were in- 179  
 vestigated with mouse cytokine and angiogenesis arrays. The 180  
 observed effects are discussed in light of the current literature 181  
 on the role of these regulatory factors on growth and differ- 182  
 entiation of mammary epithelial cells. 183

## 184 Materials and Methods

### 185 Reagents and Antibodies

Bovine insulin, ovine prolactin, and dexamethasone were 186  
 purchased from Sigma (St. Louis, MO). Recombinant murine 187  
 epidermal growth factor (EGF) was obtained from Peprotech 188  
 (Rocky Hill, NJ). Primary antibodies were applied as it fol- 189  
 lows: antiphospho-Akt (Thr308; Cell Signaling Technology, 190  
 Beverly, MA) at 1:500; anti Akt-1 (C20; Santa Cruz 191  
 Biotechnologies) at 1:500; antiphospho-p38 MAP kinase 192  
 (Thr180/Tyr182; Cell Signaling Technology) at 1:1,000; 193  
 anti-p38 MAP kinase (Cell Signaling Technology) at 194  
 1:1,000; antiphospho-signal transducer and activator of tran- 195  
 scription (STAT)-5 (Tyr694, recognizes also Tyr699 of 196  
 STAT5B; Cell Signaling Technology) at 1:900; anti-STAT5 197  
 (Cell Signaling Technology) at 1:900; anti- $\alpha$ -tubulin (Santa 198  
 Cruz Biotechnologies) at 1:1,000; and anti- $\beta$ -casein (M-14; 199  
 Santa Cruz Biotechnologies) at 1:1,000. Corresponding anti 200

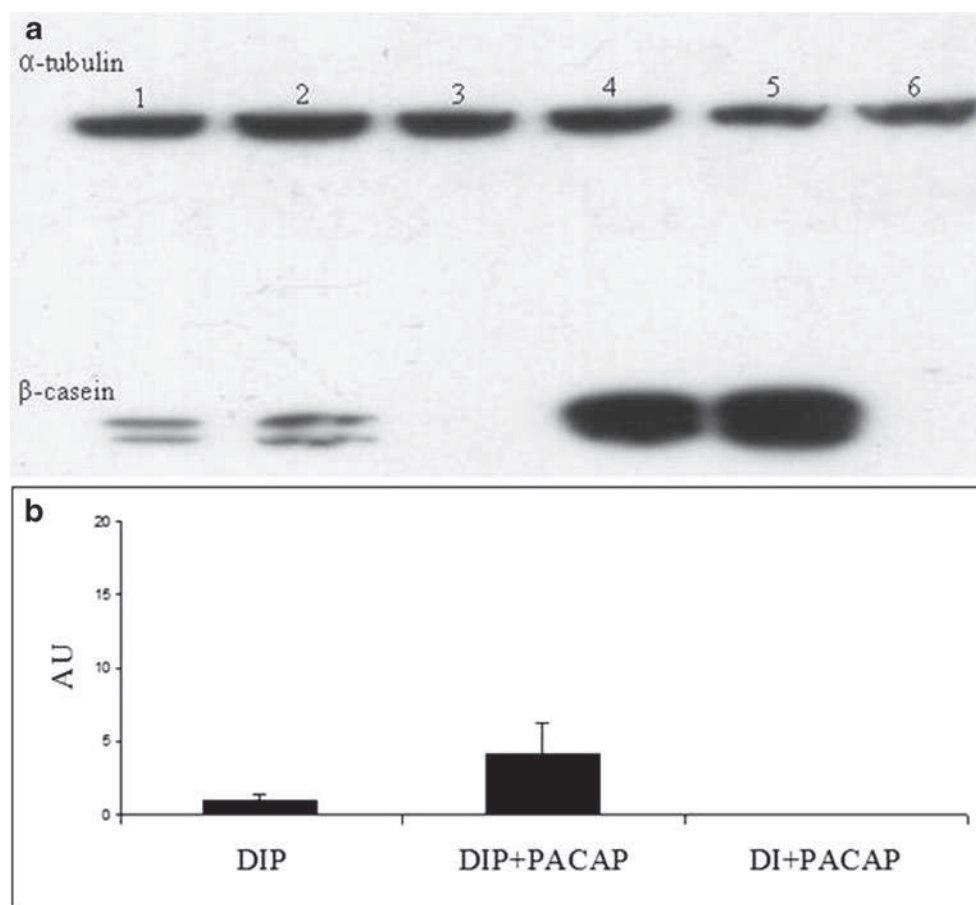
Q4	201	IgG-horseradish peroxidase (HRP) secondary antibodies were	with 5 % milk for 30 min at room temperature, incubated	238
	202	purchased from Santa Cruz Biotechnologies. PACAP38 was	overnight at 4 °C with the primary antibodies, and then further	239
	203	synthesized using a solid-phase procedure utilizing <sup>t</sup> Boc	incubated for 30 min at room temperature with the appropriate	240
	204	chemistry (Pirger et al. 2010).	secondary antibodies and the reactions were detected with the	241
			ECL Plus Western blotting detection system (GE Healthcare,	242
			Little Chalfont, UK).	243
	205	Cell Culture and Hormone Induction		
			Mouse Cytokine Array and Mouse Angiogenesis Array	244
	206	HC11 mouse mammary epithelial cells were maintained in	Secreted cytokines and angiogenesis-related proteins were	245
	207	growth medium, which contained RPMI-1640, 10 % heat-	investigated by semiquantitative Mouse Cytokine Array	246
	208	activated fetal calf serum (FCS), 5 µg/ml insulin (I), and	Panel A and Mouse Angiogenesis Array Kit (R&D Systems,	247
	209	10 ng/ml EGF supplemented with 50 µg/ml gentamicin and	Hungary). In these arrays, the investigated proteins bind care-	248
	210	glutamine. Cells were grown in 5 % CO <sub>2</sub> at 37 °C and	fully selected captured antibodies spotted in duplicate on	249
	211	passaged every 3–4 days. Cells were plated in six-well plates	nitrocellulose membranes. The kits contain all buffers, detec-	250
	212	and grown to confluence for 2–3 days. After the cells reached	tion antibodies, and membranes necessary for the measure-	251
	213	the confluent state, they were washed twice with PBS to	ments. The arrays were performed as described by the manu-	252
	214	remove EGF, and an additional 2-day incubation was carried	facturer. Briefly, after blocking the array membranes for 1 h,	253
	215	out in pre-hormone medium (PHM) containing RPMI-1640,	500 µl medium was added and incubated overnight at 2–8 °C	254
Q5	216	2 % FCS, 5 µg/ml insulin, and 50 µg/ml gentamicin and	on a rocking platform with detection antibody cocktail. After	255
	217	glutamine. After 2 days, the medium was changed to DIP	washing with buffer three times and adding HRP-conjugated	256
	218	medium containing 1 µM dexamethasone (D), 5 µg/ml I, and	streptavidin, the membranes were exposed to chemilumines-	257
	219	5 µg/ml prolactin (P) in PHM. The PHM was changed, and D,	cent detection reagent. X-ray films were scanned on transmis-	258
	220	PRL, and PACAP38 were added to the cell cultures every day	sion mode. Factors which showed changes with eye control in	259
	221	(Fig. 1). The cell cultures were co-incubated with 100 nM	each experiment were analyzed by ImageJ software. The	260
	222	PACAP38 for 4 days with and without PRL in cell differen-	positive controls at the reference spots were normalized to	261
	223	tiation experiments. In signal transduction experiments,	non-differentiated cells in order to compare the results from	262
	224	Western blot analysis was performed on confluent cell cul-	different membranes. Pixel densities were expressed in arbi-	263
	225	tures after 20 min of DIP and 100 nM PACAP38 treatment.	trary units. The released proteins of stimulated cells were	264
			compared to that of the non-differentiated untreated cells.	265
	226	Western Blot Analysis	Statistical analysis was performed by one- and two-way	266
			ANOVA test.	267
	227	Cell lysates were prepared by washing cells three times with		
	228	ice-cold PBS followed by lysis for 30 min at 4 °C in lysis	<b>Results</b>	268
	229	buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM	β-casein was expressed in HC11 cells only after DIP treat-	269
	230	EGTA, 2 mM EDTA, 25 mM β-glycerophosphate, 1.5 mM	ment, as described previously (Ball et al. 1988). PACAP co-	270
	231	MgCl <sub>2</sub> , 10 % glycerol, 1 % Triton X-100, 5 µg/ml aprotinin,	incubation without PRL did not induce β-casein expression,	271
	232	5 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride,	and it did not modify the DIP-evoked β-casein expression	272
	233	1 mM dithiothreitol, 1.19 mM Na <sub>3</sub> VO <sub>4</sub> , and 2.5 mM NaF).	either (Fig. 2). The downstream signaling via phosphorylation	273
	234	Lysates were centrifuged (15,300 rpm) at 4 °C for 10 min to		
	235	remove insoluble parts. Protein concentration was determined		
	236	by Bradford, and proteins were separated by SDS-PAGE and		
	237	blotted on Odyssey membranes. Membranes were blocked		



Q6 Fig. 1 Timeline of the experiment. GM growth medium, PHM pre-hormone medium

Q7

**Fig. 2** Western blot analysis of  $\beta$ -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  $\beta$ -casein expression (1 and 4). The band of  $\beta$ -casein does not appear after PACAP treatment without PRL (3 and 6), and PACAP has no modulatory effect on DIP-induced  $\beta$ -casein expression either (2 and 5). **b** Densitometric analysis of three independent experiments on B22 clone. Results are shown mean  $\pm$  SE, and  $\alpha$ -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).

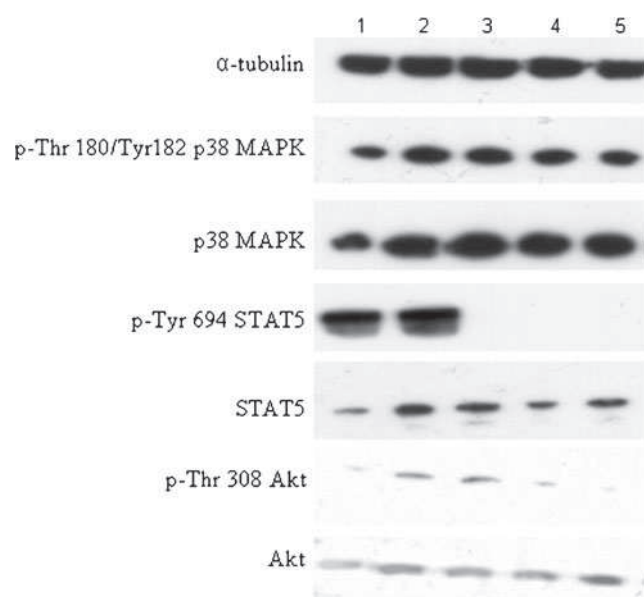
On the angiogenesis array, the IGFBP10 signal was well detectable, but did not show changes in intensity after DIP or PACAP treatment, while other insulin-like growth

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 alveogenesis/





**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 (Briskin 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  $\beta$ -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). TNF $\alpha$  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  $\beta$ -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

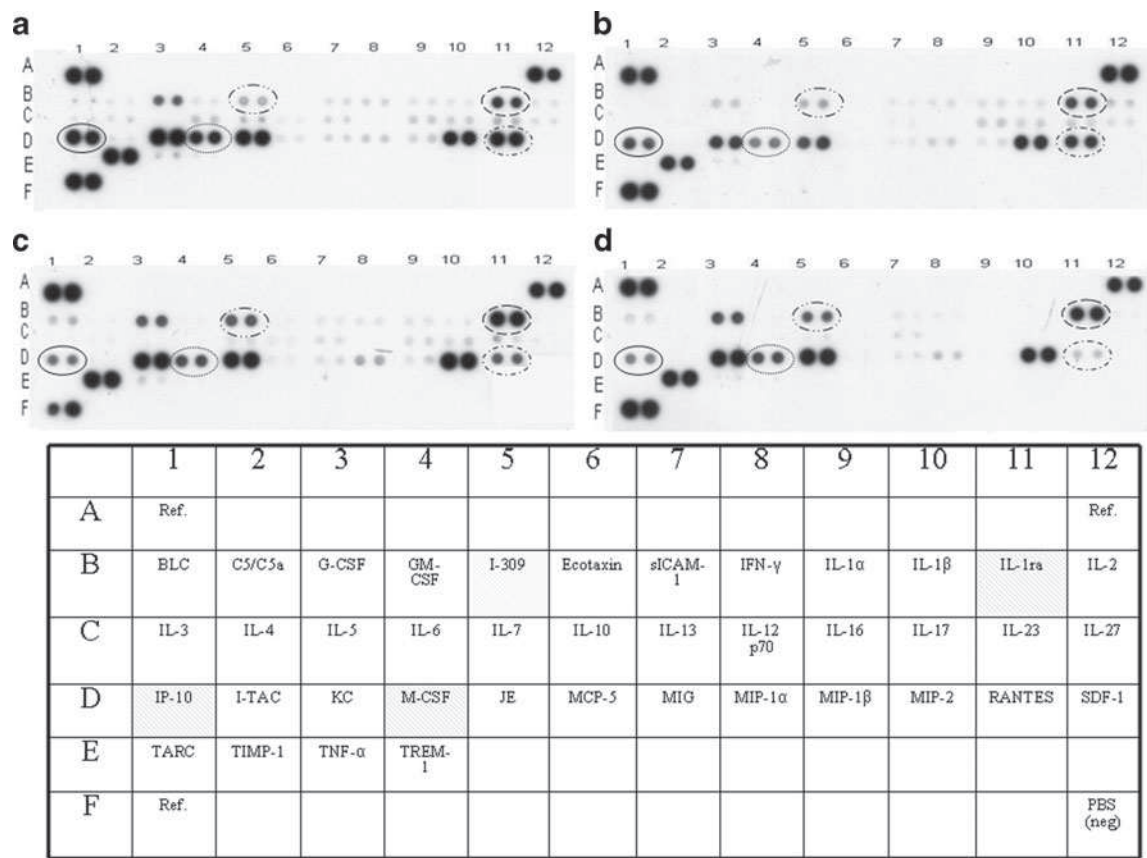
of  $\beta$ -casein gene expression in our study. We extended the characterization of lactogenic hormone-induced differentiation by determining secretion of almost 50 chemokines/cytokines, several growth/angiogenesis-related proteins, and some other factors with the mouse cytokine array panel and angiogenesis 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 TNF $\alpha$  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



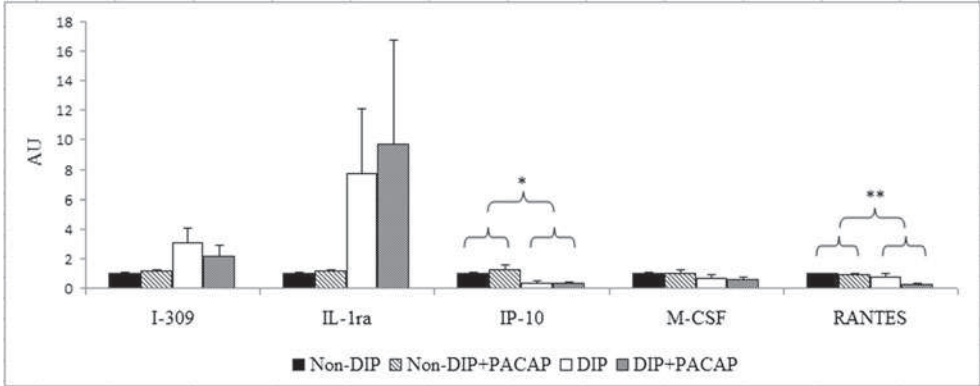
**Fig. 4** Mouse cytokine array panel **a** of non-differentiated (**a, b**) and differentiated HC11 cells (**c, d**) without PACAP (**a, c**) and with PACAP co-incubation (**b, d**). Proteins which show obvious changes in expression

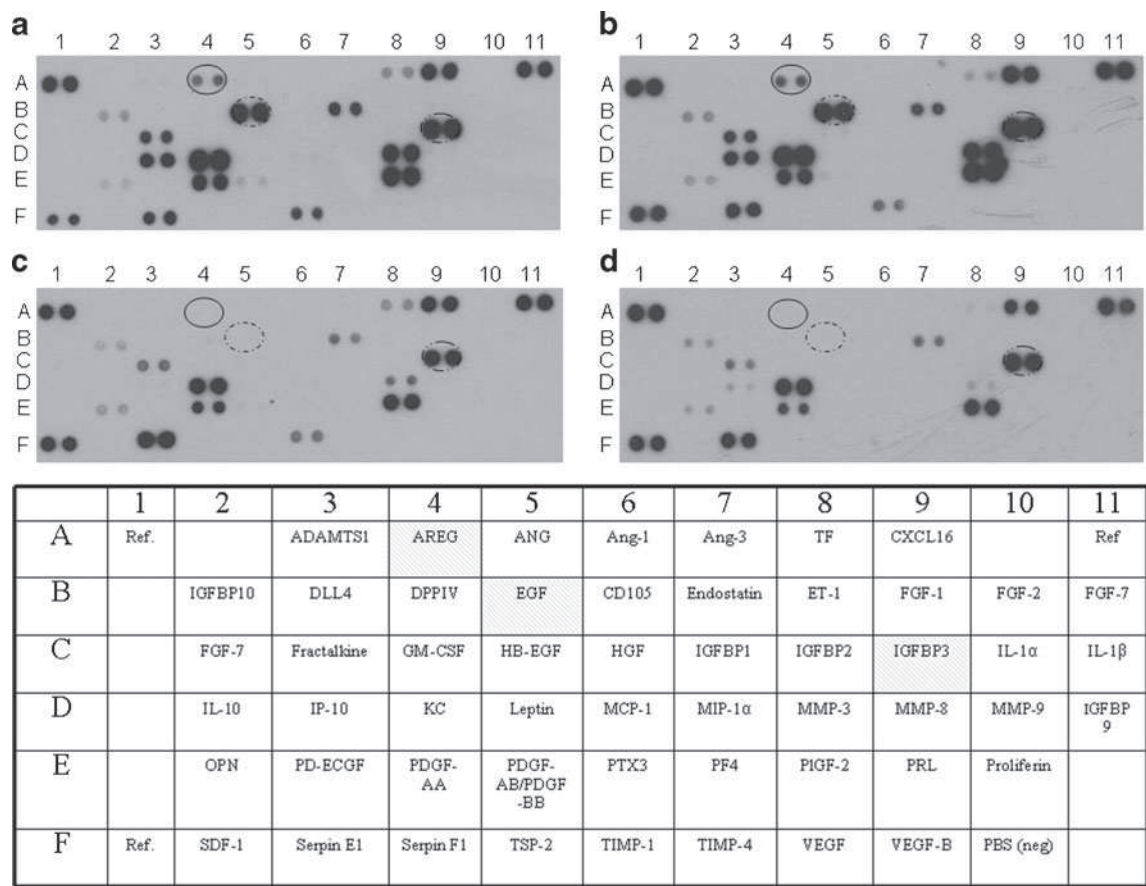
after DIP and/or PACAP treatment are marked by *circles* and comprise B5 = I-309; B11 = IL-1ra; D1 = IP-10; D4 = M-CSF; and D11 = RANTES

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) $\alpha$  are structurally related proteins. While AREG is

specifically required for ductal morphogenesis, EGF and TGF $\alpha$  are dispensable for this process (Luetke et al. 1999). EGF blocks functional differentiation ( $\beta$ -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





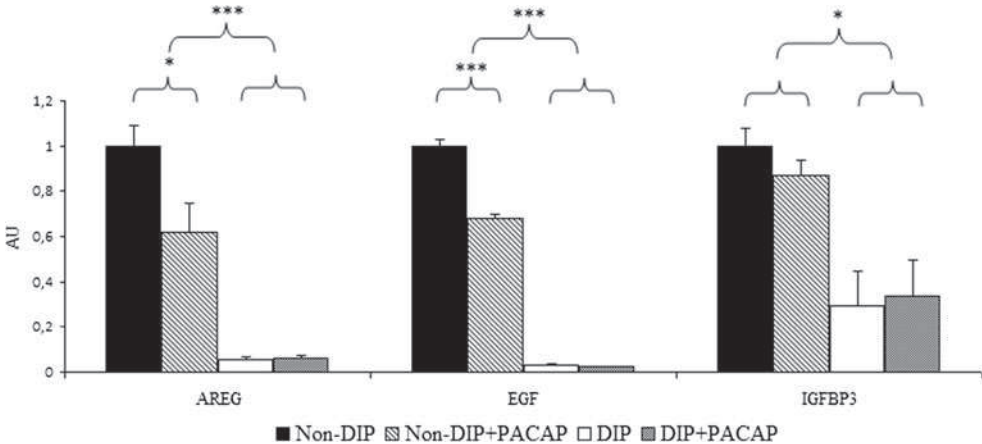
**Fig. 6** Mouse angiogenesis array of non-differentiated (a, b) and differentiated HC11 cells (c, d) without PACAP (a, c) and with PACAP co-incubation (b, d). Proteins which show obvious changes after DIP and/or PACAP treatment are indicated by circles and comprise A4 = AREG, B5 = EGF, and C9 = IGFBP3

the differentiation of HC11 cells either at the level of  $\beta$ -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- $\beta$  expression on PACAP, as evident from TGF- $\beta$  downregulation in PACAP KO mice (Tan et al. 2009), and the role of TGF- $\beta$  in inhibition of ADAM metalloproteinase domain 17 (ADAM17), a metalloproteinase implicated in shedding of AREG, EGF, TGF- $\alpha$ , and activation of EGFR (Sternlicht et al. 2005). TGF- $\beta$  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





435 Whether TGF- $\beta$  is expressed in HC11 cells and modified by  
 436 PACAP remains to be shown in further studies.

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

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

459 In summary, we demonstrated that PACAP had no direct  
 460 effect on the lactogenic hormone-induced terminal differenti-  
 461 ation of HC11 mouse mammary epithelial cells. A significant  
 462 decrease in the release of IP-10/RANTES was detected during  
 463 differentiation which might be relevant for influencing the  
 464 altered recruitment of lymphocytes in the terminal differenti-  
 465 ated gland as it is observed under “in vivo” conditions. The  
 466 decreased secretion of AREG/EGF is considered to contribute  
 467 to the proliferative to lactogenic phase switch in terminal  
 468 differentiated gland. Interestingly, PACAP co-incubation sig-  
 469 nificantly decreased the levels of AREG and EGF secreted  
 470 from non-differentiated mammary cells, which may have  
 471 physiological implications. Furthermore, in the light of the  
 472 prominent role of EGFR signaling in breast cancer, this inhib-  
 473 itory effect of PACAP could be relevant in influencing the  
 474 development and progression of this disease.

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