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EMMPRIN (basigin/CD147) Expression is not Correlated With MMP Activity During Adult Mouse Mammary Gland Development

Malgorzata Szymanowska, Kay A.K. Hendry, Claire Robinson, and Andreas F. Kolb*

Molecular Recognition Group, Hannah Research Institute, Ayr KA6 5HL, Scotland

ABSTRACT

Extracellular matrix metalloproteinase inducer (EMMPRIN/basigin/CD147) is a cell surface protein, which has been associated with the induction of matrix metalloproteinase (MMP) genes during cancer metastasis. EMMPRIN plays a role in a variety of physiological processes as is evident by the diverse deficiencies detectable in EMMPRIN knockout mice. We have analysed the role of EMMPRIN in the induction of MMP genes during mammary gland differentiation and involution. Co-transfection studies showed that EMMPRIN has diverse effects on MMP promoter activity in different mammary and non-mammary cell lines. Expression of EMMPRIN mRNA is enhanced markedly by insulin in a mammary gland cell line but appears to have no direct effect on MMP gene expression in these cells. Microarray analysis and quantitative PCR show that EMMPRIN is expressed throughout mammary gland differentiation in the mouse. Its expression decreases during early pregnancy and briefly after induction of mammary gland involution by litter removal. Immunohistochemical analysis shows that EMMPRIN expression is limited to the stromal compartment during pregnancy, whereas it is strongly expressed in the epithelium during lactation. In summary the data argue against a causal role for EMMPRIN for the induction of MMP gene expression during adult mammary gland development. These data therefore support a physiological role for EMMPRIN other than MMP induction in mammary gland biology. J. Cell. Biochem. 106: 52–62, 2009.

KEY WORDS: MAMMARY GLAND; DEVELOPMENT; MATRIX METALLOPROTEINASE

The mammary gland undergoes extensive tissue remodelling during each lactation cycle. During pregnancy, the epithelial compartment of the gland is vastly expanded [Benaud et al., 1998]. At the end of lactation the epithelial cells undergo apoptosis and adipocyte differentiation is induced [Lilla et al., 2002]. Ductal and alveolar growth during puberty and pregnancy, and the involution process after weaning require the action of proteolytic enzymes including matrix metalloproteinases (MMP), plasminogen and membrane peptidases and the corresponding genes are activated during these periods [Benaud et al., 1998; Alexander et al., 2001; Sorrell et al., 2005].

MMPs are a family of approximately 25 zinc dependent proteinases [Green and Lund, 2005; Sorrell et al., 2005]. Most MMPs are secreted from the cell as zymogens and then activated by proteolytic cleavage. Some MMPs however are membrane bound proteins. The activity of MMPs is regulated at a variety of levels including gene transcription, protein activation (cleavage of MMP precursor zymogens to an active form) and protein activity, which is blocked in the extracellular space by set of cognate inhibitor proteins (TIMPs: tissue inhibitors of MMPs). MMPs play a crucial role in a variety of processes but display a level of redundancy. Inactivation of a single MMP gene therefore rarely causes a life-threatening deficiency. However, inactivation of specific MMPs (e.g. MMP3 or MMP2) has serious effects on ductal branching in the developing mammary gland [Wiseman et al., 2003]. The MMP system cooperates with the plasminogen system in variety of

Abbreviations used: EMMPRIN, extracellular matrix metalloproteinase inducer; GAPDH, glycerol-aldehyde-phosphate-dehydrogenase; MMP, matrix metalloproteinase.

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Malgorzata Szymanowska's present address is Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G1 1XW, Scotland.

Kay A.K. Hendry's present address is AvantiCell Science Limited, Auchincruive, Ayr KA6 5HW, Scotland.

*Correspondence to: Andreas F. Kolb, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, United Kingdom. E-mail: a.kolb@rowett.ac.uk

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situations (e.g. mammary development and wound healing), and concurrent inhibition of both, the MMP and the plasmin system, leads to a complete block of tissue remodelling activity [Bullard et al., 1999; Lund et al., 1999, 2000; Green et al., 2006]. During pregnancy, MMP gene expression is critically induced by signalling through the EGF receptor and inactivation of the receptor leads to an impaired development of the mammary ductal tree [Wiesen et al., 1999].

A number of molecules have been implicated in the regulation of MMP expression and activation during mammary gland development. One of the candidate proteins, which may regulate MMP gene expression in the mammary gland, is EMMPRIN/basigin/CD147 [Nabeshima et al., 2006]. EMMPRIN is a cell surface glycoprotein containing two immunoglobulin-like domains. The crystal structure of its extracellular domain has recently been resolved [Yu et al., 2008]. It was identified as a factor in tumour cells that is able to induce collagenase activity in co-cultivated fibroblasts [Biswas, 1982]. Molecular studies revealed that the protein is identical to other proteins (M6, CD147, basigin, OX47, HT7), which had been identified on the basis of different physiological activities. Purified recombinant human EMMPRIN expressed in CHO cells was able to stimulate MMP1, MMP2 and MMP3 expression in primary human fibroblasts [Guo et al., 1997]. In addition, expression of mouse EMMPRIN using an adenovirus vector also stimulated MMP1 and MMP3 expression in human CCD-16 and NHF-III fibroblasts [Li et al., 2001]. EMMPRIN is involved in a variety of processes in vivo as has been shown by inactivation of the corresponding gene in transgenic mice [Igakura et al., 1998]. The observed defects include failed intercellular recognition during implantation and consequential loss of most homozygous embryos, interstitial pneumonia in the surviving mice, arrested spermatogenesis and resulting infertility. All of these processes could involve the action of MMPs. Nevertheless, it is not clear at present whether these deficiencies are a direct result of impaired MMP gene activation. EMMPRIN is mainly found embedded in the cell membrane but a small proportion of EMMPRIN expressed in breast cancer cells is secreted into the cell culture medium [Taylor et al., 2002]. EMMPRIN is not only able to activate MMP gene expression but is also able to mediate secretion or activation of pre-existing MMP protein [Taylor et al., 2002]. EMMPRIN has also been identified as a prognostic marker associated with poor disease outcome for ovarian and breast cancer [Davidson et al., 2003; Reimers et al., 2004] and small interfering RNAs directed against EMMPRIN inhibit the proliferation, invasiveness and metastatic activity of malignant melanoma cells [Chen et al., 2006]. At present the precise mechanism by which EMMPRIN induces MMP gene expression is not clear. However, EMMPRIN has been found to interact with a number of molecules including $\alpha 3\beta 1$ integrin and $\alpha 6\beta 1$ integrin [Berditchevski et al., 1997], lactate transporters MCT1 and MCT4 [Kirk et al., 2000], cyclophilin-A and B [Yurchenko et al., 2002], caveolin-1 [Tang and Hemler, 2004]. Caveolin inhibits the extensive glycosylation of EMMPRIN to a high glycosylation (HG) isoform, which self-aggregates more efficiently and is more potent in stimulating MMP1 expression [Tang et al., 2004]. Caveolin has previously been implicated as a tumour suppressor protein and the prevention of excessive EMMPRIN glycosylation may represent one mechanism by which it fulfils this role. EMMPRIN has also been reported to undergo homophilic interaction with itself [Sun and Hemler, 2001; Belton et al., 2008] to induce MMP expression and this ability is correlated with the extent of glycosylation. Deglycosylated EMMPRIN antagonizes the induction of MMP gene expression [Sun and Hemler, 2001].

We wanted to determine whether EMMPRIN is an inducer of MMP gene expression during mammary gland development. We therefore analysed the effect of EMMPRIN expression in several mammary and non-mammary cell lines. We also monitored patterns of EMMPRIN expression during the process of adult mammary gland development by using quantitative PCR and immunohistochemistry. Our results demonstrate that in mouse mammary tissue MMP gene expression is not correlated with the abundance of EMMPRIN.

MATERIALS AND METHODS

CELLS

HC11 murine mammary gland cells [Ball et al., 1988] were grown in RPMI 1640 medium containing 10% foetal calf serum (FCS), 2 mM glutamine, 5 μg/ml insulin, 10 ng/ml EGF and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Baby hamster kidney cells (BHK-21, ECACC Ref. No. 85011433) were grown in DMEM medium containing 10% FCS, 2 mM glutamine and antibiotics. Human embryonic kidney cells (HEK 293; ECACC No.: 85120602) were cultivated in DMEM medium supplemented with 10% FCS, antibiotics and 2 mM glutamine. MacT bovine mammary epithelial cells [Huynh et al., 1991] were grown in DMEM medium containing 10% FCS, 2 mM glutamine, 5 μg/ml insulin and antibiotics. HepG2 cells (ATCC order number: HB-8065) were cultivated in minimum essential medium (Eagle) with 10% foetal bovine serum, 2 mM L-glutamine 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Transfections were done in a 24-well plate format using a calcium phosphate protocol. Two micrograms of plasmid DNA were transfected per well. Luciferase activity was determined as described previously [Kolb, 2002]. At least three independent measurements were made for each data point. Transfection efficiency in transient transfections was controlled by Southern blot analysis of cellular DNA using a probe corresponding to the luciferase gene. Transfections were done with identical amounts of DNA. For co-transfections negative control experiments contained empty vector plasmid. Lactogenic hormone induction in HC11 cells was done in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 5 μg/ml insulin, 5 μg/ml prolactin, 1 μg/ml hydroxicortisone and antibiotics. HC11 cells were grown to density and incubated at confluence for 56 h before the induction medium was added.

DNA PLASMIDS

The generation of the plasmid pT-MMP3-luc was described before [Flint et al., 2006]. The plasmids pT-MMP3-luc1, pT-MMP3-luc2 and pT-MMP3-luc3 were derived from pT-MMP3-luc after excision of promoter fragments using *Bam*HI/*Nsi*I, *Bam*HI/*Xmn*I and *Bam*HI/*Bst*XI digestion, respectively. This results in luciferase reporter plasmids in which expression of the marker gene is driven by fragments of 732 bp (pT-MMP3-luc1), 176 bp (pT-MMP3-luc2) or 59 bp (pT-MMP3-luc3) of the MMP3 promoter (cf. Fig. 2a). The plasmid pCR3-hEMMPRIN was a gift from Bryan Toole (Department

of Anatomy and Cellular Biology, Tufts University, School of Medicine, Boston, MA).

RNA ANALYSIS

Total RNA was extracted from the mammary gland and HC11 cells samples and 2 μg of RNA were reverse transcribed as described previously [Kolb, 2002; Flint et al., 2006]. Quantitative PCR was carried out using a Roche Light Cycler system and a Fast Start DNA Master SYBR Green I Kit (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. PCR amplifications were done with a final primer concentration of 0.5 μM and a $MgCl_2$ concentration of 3 mM. Primer design was done using the Primer Select program of the DNA Star software suite. The sequences, annealing temperatures and amplicon sizes of the oligonucleotides used in this study are provided in Table I. The PCR products obtained by quantitative PCR were evaluated by melting point analysis and agarose gel electrophoresis. Thermal cycling was carried out using the following conditions: initial denaturation was done by incubation at 95°C for 10 min; subsequently 40 or 50 cycles of amplification were done with 15 s at 95°C, 5 s at the specific annealing temperature for each gene (Table I), 30 s at 72°C. A single fluorescence acquisition point was specified for each gene at the end of each cycle. At the end of the PCR amplification the melting curve of the products was analysed by incubation at 95°C for 0 s, 57°C for 15 s and a linear temperature transition at 0.05°C/s from 57 to 95 °C with continuous fluorescence acquisition. Calibration curves for the MMP-3, MMP-12, β-casein, GAPDH and β-actin genes were generated from serial dilutions of a plasmid containing the cDNA for each gene.

Microarray data were provided by Dr. Torsten Stein (Institute of Pathology, University of Glasgow, UK). Data were derived from triplicate experiments after hybridisation to the Affymetrix MG-U74Av2 chip and raw data analysis using the Microarray Suite 5.0 software as described [Stein et al., 2004].

IMMUNOHISTOCHEMISTRY

Sections of mouse mammary tissue (5 μM thickness) were transferred to glass slides, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) and washed twice in 1× PBS. The tissue section was the incubated in the presence of 25% horse serum for 30 min at room temperature. Subsequently the tissue was exposed to a 1:100 dilution of the primary antibody at 37°C for 90 min, washed three times for 10 min in 1× PBS-Tween and then incubated in the presence of a 1:100 dilution of the secondary antibody (FITC-linked rabbit anti-goat IgG, Vector Laboratories) at room temperature for 90 min The samples were then washed again three times in 1× PBS-Tween and mounted in 20 μl of a Vectashield solution (Vector Laboratories) containing DAPI. Slides were then photographed under a Leica inverted microscope at the indicated magnifications. A 1:100 dilution of a pre-immune goat serum was used as control for the primary antibody treatment. Tissue culture cells were seeded on sterile glass-coverslips and treated in the same way as tissue sections.

WESTERN BLOT ANALYSIS

In order to prepare cellular protein extracts for Western blot analysis cells were scraped in a buffer containing 25 mM K_2HPO_4/KH_2PO_4 pH 8.0, 8 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 15% glycerol and a broad spectrum proteinase inhibitor mix (SIGMA P-8340) at a concentration of 1 ml/10^9 cells. Cell fragments including nuclei were pelleted by short centrifugation (1 min at 12,000 rpm) at room temperature in a bench-top centrifuge and the supernatant was mixed with 1/4 volume of a 4× concentrated protein sample buffer (final concentration: 60 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 25 mM DTT, 0.002% bromophenol blue) for SDS–polyacrylamide gel analysis. The samples were aliquoted and stored at −80°C. Protein samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose as described [Kolb et al., 1996]. Western blots were developed using a chemiluminescent detection system (Pierce) and X-ray films (Fuji). A goat anti-human EMMPRIN antiserum (Santa Cruz: sc-9756) and a goat-anti-mouse EMMPRIN antiserum (Santa Cruz: sc-9757) were used as primary antibodies. A horseradish peroxidase-linked rabbit-anti-goat IgG antiserum was used as secondary antibody. A biotinylated ladder of molecular weight marker proteins (Cell Signaling Technology) was used in conjunction with a horseradish peroxidase linked rabbit-anti-biotin antiserum to determine the molecular weights of the resolved proteins.

RESULTS

ANALYSIS OF MMP EXPRESSION IN RESPONSE TO EMMPRIN CO-TRANSFECTION IN MAMMARY AND NON-MAMMARY CELL LINES

As a first step of our analysis we investigated the ability of EMMPRIN to augment the activity of the MMP3 promoter activity in

TABLE I. Oligonucleotides Used for Quantitative PCR

Name	Length	Sequence	Annealing temperature (°C)	Amplicon length (bp)
b-actin1	23mer	5′ GTC GAC AAC GGS TCC GSC ATG TG 3′		
b-actin2	23mer	5′ CTG TCR GCR ATG CCW GGG TAC AT 3′	60	908
EMMPRIN-1	24mer	5′ GTC CGA TGC ATC CTA CCC TCC TAT 3′		
EMMPRIN-2	20mer	5′ CCC GCC TGC CCC ACC ACT CA 3′	59	459
bcas21	23mer	5′ GAT GCC CCT CCT TAA CTC TGA AA 3′		
bcas22	22mer	5′ TTG TGG AAG GAA GGG TGC TAC T 3′	56	226
GAPDH1	23mer	5′ GCT TTC CAG AGG GGC CAT CCA CA 3′		
GAPDH2	25mer	5′ ACG GCA AAT TCA ACG GCA CAG TCA A 3′	61	412
MMP3-3	24mer	5′ ACC CAG TCT ACA AGT CCT CCA CAG 3′		
MMP3-4	21mer	5′ GCA GCA TCG ATC TTC TTC ACG 3′	56	463
MMP12-1	20mer	5′ CCC AGC ACA TTT CGC CTC TC 3′		
MMP12-2	24mer	5′ AGC TCC TGC CTC ACA TCA TAC CTC 3′	54	494

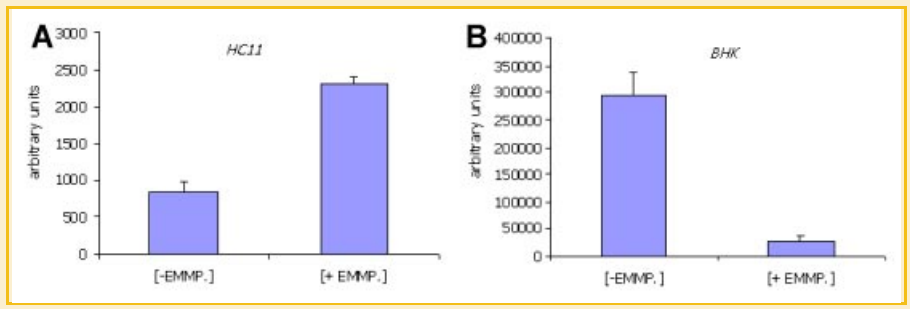

Fig. 1. Influence of EMMPRIN co-transfection on the expression of a MMP3-luc reporter gene in HC11 (panel A) and BHK cells (panel B). Cells were co-transfected with 1 μg of the pCR3-hEMMPRIN expression vector [+EMMP] or an empty control vector [−EMMP] together with 1 μg of the reporter construct (pT-MMP3-luc). Luciferase activity was measured in the cellular protein extracts 48 h post transfection. Data are presented as arbitrary units derived from 3 experiments.

transfected cell lines. For that purpose we expressed the human EMMPRIN gene from an expression vector (a gift from Bryan Toole, Tufts University) in a number of mammary gland and non-mammary cell lines together with a MMP3-promoter driven luciferase reporter gene (pT-MMP3-luc) and measured the induction of luciferase activity.

In HC11 mouse mammary gland cells we observed a moderate (2.5fold) increase in pT-MMP3-luc expression upon co-transfection of the EMMPRIN expression construct (Fig. 1a). In contrast, repression of MMP3 promoter activity was detected under identical experimental conditions in BHK cells (to 10% of control levels; Fig. 1b) and the bovine mammary gland cell line MacT (to 15% of control levels, Fig. 2b). In HEK 293 cells, which have been shown to express EMMPRIN [Tang et al., 2004], the reporter construct was insensitive to the co-expression of the EMMPRIN gene (data not shown).

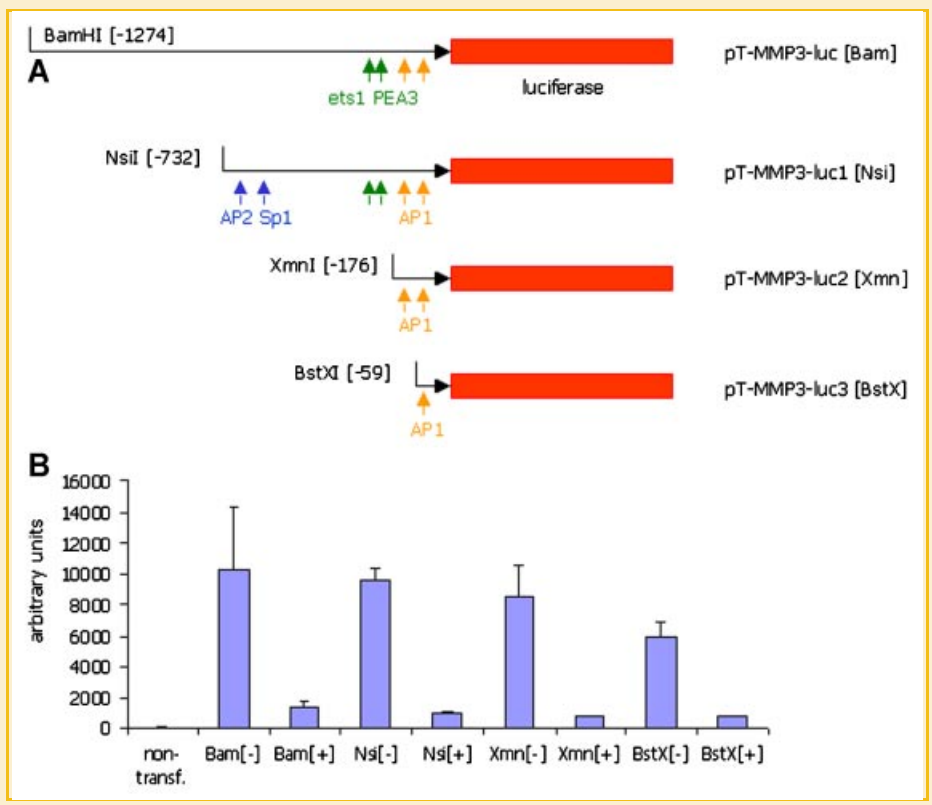

Fig. 2. Influence of EMMPRIN co-transfection on the expression of a MMP3-luc reporter gene in MacT bovine mammary gland cells. Panel A: Schematic representation of the reporter gene constructs pT-MMP3-luc, pT-MMP3-luc1, pT-MMP3-luc2, pT-MMP3-luc3. Restriction sites used to generate the promoter deletion mutants are indicated. The number in brackets indicates the position of the restriction site relative to the predicted MMP3 transcriptional initiation site. Vertical arrows mark predicted binding sites for important transcription factors. Panel B: Luciferase expression in MacT cells transfected with 1 μg of the reporter constructs pT-MM3-luc (*Bam*), pT-MMP3-luc1 (*Nsi*), pT-MMP3-luc2 (*Xmn*) and pT-MMP3-luc3 (*Bst*X). Cells were co-transfected with 1 μg of the pCR3-hEMMPRIN expression vector [+] or an empty control vector [−]. Luciferase activity was measured in cellular protein extracts isolated 48 h post transfection. Data are presented as arbitrary units derived from three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

A series of deletion mutants of the MMP3 promoter was generated to delineate the sequences involved in the EMMPRIN mediated suppression of MMP3 activity. The positions of ets and AP1 transcription factor binding sites are indicated in Figure 2a. These sites have been shown to mediate most of the regulatory events at MMP promoter elements [Sorrell et al., 2005]. In MacT cells all four constructs show an EMMPRIN mediated down-regulation; even the construct pT-MMP3-luc3, which contains only a 59 bp minimal MMP3 promoter. This suggests that EMMPRIN interferes with the activity of the MMP3 promoter via regulatory circuits converging at the AP1 transcription factor-binding site. The suppression of the MMP3 promoter activity by EMMPRIN could also be reproduced in the context of a different reporter construct based on the vector pBK-CMV confirming that the regulatory effects are independent of vector sequences (data not shown).

Immunohistochemical analysis demonstrated that the EMMPRIN protein in transfected cells was localised to membranes (cell membrane and intracellular membranes) as shown for the exemplary BHK cells in Figure 3b. No staining is visible in cells transfected with a control vector (Fig. 3d). In HepG2 cells EMMPRIN localisation was more diffuse (Fig. 3a). EMMPRIN's ability to induce MMP gene expression has been shown to depend on its glycoslation pattern. Only the highly glycosylated isoform of the protein is able to polymerise in the membrane and induce MMP expression in neighbouring cells [Sun and Hemler, 2001; Tang and Hemler, 2004]. The transfection of BHK, HC11, MacT and HEK 293 cells only leads to the synthesis of a low glycosylation isoform of EMMPRIN (with an apparent molecular weight of around 37 kDa) as evidenced by Western blotting (Fig. 4). In contrast, HepG2 cells express a highly glycosylated isoform of EMMPRIN (with an apparent molecular weight of 60 kDa; Fig. 4a). In HEK 293 and BHK cells a more glycosylated isoform of EMMPRIN can also be detected (apparent molecular weights of 50 and 58 kDa, respectively), whose concentration is not augmented by transfection of the EMMPRIN expression construct (Fig. 4a). This may due to a limited glycosylation capacity for EMMPRIN, which restricts the generation of a higher concentration of highly glycosylated EMMPRIN. It should be noted that the construct used in these experiments was previously used to stably transfect CHO cells where it directed synthesis of a high glycosylation isoform of EMMPRIN [Guo et al., 1997].

ANALYSIS OF MMP AND EMMPRIN EXPRESSION IN RESPONSE TO HORMONE TREATMENT IN VITRO

The transfection data do not support a role for EMMPRIN in the induction of MMP genes in mammary cell lines. In order to confirm these data we investigated the regulation of the endogenous MMP and EMMPRIN genes in the mouse mammary gland cell line HC11. MMP gene expression is sensitive to glucocorticoid hormones and

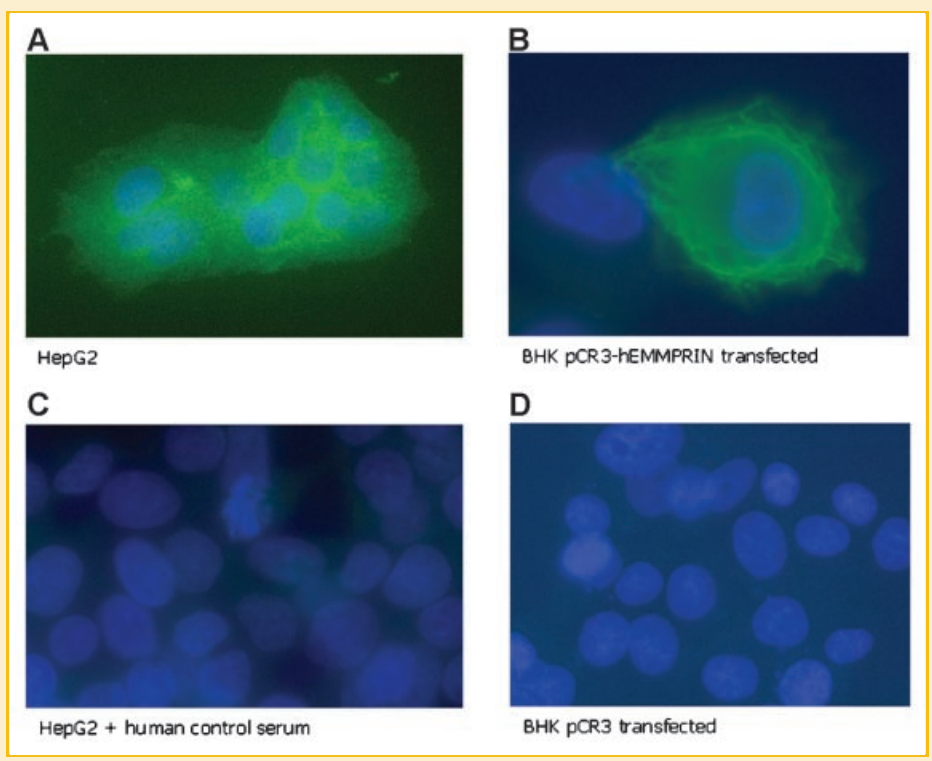

Fig. 3. Immunohistochemical analysis of EMMPRIN expression in HepG2 cells (panels A and C) and BHK cells transfected with the plasmid pCR3-hEMMPRIN (panel B) or empty control vector (panel D). Cells were seeded on glass slides and transfected (in the case of BHK cells). Forty-eight hours post transfection cells were analysed for EMMPRIN expression. A 1:100 dilution of a goat anti-human EMMPRIN antiserum (Santa Cruz: sc-9752) was used as primary antibody on cells in panel A, B and D. A 1:100 dilution of a goat anti-human control antiserum was used as primary antibody on cells in panel C. All samples were then incubated with a rabbit anti-goat-FITC antiserum (Vector Laboratories) as secondary antibody, mounted in Vectashield mounting reagent with DAPI and then photographed on a Leica inverted microscope at a 1,000fold magnification.

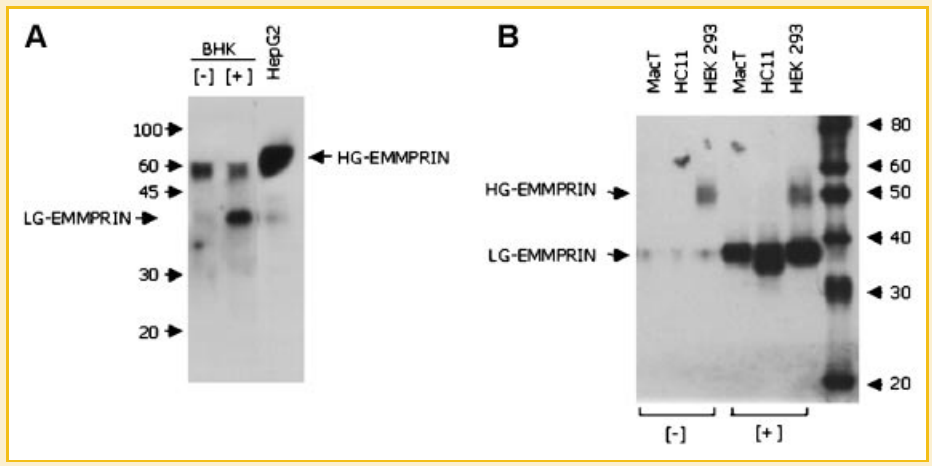

Fig. 4. Western blot analysis of EMMPRIN expression. Panel A: Cellular protein extracts isolated from HepG2 cells and BHK cells transfected with the expression vector pCR3-EMMPRIN [+] or empty vector [−] were separated on a 10% polyacrylamide gel and blotted to nitrocellulose. EMMPRIN protein was detected with an anti-human EMMPRIN antiserum (Santa Cruz: sc-9756). The positions of the low glycosylation (LG-EMMPRIN) and high glycosylation (HG-EMMPRIN) isoforms of EMMPRIN are indicated by arrows. Panel B: Cellular protein extracts isolated from MacT, HC11 and HEK 293 cells transfected with the expression vector pCR3-hEMMPRIN [+] or empty vector [−] were separated on a 10% polyacrylamide gel and blotted to nitrocellulose. EMMPRIN protein was detected as above.

prolactin [Lund et al., 1996; Flint et al., 2006]. In order to test whether these influences are mediated by EMMPRIN we studied the expression of MMP and EMMPRIN in response to hormone induction. HC11 cells can be induced to express the milk protein gene β-casein when treated with a combination of hormones including prolactin, glucocortiocoids and insulin. The response of β-casein transcription to stimulation with individual hormones or combinations thereof is shown in Figure 5a and demonstrates a large

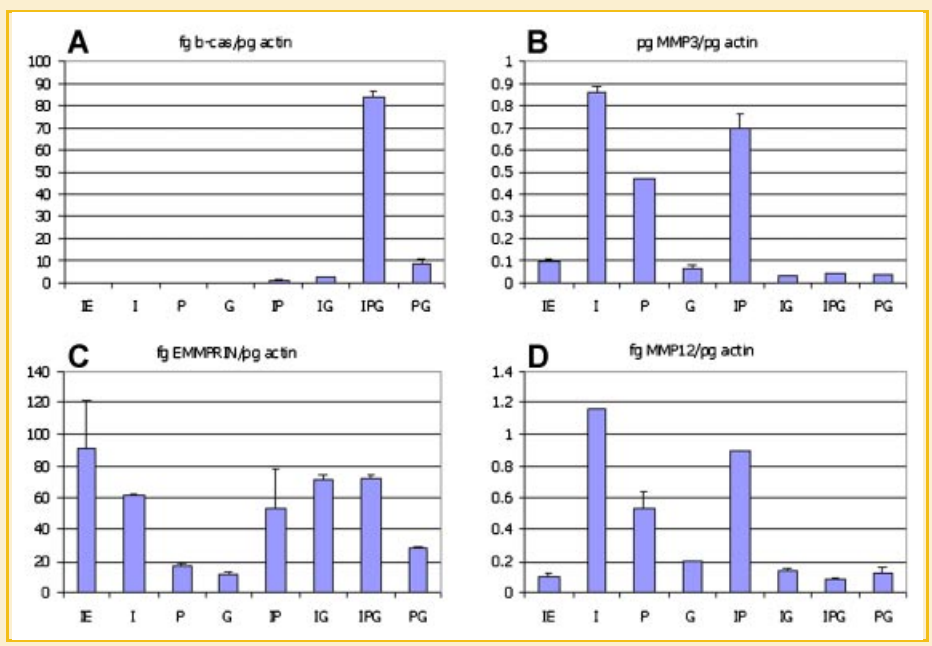

Fig. 5. Hormone responsiveness of mouse EMMPRIN (basigin), MMP12, MMP3 and β-casein expression in the mouse mammary gland cell line HC11: Cells were grown to confluence for 3 days and then treated with combinations of the following hormones and growth factors: Insulin (I; final concentration 5 μg/ml), EGF (E; final concentration 10 ng/ml), prolactin (P; final concentration 5 μg/ml), hydrocortisone (G; final concentration 1 μg/ml). Total RNA was isolated and reverse transcribed. Aliquots of the cDNA reactions were used as template for quantitative PCR with primer sets for mouse EMMPRIN (primer pair: EMMPRIN-1/EMMPRIN-2), MMP3 (primer pair: MMP3-3/MMP3-4), β-casein (primer pair: bcas21/bcas22) and MMP12 (primer pair: MMP12-1/MMP12-2) (Table I). Expression of MMP3, EMMPRIN, MMP12 and β-casein was correlated with actin expression (primer pair: b-actin1/b-actin2). Panel A: Expression of β-casein shown as fg β-casein cDNA/pg of β-actin cDNA. Panel B: Expression of MMP3 shown as pg MMP3 cDNA/pg of β-actin cDNA. Panel C: Expression of EMMPRIN shown as fg EMMPRIN cDNA/pg of β-actin cDNA. Panel D: Expression of MMP12 shown as fg MMP12 cDNA/pg of β-actin cDNA.

increase in β-casein expression when cells are treated with all three hormones. Expression of MMP3 and MMP12 is dramatically reduced as soon as a glucocorticoid hormone (hydroxycortisone) is included in the medium. The inhibitory effect of glucocorticoids is also seen in various cell lines transfected with a MMP3-promoter driven luciferase reporter construct (data not shown), which is consistent with published data [Lund et al., 1996; Flint et al., 2006]. Prolactin induces a slight reduction in expression of both MMP3 and MMP12. EMMPRIN expression, in contrast, is significantly reduced (to about 30%) if insulin is omitted from the cell culture medium, irrespective of the other medium components. Other hormone combinations have no significant influence on EMMPRIN expression (Fig. 5c). These data demonstrate that expression of MMP3 and MMP12 is not directly correlated with the expression of EMMPRIN in the mammary gland cell line HC11. The data also establish insulin as an inducer of EMMPRIN expression in HC11 cells.

ANALYSIS OF EMMPRIN EXPRESSION DURING MOUSE MAMMARY GLAND DEVELOPMENT

Next we analysed the expression of EMMPRIN during mammary gland development in the mouse using quantitative PCR. The data, which correlate mouse EMMPRIN expression with that of the house-keeping gene GAPDH, demonstrate that EMMPRIN mRNA is detectable throughout mammary development (Fig. 6a). Expression is decreased to about 50% at the onset of lactation and increased again during peak lactation. Induction of mammary involution by litter removal leads to a slight decrease in expression at day 1 and day 2, post-litter removal, which is partly reversed at later stages of involution. In contrast there are characteristic peaks of expression for the representative proteinase MMP3 (Fig. 6b) with peaks of expression during pregnancy and involution. A large number of other MMPs and other proteolytic enzymes mimic this expression pattern [Sorrell et al., 2005; Flint et al., 2006]. If the expression of MMP3 and EMMPRIN are put in relationship to one another, the expression pattern largely follows that of MMP3 (Fig. 6d). These data are also corroborated when the data set from a recently published microarray experiment [Stein et al., 2004] was examined for expression patterns of EMMPRIN and MMP genes during mammary gland development (at 17 time points). The microarray data demonstrate that when the expression of MMP3 is correlated with that of EMMPRIN, the pattern of MMP3 expression is largely conserved (Fig. 7). If the expression profile of MMP3 were directly dependent on the expression of EMMPRIN one would expect a constant value throughout mammary development.

We subsequently analysed the expression of EMMPRIN in the process of mammary gland development by using immunohistochemistry. In the virgin mammary gland, expression of EMMPRIN is localised to the tissue underlying the mammary epithelium but absent from the epithelial cell population itself (Fig. 8a). Likewise during pregnancy, expression of EMMPRIN is weak in the epithelial portion of the gland but detectable in the underlying stromal tissue (Fig. 8b,c). During late pregnancy and lactation, expression of EMMPRIN is abundant in the epithelial cells of the mammary gland (Fig. 8d). This is consistent with data published for the expression of human EMMPRIN in mammary tissue [Caudroy et al., 1999]. Double labelling of mammary tissue with the antiserum against EMMPRIN

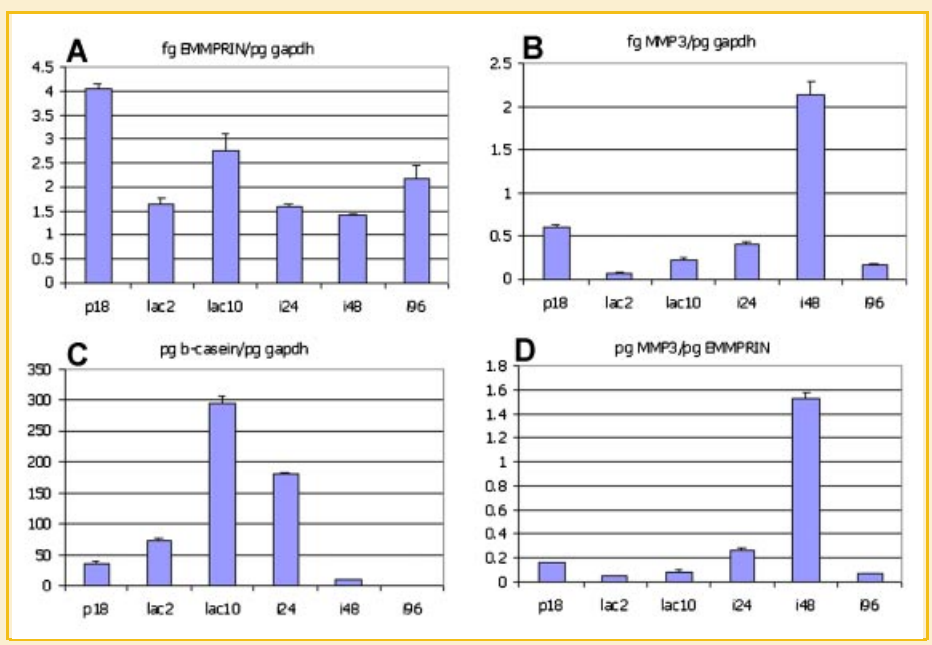

Fig. 6. Expression profile of mouse EMMPRIN (basigin), MMP3 and β-casein during mouse mammary gland development. Total RNA was isolated from tissue samples at day 18 of pregnancy (p18), day 2 and 10 of lactation (lac2, lac10) and 24, 48 and 96 h post induction of involution by litter removal. RNA was reverse transcribed and aliquots of cDNA used for quantitative PCR using the Light Cycler system (Roche) using primer sets for mouse EMMPRIN (basigin), MMP3, β-casein and glycerol-aldehyde-phosphate-dehydrogenase (GAPDH; primer pair: GAPDH1/GAPDH2). Panel A: Expression of EMMPRIN shown as fg EMMPRIN cDNA/pg of GAPDH cDNA. Panel B: Expression of MMP3 shown as fg MMP3 cDNA/pg of GAPDH cDNA. Panel C: Expression of β-casein shown as pg β-casein cDNA/pg of GAPDH cDNA. Panel D: Expression of MMP3 shown as pg MMP3 cDNA/pg of EMMPRIN cDNA.

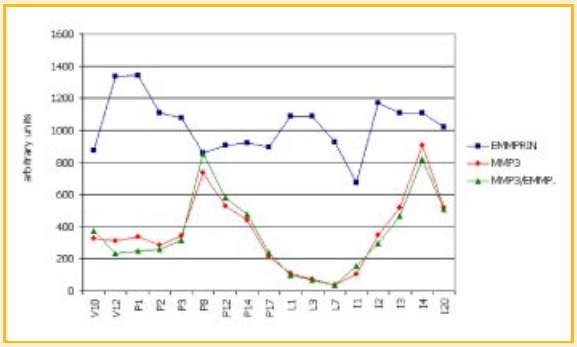

Fig. 7. Microarray data of mouse EMMPRIN (basigin) and MMP3 expression (in arbitrary units) during mammary gland development: [v] virgin (age in weeks), [p] pregnant, [l] lactating, [i] involuting (all in days) [Stein et al., 2004]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and an antiserum directed against mouse milk proteins stains the same cells (data not shown). During the early stages of involution (day 1 and 2 post-litter removal) expression is localised to the remaining epithelial cells, whereas at later stages (day 4 post-litter removal) EMMPRIN protein is detectable in most cells of the gland (Fig. 8e). The EMMPRIN protein was found localised to the cell membrane and intracellular membranes (similar to its distribution in transfected cells, e.g. in Fig. 2b).

Western blot analysis of protein extracts derived from several stages of mammary gland development confirms the histochemical findings in that protein expression levels are low during pregnancy but high during lactation and involution (Fig. 9a). In mammary tissue a double band of 41 and 42 kDa can be detected whereas in liver extracts, three isoforms of the EMMPRIN protein with molecular weights of 37, 42 and 45 kDa are detected (with the 42 kDa form prevalent). The EMMPRIN glycosylation isoforms detected in mammary tissue display a molecular weight that is different to both the low glycosylation form of human EMMPRIN detected in transfected cells (37 kDa, cf. Fig. 4b) and the high glycosylation form detected, for example in HepG2 cells (60 kDa, Fig. 4a). A mouse EMMPRIN gene expressed in NHF-III fibroblasts leads to the synthesis of two glycosylation isoforms with apparent molecular weights of 45 and 59 kDa [Li et al., 2001] the smaller of which has a weight similar to the protein detected in mammary tissue. The 37-kDa glycosylation isoform detected in liver has approximately the same molecular weight as the isoform detected in transfected cells.

DISCUSSION

EMMPRIN has been characterised as an important inducer of MMP expression in tumour tissue [Biswas et al., 1995] and is active in a number of cell culture systems [Li et al., 2001]. It can act as a homophilic receptor for itself in cell culture experiments [Sun and Hemler, 2001] and glycosylation is a major contributor to EMMPRIN's ability to induce MMP gene expression [Tang and Hemler, 2004]. However, in some cell culture systems bacterially expressed (and hence non-glycosylated) EMMPRIN is also able to induce expression of MMP genes [Belton et al., 2008].

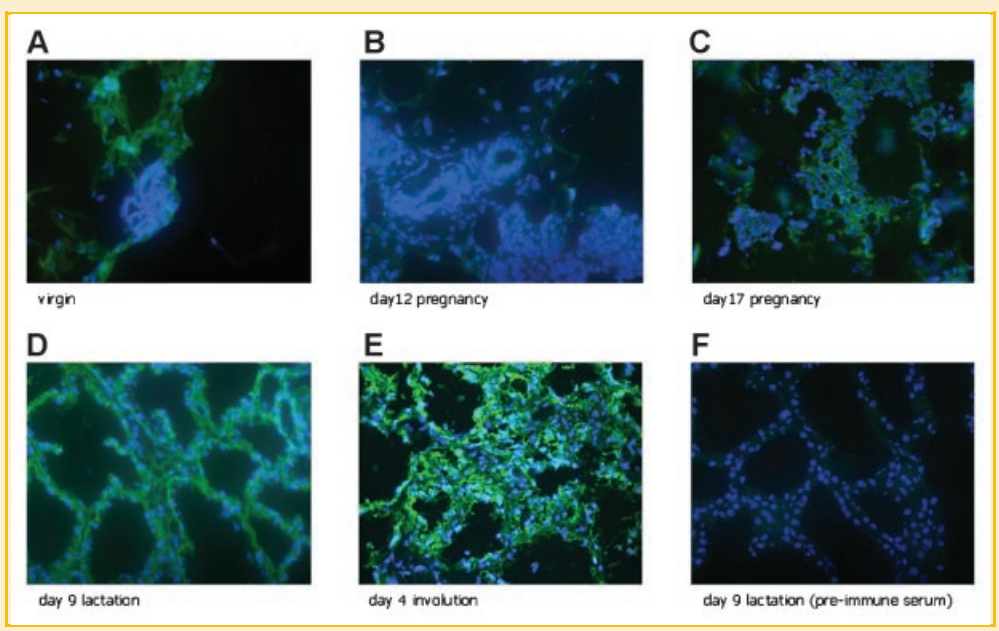

Fig. 8. Immunohistochemical analysis of EMMPRIN expression. Sections from mammary gland tissue taken from virgin (panel A), 12 days pregnant (panel B), 17 days pregnant (panel C), 9 days lactating mice (panel D, F) and mice 4 days after involution was induced by litter removal (panel E) were analysed using a goat anti-mouse EMMPRIN serum (Santa Cruz: sc 9757; panels A–E) or a goat pre-immune serum (panel F) and a FITC linked rabbit anti-goat IgG serum as secondary antibody. Sections were processed as described in Materials and Methods Section and photographed at 400fold magnification.

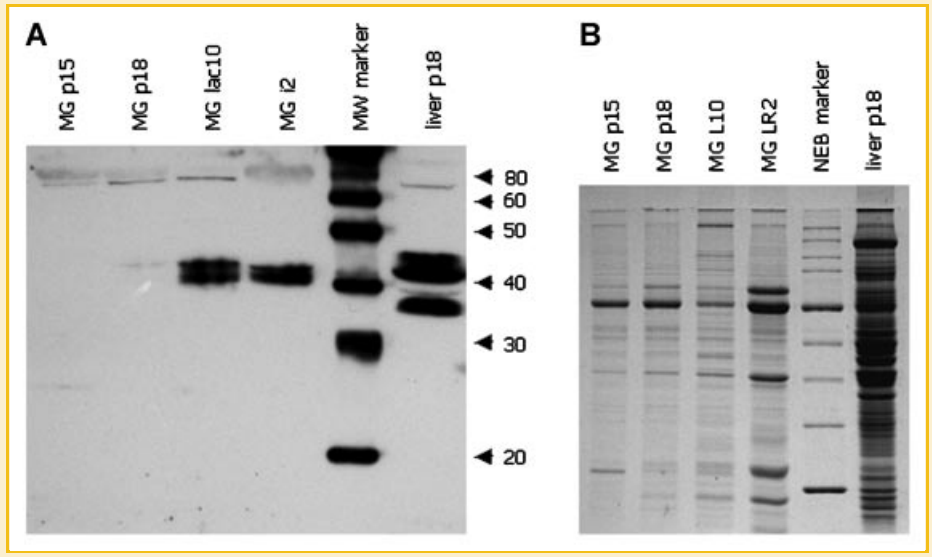

Fig. 9. Western blot analysis of mouse EMMPRIN expression: Cellular protein extracts were isolated from 50 μg of mammary gland tissue from 15 days pregnant (MG p15), 18 days pregnant (MG p18) and 10 days lactating mice (MG lac10) and mice 2 days after involution was induced by litter removal (MG i2). As control, cellular protein extracts were isolated from 50 μg of mouse liver tissue (also isolated at day 18 of pregnancy (liver p18)). Panel A: 10 μl of extract were separated on a 10% polyacrylamide gel and blotted to nitrocellulose. EMMPRIN protein was detected using a goat-anti mouse EMMPRIN antiserum (Santa Cruz: sc 9757). Panel B: 10 μl of extract were separated on a 10% polyacrylamide gel and stained with Coomassie Blue.

Inactivation of the EMMPRIN gene in mice leads to a variety of defects and only a small proportion of homozygous knockout mice survive [Igakura et al., 1998]. Presumably due to deficiencies in embryo implantation, homozygous EMMPRIN/basigin deficient mice are not able to reproduce. However, as EMMPRIN fulfils a variety of functions it is not clear whether implantation failure is a direct result of defective induction of MMP genes.

Two findings have implicated EMMPRIN in the regulation of MMPs in the mammary gland. Firstly, EGF a major regulator of MMP activity and mammary gland development induces EMMPRIN expression [Wiesen et al., 1999; Menashi et al., 2003]. Secondly, caveolin-1 has been identified as a factor determining the amount of highly glycosylated EMMPRIN that is transported to the cells surface [Tang and Hemler, 2004]. During mammary gland development the rate of caveolin expression is high in the virgin gland but decreases during pregnancy. It remains low during lactation and only increases during the very late stages of involution [Master et al., 2002]. Prolactin has been identified as a major suppressor of caveolin expression and caveolin knockout mice show an increased stem cell population which in turn leads to the development of a pre-malignant phenotype of primary mammary epithelial cells [Williams et al., 2004; Sotgia et al., 2005, 2006]. Especially the increased ability of the caveolin deficient mammary acini to undergo branching may point to an increase of MMP activity [Sotgia et al., 2006]. EMMPRIN therefore is a plausible candidate protein, which may mediate the effect of caveolin on MMP expression.

This prompted us to directly assess the correlation between EMMPRIN expression and MMP expression in mammary tissue. We have addressed this question by comparing the expression profiles of EMMPRIN and several MMP genes during mammary gland development in vivo and in a mammary gland cell line in vitro using microarray analysis, quantitative PCR and immunohistochemistry. In both cases we were unable to find any correlation of EMMPRIN and MMP expression at the RNA or protein level. In co-transfection experiments MMP gene expression is slightly activated in one mammary gland cell line and significantly reduced in another. We could also show that the protein localises to the membranes of transfected cells.

The predominant EMMPRIN protein generated after transfection in all cell lines analysed (including BHK, HEK 293, HC11 and MacT cells) is of low molecular weight that is around 37 kDa. Transfection derived EMMPRIN also fails to augment the existing levels of highly glycosylated EMMPRIN in HEK 293 cells. This confirms published data, which indicate that in many cell types (including CHO and COS cells), transfection of an EMMPRIN expression construct leads to the synthesis of a low glycosylation isoform [Guo et al., 1997], which is unable to stimulate MMP gene expression. Synthesis of the high glycosylation form of around 50–60 kDa has been reported after stable transfection of an EMMPRIN expression construct (which leads to the synthesis of the highly glycosylated isoform in some cell clones) [Guo et al., 1997] or after delivery of the EMMPRIN expression cassette in the context of an adenovirus vector [Li et al., 2001]. At present it is unclear how the method of DNA transfer might influence EMMPRIN glycosylation. The variation may be due to the different expression levels achieved in transient and stable transfections; only the latter generating the abundant levels of EMMPRIN protein required to overcome the inhibition that caveolin exerts on EMMPRIN glycosylation. Alternatively, the variation in glycosylation may be a consequence of the requirement for co-expression of several EMMPRIN splice-variants, which can be derived from the endogenous gene but not from a transfected cDNA [Belton et al., 2008].

Western blot analysis of mammary gland tissue shows abundant expression of EMMPRIN protein during lactation and early involution. However, the apparent size of the detected protein indicates an intermediate level of glycosylation. The highly glycosylated isoform of EMMPRIN (of around 58 kDa) associated with its ability to induce MMP expression was not detected in mammary tissue.

In summary, we find no correlation between EMMPRIN and MMP expression in the adult mouse mammary gland in our experimental systems. The MMP-inducing activity of EMMPRIN may therefore not be required for normal mammary gland development although other activities, like the interaction with cell surface integrins, may be crucial [Berditchevski et al., 1997]. However, glycosylation patterns may be altered in breast cancer allowing a high glycosylation isoform to be generated (maybe influenced by availability of caveolin). Under these circumstances EMMPRIN may assume a tumour-promoting role.

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