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Regulation of genes encoding proteolytic enzymes during mammary gland development

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Introduction

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 require the action of proteolytic enzymes (including matrix metalloproteinases, plasminogen and membrane-peptidases) and the corresponding genes are activated during these periods (Benaud et al. 1998; Alexander et al. 2001). Matrix metalloproteinases (MMP) are expressed in several cell types of the mammary gland including stromal fibroblasts (e.g., MMP3, MMP2), epithelial cells (e.g., MMP7 or MMP9), adipocytes (e.g., MMP2) and lymphoid cells (e.g., MMP9) (Crawford et al. 1996; Lund et al. 1996; Wiseman et al. 2003). A number of knock-out mice, which are deficient for individual MMP genes (e.g., MMP2, MMP3) or plasminogen, display alterations to mammary gland structure and impairment of lactation (Lund et al. 1999; Wiseman et al. 2003).

Mammary gland involution proceeds in two distinct phases: a first, potentially p53-dependent, partly reversible phase characterized by epithelial apoptosis; and a second, p53-independent, irreversible phase characterized by extensive tissue remodelling and adipocyte differentiation (Li et al. 1996; Lund et al. 1996; Jerry et al. 1998). Proteolytic enzymes are induced during the second phase of involution suggesting that their expression is a consequence of epithelial cell apoptosis rather than a prerequisite. Nevertheless matrix interactions provide survival signals to the mammary epithelium, such that removal of these signals can enhance apoptosis (Farrelly et al. 1999; Gilmore et al. 2000). Proteolytic breakdown products of matrix components such as fibronectin can induce apoptosis in some mammary cell lines (Schedin et al. 2000). Proteinase activity may therefore accelerate apoptosis of the epithelium, whilst stimulating differentiation of adipocytes *via* matrix remodelling (Alexander et al. 2001).

We analysed two micro-array datasets (Clarkson et al. 2004; Stein et al. 2004), derived from C57Bl/6 and Balb/C mice, respectively, to determine which genes encoding proteolytic enzymes are differentially regulated during pregnancy and involution. In addition, we interrogated the datasets for expression of potential regulators of MMP gene expression. Subsequently we analysed the expression pattern of several genes encoding proteolytic enzymes by quantitative PCR and compared the results with the micro-array data. In addition, we analysed the promoters of three MMP genes *in silico* to identify transcription factor binding sites that might correlate with promoter potency.

Gene expression of proteolytic enzymes

A number of genes encoding proteolytic enzymes are activated during puberty, pregnancy and involution, whereas expression of most proteolytic enzymes is inhibited during lactation. This regulatory pattern can be demonstrated by plotting the mRNA expression data of a typical proteolytic enzyme (aminopeptidase N, APN) as detected by micro-array analysis of mammary tissue isolated against the various stages of development (Fig. 1a). We analysed two recent affymetrix oligonucleotide micro-array datasets for the expression pattern of genes encoding matrix metalloproteinases, amino- and carboxypeptidases and members of the ADAM (a disintegrin and metalloproteinase domain) protein family and the plasminogen system. In general, micro-array data above 100 arbitrary units that showed a *t* test *P* value $<0{\cdot}1$, and changes in expression levels of more than 1·75-fold were considered to be significant.

The results for selected genes were then confirmed by quantitative PCR analysis. For this purpose mouse mammary tissue was isolated from CBA × C57Bl/6 mice at the indicated time points and RNA was isolated from

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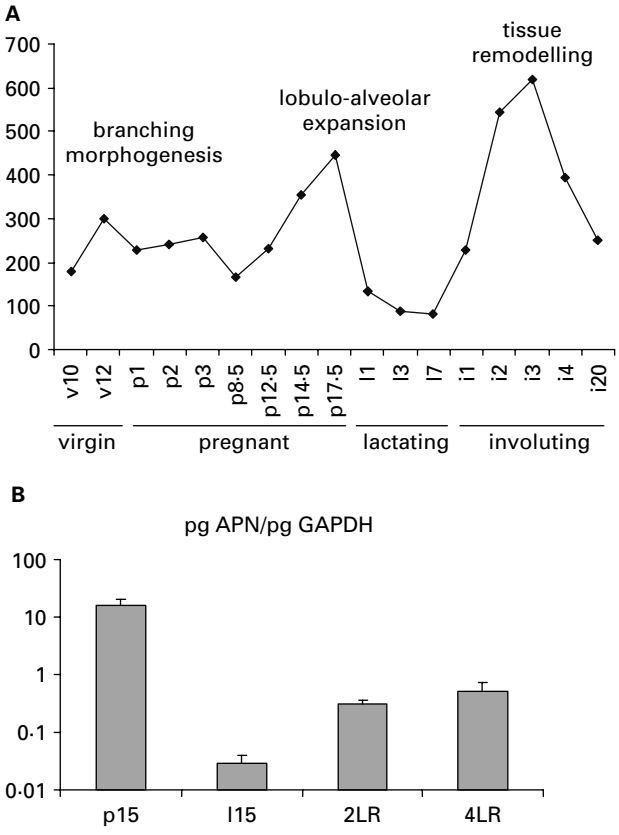

Fig. 1. Expression pattern of a typical proteinase, aminopeptidase N (APN), during mammary gland development. Panel A: Micro-array data of APN expression (in arbitrary units) during mammary gland development: Virgin [v] time points are in weeks; all other time points, [p] pregnant [l] lactating [i] involuting, are in days (Stein et al. 2004). Panel B: APN steady state mRNA expression as measured by quantitative PCR. APN expression was correlated with the expression of the control transcript GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [pg APN/pg GAPDH].

transfected cells using the Ambion RNAwiz reagent following the supplier's protocol. Reverse transcription of RNA was done with MLV RNAse(−) reverse transcriptase (Promega) following the supplier's recommendations using 2 µg of total RNA as template. A 1 in 200 dilution of the cDNA synthesis reaction was subsequently used as template for quantitative PCR (Light-Cycler, Roche). Amplifications were done using the primer combinations shown in Table 1 at 40 cycles of 15 s at 95 °C, 5 s at the indicated annealing temperature (Table 1) and 10 s at 72 °C. Data were collected at the end of each PCR cycle.

The murine and the human genome both contain 23 *bona fide* MMP genes. Ten MMP genes (1a, 1b, 3, 7, 8, 10, 12, 13, 20 and 27) are clustered at a single locus on murine chromosome 9 (Fig. 2; Table 2) and on human chromosome 11. We analysed two micro-array datasets for the expression of MMP genes during mammary development (Clarkson et al. 2004; Stein et al. 2004). Fourteen MMP genes are represented in the two datasets (Table 2). As shown in Fig. 3a, two MMP genes (MMP14 and MMP3) show a marked up-regulation during pregnancy and involution, whereas one other MMP (MMP12) is only up-regulated during involution. This finding is replicated in both micro-array datasets. The expression levels of most other MMP genes are low (most of them below the statistical significance level required by the affymetrix software) and appear to be constant during mammary development. MMP11 expression is slightly elevated during involution. MMP9 shows peaks of expression in early pregnancy and involution in one dataset but not in the other. We then analysed the expression of three MMP genes (MMP3, MMP12 and MMP7) located within the MMP locus for their expression during mammary gland involution by quantitative PCR. All three genes are induced during mammary involution (Fig. 3c–e). However, MMP3 transcripts are 20-times as abundant as MMP12 transcripts and 2000-times as abundant as MMP7 transcripts in the involuting mammary gland. However, expression of all three MMP is significantly enhanced during mammary gland involution (MMP3, 140-fold induction from lactating to day 4 post litter removal; MMP12, 19-fold induction and MMP7, 8-fold induction). This suggests that MMP7 gene expression during mammary gland involution was too low to be reliably detected by micro-array analysis. The relative expression levels of these three MMP are also replicated in the mouse mammary gland cell line HC11 (A. Kolb, unpublished observations), suggesting that the differences in expression levels may be a consequence of differing promoter potency.

The plasminogen system is essential for the proper development of the mammary gland. Transgenic mice carrying a deficiency of the plasminogen gene are severely impaired in their ability to lactate and up to 75% of the mice (depending on the genetic background) fail to support their litter (Lund et al. 2000). In addition, the plasminogen system appears to be required for proper tissue remodelling during involution, as nearly all of those plasminogen-deficient mice that were able to lactate did not support a second litter (Lund et al. 2000). Tissue plasminogen activator (tPA) expression has been shown to increase during mammary gland involution (Ossowski et al. 1979; Tonner et al. 2000). This is also reflected in the micro-array datasets, which show peaks of tPA expression during pregnancy and involution. These findings are supported by our quantitative PCR data, which demonstrate a four-fold induction of tPA during involution (M Boutinaud, unpublished observations). In addition, the urokinase plasminogen activator (uPA)-receptor is induced by about 3-fold during involution in both datasets. On the whole, the fluctuation of gene expression during mammary gland development is not as pronounced for members of the plasminogen system as it is for MMP when assayed by micro-array analysis (Fig. 3b). Urokinase plasminogen activator (uPA) expression was induced 2-fold at 3 d post litter removal. However, a strong induction of uPA was previously demonstrated from day 4 to day 7 post litter removal in Balb/C mice (Lund

Table 1. List of oligonucleotides used in this study

Name	Length	Sequence	Annealing temperature	Amplicon length
b-actin1	23mer	5′ GTC GAC AAC GGS TCC GSC ATG TG 3′	60 °C	908bp
b-actin2	23mer	5′ CTG TCR GCR ATG CCW GGG TAC AT 3′		
GAPDH1	25mer	5′ ACG GCA AAT TCA ACG GCA CAG TCA A 3′	61 °C	426 bp
GAPDH2	23mer	5′ GCT TTC CAG AGG GGC CAT CCA CA 3′		
cyclophilin-f.	23mer	5′ CAT CCT AAA GCA TAC AGG TCC TG 3′	58 °C	165 bp
cyclophilin-r.	20mer	5′ TCC ATG GCT TCC ACA ATG TT 3′		
MMP3-3	24mer	5′ ACC CAG TCT ACA AGT CCT CCA CAG 3′	56 °C	463 bp
MMP3-4	21mer	5′ GCA GCA TCG ATC TTC TTC ACG 3′		
MMP12-1	20mer	5′ CCC AGC ACA TTT CGC CTC TC 3′	54 °C	494 bp
MMP12-2	24mer	5′ AGC TCC TGC CTC ACA TCA TAC CTC 3′		
MMP7-1	24mer	5′ TAC CCT CAT GAC TCT AAA ACA AAG 3′	54 °C	486 bp
MMP7-1	20mer	5′ TGC GTC CTC ACC ATC AGT CC 3′		
mAPN 2488	21mer	5′ CAA CTC TGG TGA ACG AAG CGG 3′	55 °C	144 bp
mAPN 2631R	21mer	5′ CAA TGC TGA TGA TGG TGG AGG 3′		

et al. 1996) indicating that uPA induction may be a late event in mammary gland involution. Interestingly, expression of PAI-1 (plasminogen activator inhibitor 1) is also increased during involution (Fig. 3b). This may serve to limit the activity of plasmin-induced proteolysis. However, PAI-1 protein can be neutralized by binding to IGFBP5, which is strongly induced during involution (Nam et al. 1997; Tonner et al. 2000). Similarly, expression of TIMP3 (tissue inhibitor of metalloproteinases) appears to be co-regulated with MMP3 expression and increases significantly during mid-pregnancy and involution, whereas expression of TIMP1 decreases during these developmental stages (data not shown).

Members of the ADAM family of proteins play a biological role in cell adhesion (via their adhesive domain) and morphogenesis (mainly *via* their protease domain) (White, 2003). ADAM function includes the shedding of membrane-bound growth factors, cytokines, cytokine-receptors and cell adhesion molecules. Cell proliferation and migration of human mammary epithelial cells was found to be dependent on the activity of ADAM proteins, which release EGF from the cell membrane to enable activation of the EGF receptor (Dong et al. 1999). Expression of an ADAM12 transgene under the control of a muscle-specific promoter induced adipogenesis in skeletal muscle of transgenic mice (Kawaguchi et al. 2002). An ADAM12 mutant lacking the protease domain had no effect indicating that proteolytic processes can be decisive differentiation stimuli. The expression pattern of ADAM9 during mammary gland development was similar to that of MMP3 and MMP14, peaking during pregnancy and involution (Fig. 4a). Interestingly, the expression pattern of the most abundantly expressed ADAM genes (ADAM5 and ADAM28) was similar to that of milk protein genes in that

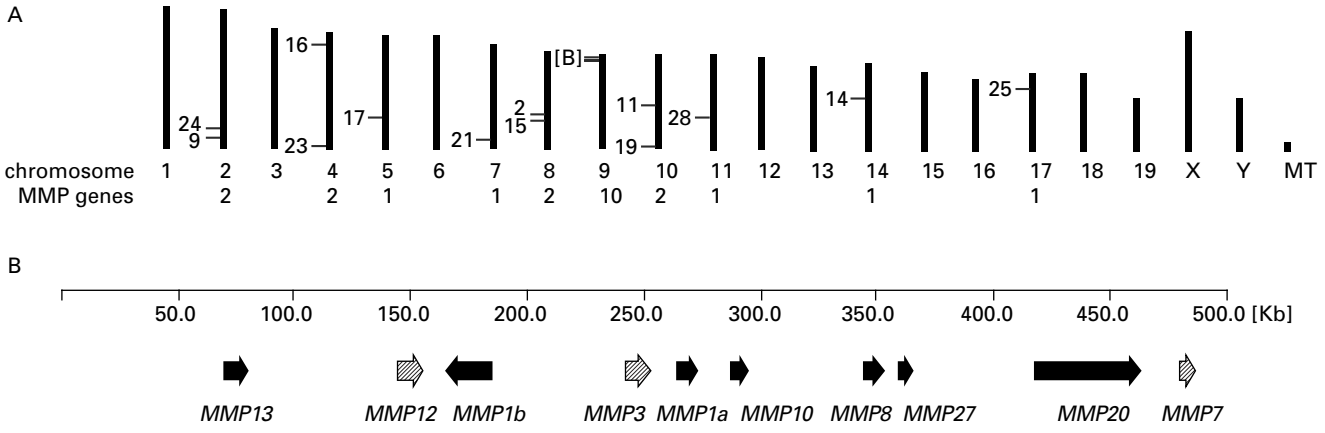

Fig. 2. Chromosome locations of murine MMP genes. Panel A: Schematic representation of the distribution of MMP genes in the mouse genome. The vertical lines represent chromosomes and the short horizontal lines mark the approximate positions of the MMP genes. Chromosome numbers and the number of MMP genes found on the chromosomes are indicated below the lines. The numbers of the individual MMP genes are indicated. The ten MMP genes present on chromosome 9 are indicated as [B] and fully represented in Panel B. Panel B: The relative locations and orientations of the MMP genes on mouse chromosome 9 are indicated as block arrows. The names of the enzymes are indicated.

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Table 2. Chromosome locations of murine MMP genes. The MMP genes represented on the Affymetrix oligonucleotide micro-array are indicated [repr.].

Chromosome	Gene	Other names	Micro-array
2	MMP9	92 kDa gelatinase, 92 kDa type IV collagenase, B/MMP9, Clg4b, Gel B, gelatinase B	repr.
2	MMP24	Membrane type 5-MMP, MT5-MMP	repr.
4	MMP16	Membrane type 3-MMP, MT3-MMP	repr.
4	MMP23	CA-MMP, cysteine array matrix metalloproteinase	
5	MMP17	Membrane type-4 matrix metalloproteinase, MT4-MMP	repr.
5	MMP21		
8	MMP2	72 kDa gelatinase, 72 kDa type IV collagenase, Clg4a, GelA, gelatinase A,	repr.
8	MMP15	Membrane type 2-MMP, MT2-MMP	repr.
9	MMP3	Progelatinase, SLN-1, SLN1, Stmy1, Str1, stromelysin 1, transin	repr.
9	MMP12	Macrophage elastase, metallo-elastase	repr.
9	MMP7	MAT, matrilysin	repr.
9	MMP27		
9	MMP1b	Mcol-B, interstitial collagenase B	
9	MMP1a	Mcol-A, interstitial collagenase A	
9	MMP20	Enamelysin	
9	MMP8	Collagenase-2	repr.
9	MMP13	Clg, collagenase-1, Collagenase-3, interstitial collagenase	repr.
9	MMP10	Stromelysin 2	repr.
10	MMP11	ST3, Stmy3, stromelysin 3	repr.
10	MMP19		
11	MMP28		
14	MMP14	Membrane type 1-MMP, MT1-MMP	repr.
17	MMP25	F730048C11Rik, Leukolysin, MT6-MMP	

it steadily increased during late pregnancy and lactation and dropped at the onset of involution (Fig. 4a).

A number of genes encoding amino- and carboxy-peptidases are up-regulated during pregnancy and involution (data not shown). One example is the gene encoding aminopeptidase N (APN) whose expression pattern is similar to that of MMP3 (Figs 1a and 4c). The pattern of APN expression could also be confirmed by quantitative PCR (Fig. 1b), although the induction of APN expression during involution appears to be more modest than the micro-array analyses would suggest. No other amino-peptidases show a similar significant induction pattern during mammary involution (in both datasets), but a number of carboxypeptidases and several cathepsin proteinases are significantly up-regulated during involution. APN is also highly expressed during late pregnancy as are glutamyl aminopeptidase and several carboxypeptidases.

MMP gene promoter elements

To determine whether the differences in the expression levels of the three different MMP genes (7, 12 and 3) are due to evident differences in transcription factor binding sites in the promoter, we analysed 1 kb upstream of the transcriptional start site *in silico* using the GeneQuest program in the DNA-Star software suite and found a number of features to be conserved in the three promoter regions. Although these computer predictions do not demonstrate function of the detected binding sites (e.g., the sites may be inaccessible in the chromatin structure of the nucleus *in vivo*) they can give important clues for the investigation of promoter regulation.

In accordance with published data the TATA box is closely associated with a consensus AP1 binding site in all three promoters (Borden et al. 1997). A dramatic increase in AP1 binding activity is detectable in nuclear extracts isolated from involuting mammary tissue (Marti et al. 1994). A number of signalling pathways have been shown to converge at the AP1 site (McDonnell et al. 1990; McDonnell et al. 1994) and a variety of agents have been found to stimulate MMP gene transcription *via* this site (Crawford et al. 1996; Borden et al. 1997). MMP promoter elements in which the AP1 site has been deleted were found to be largely inactive. Over-expression of the transcription factor fos has been found to be sufficient to induce MMP1 but not MMP3 expression in some organs indicating the different contribution the AP1 activation makes to different MMP promoter elements. Conversely, MMP1 expression is no longer TPA (12-O-tetradecanoyl-phorbol-13-acetate)-inducible in fos deficient mice, whereas MMP3 expression and inducibility is only affected under certain conditions (Crawford et al. 1996). Transcription factors are usually regulated post-transcriptionally, typically *via* phosphorylation. However, a critical amount of protein must be available in the cell to enable a transcriptional response to exogenous stimuli. An increase in transcription of c-jun (Clarkson et al. 2004) and jun-B (Stein et al. 2004) was detected by micro-array analysis in the first 24 h after initiation of mammary gland involution by litter removal (data not shown).

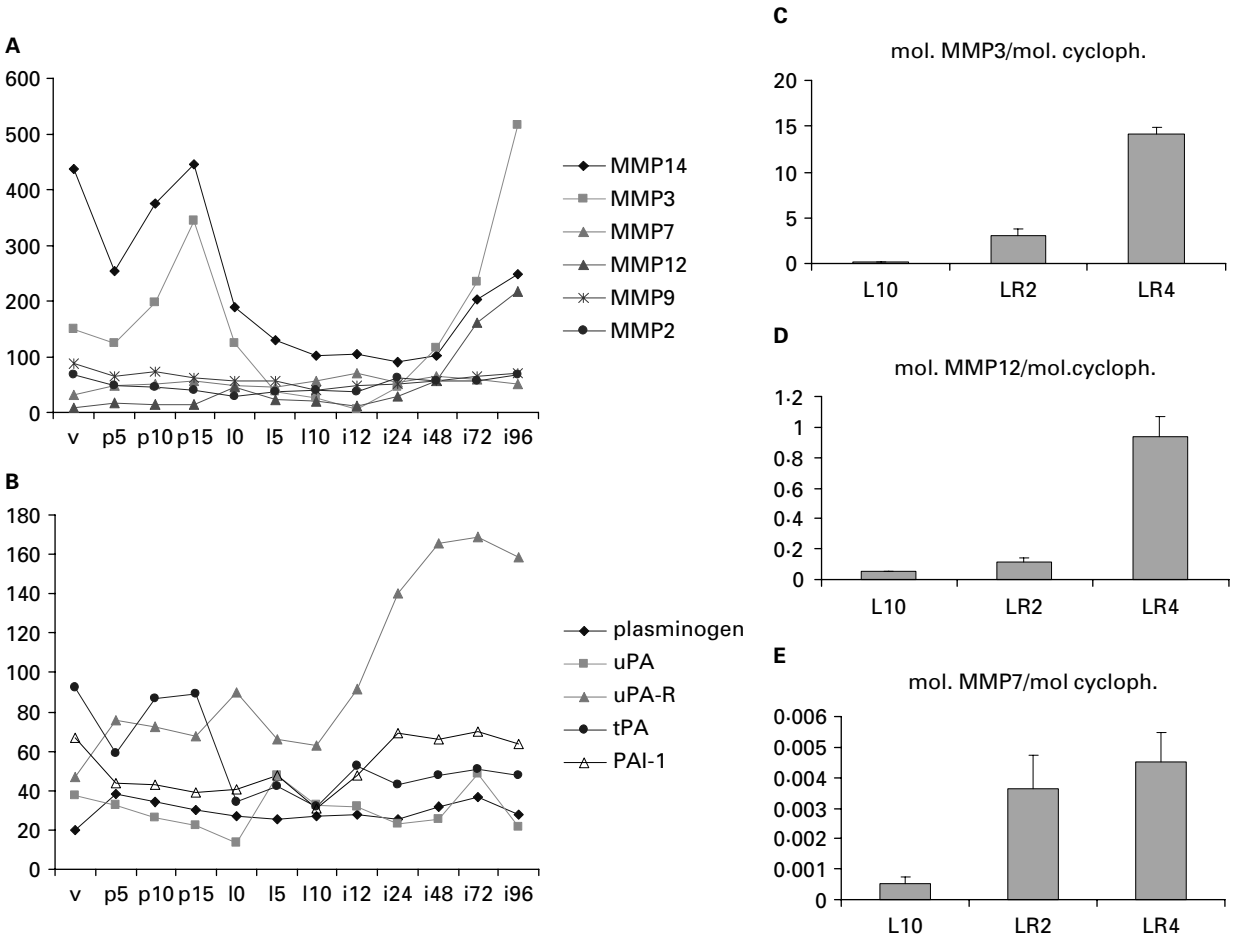

Fig. 3. Analysis of MMP gene expression during mammary gland development. Panel A: Micro-array data of MMP2, 3, 7, 9, 12 and 14 expression derived from (Clarkson et al. 2004). [v] virgin, [p] pregnant (in days) [l] lactating (in days) [i] involuting (in hours). Panel B: Micro-array data for members of the plasminogen system (Clarkson et al. 2004). Panel C–E: Quantitative PCR of MMP3, 7 and 12 during mammary involution. [L10] lactation day 10, [LR2] day 2 after litter removal, [LR4] day 4 after litter removal. MMP expression is shown relative to cyclophilin-A expression [molecules of MMP/molecules of cyclophilin].

Numerous binding sites for transcription factors of the ets family (e.g., PEA3) are found in all three promoter elements. The PEA3 binding sites in the MMP12 promoter are located further away from the transcriptional start site than in the MMP3 and MMP7 promoter. Binding sites for members of the ets transcription factor family have been detected in almost all MMP promoters. The sites are mostly found in close association with the AP1 site (Borden et al. 1997). Promoter-reporter constructs containing a fragment with the AP1 and the ets site often behave like the whole MMP promoter in cell lines (Crawford et al. 1996). In some promoters AP1 and ets have been found to act cooperatively whereas only additive effects have been detected in others. A number of ets transcription factors are expressed in the mammary gland (Shepherd et al. 2001) and different ets-family members influence the expression of different proteolytic enzymes (Galang et al. 2004). A single nucleotide polymorphism, associated with increased expression of MMP1 and enhanced cancer susceptibility, has been shown to generate an additional ets-factor binding site in the MMP1 promoter (Brinckerhoff et al. 2002). A sharp increase in the expression of ELK4 (an ets transcription factor) could be detected shortly after initiation of mammary gland involution by litter removal (data not shown). ELK4 is one of the most abundant ets transcription factors in mammary tissue (Shepherd et al. 2001; Galang et al. 2004). However, its expression is mainly detected in epithelial and myeloid cells. The sharp increase in ELK4 expression may therefore reflect the general increase in lymphoid marker gene expression during mammary gland involution (Stein et al. 2004). In addition, ELK4 expression in mammary tissue and mammary gland cell lines was found not to be correlated with the expression of MMP3, MMP7 and MMP9 (Galang et al. 2004). This may suggest that the influence of ELK4 on the transcription of MMP genes in the mammary stromal cells is indirect.

All three promoter regions (MMP3, 7 and 12) carry alternative TATA boxes and a number of additional

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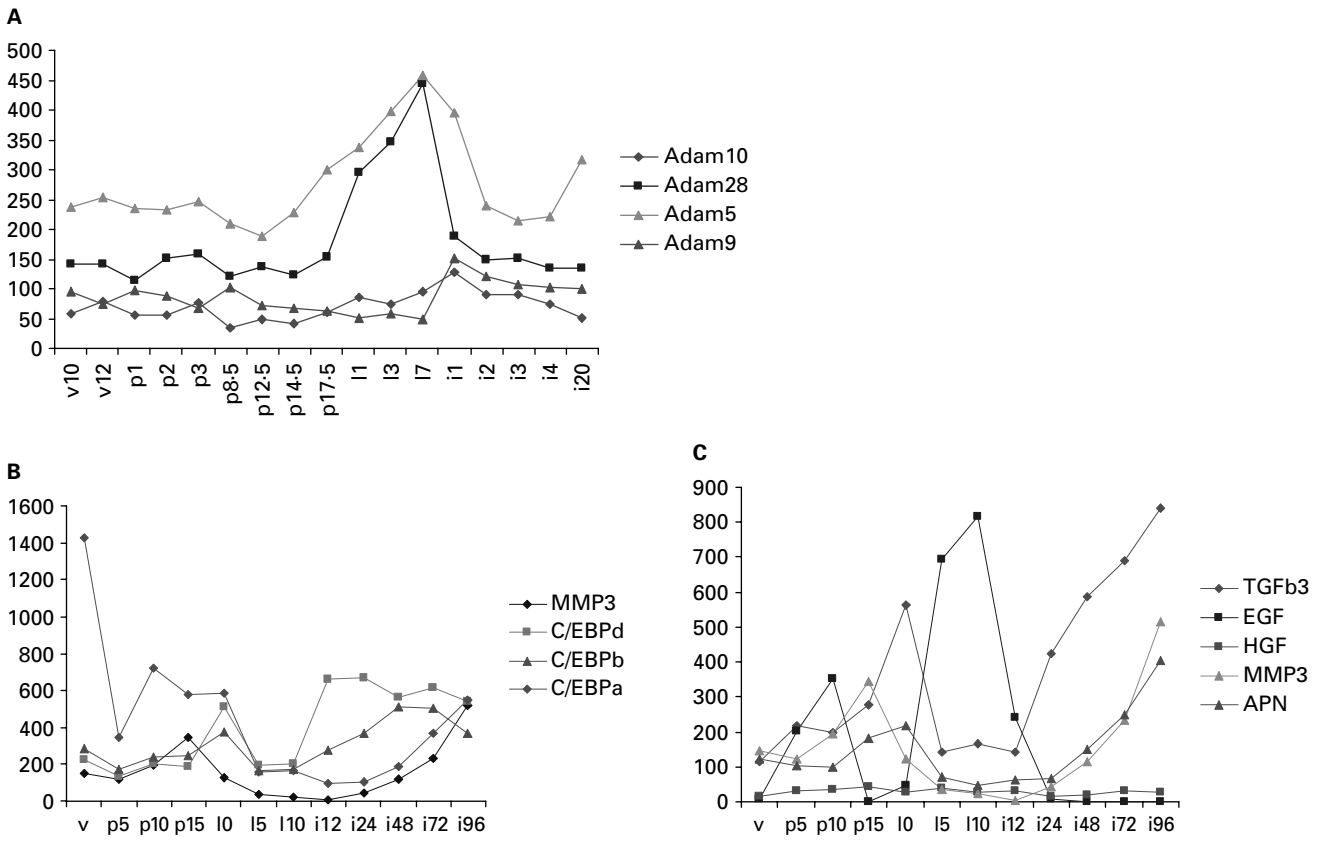

Fig. 4. Panel A: Gene expression patterns of ADAM5, 9, 10 and 28 during mammary gland development, derived from (Stein et al. 2004). Virgin [v] time points are in weeks; all other time points are in days. Panel B: Gene expression patterns of CAAT enhancer binding protein [C/EBP] isoforms delta, beta and alpha; data derived from (Clarkson et al. 2004). Involution time points are in hours. Panel C: Expression patterns of transforming growth factor beta3 [TGFb3], hepatocyte growth factor [HGF], epidermal growth factor [EGF], MMP3 and APN; data derived from (Clarkson et al. 2004). Involution time points are in hours.

promoter-distal AP1 sites. Notably, binding sites for the general transcription factors Sp1 and C/EBP are found adjacent to the promoter proximal AP1 site in the MMP3 promoter but not in the MMP12 and MMP7 promoters. This may explain the significantly higher expression levels elicited by this promoter (in comparison with other MMP promoter elements) in mammary tissue. C/EBP transcription factors do not have a well defined consensus sequence and binding site predictions are based on sequence comparisons to other C/EBP-responsive promoter elements (Doppler et al. 1995; Shelest et al. 2003). Transcription of three C/EBP family members (alpha, beta and delta) is increased during pregnancy and involution but the timing of induction is different: transcription of C/EBPdelta is rapidly induced at the onset of involution (Clarkson et al. 2003), whereas C/EBPalpha expression gradually increases in parallel with MMP3 expression. This may suggest C/EBPalpha as a candidate inducer of MMP3 transcription (Fig. 4b). C/EBP has also been identified as a regulator of the IGFBP5 gene (Ji et al. 1999) and the timing of the activation of IGFBP5 gene expression during mammary involution suggests that C/EBPdelta is potentially involved in this activation. IGFBP5 plays a central role in the process of mammary involution and over-expression of IGFBP5 in the mammary gland of transgenic animals impairs lactation and induces apoptosis in mammary epithelial cells *in vivo* (Tonner et al. 2002). The C/EBPdelta gene itself is responsive to induction by Stat3, another key regulator of mammary involution (Clarkson et al. 2003).

All three MMP promoter regions have a binding site for members of the glucocorticoid receptor family. Glucocorticoid hormones like hydroxycortisone have been shown to have an inhibiting effect on MMP gene expression *in vivo* (Lund et al. 1996) and *in vitro* (Eberhardt et al. 2002; A Kolb, unpublished observations).

Inducers of proteinase gene expression

A large number of hormones and growth factors have been implicated in the regulation of proteolytic enzymes. Some of them are systemic hormones (e.g., progesterone or prolactin, which have, respectively, a stimulatory and an inhibitory effect on MMP expression (Lee et al. 2001)) and

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would not be detected in a micro-array study using mammary tissue. We analysed the micro-array datasets for the expression patterns of genes encoding candidate activators of MMP and mammary apoptosis, namely EGF (epidermal growth factor), HGF (hepatocyte growth factor), TGF (transforming growth factor) and LIF (leukaemia inhibitory factor).

MMP gene expression during pregnancy has been shown to be highly dependent on EGF. Tissue recombination experiments have shown that mammary stroma, which is deficient for the EGF receptor cannot be induced to express MMP genes during pregnancy (Wiesen et al. 1999). A huge increase in EGF expression was detectable during the tissue-remodelling phase of early pregnancy suggesting that the EGF required for MMP gene activation is produced in the mammary gland. Expression levels then drop precipitously during late pregnancy (Fig. 4c). During lactation, another peak of EGF transcription is evident. However, no EGF expression is detectable during mammary gland involution indicating that EGF is not a major stimulator of MMP gene expression during that developmental phase. These results also suggest that the signalling pathways involved in MMP gene activation differ between pregnancy and involution. Signalling through the EGF receptor may also play a role during mammary involution as a proetolytic cleavage fragment of laminin-5 (DIII) can stimulate EGF receptor mediated phosphorylation events (Schenk et al. 2003).

Members of the TGFbeta family have a marked effect on tissue homeostasis and signal though the Smad family of transcription factors (Hall et al. 2003). TGFbeta isoforms can inhibit or stimulate MMP gene expression. TGFbeta1 inhibits the TPA-mediated activation of the MMP1 promoter in murine C3H fibroblasts (Hall et al. 2003). MMP13 expression is significantly reduced in the palate of TGFbeta3 knock-out mice suggesting that TGFbeta3 is a major activator of the MMP13 gene (Blavier et al. 2001). TGFbeta3 is also a major local inducer of cell death in mammary tissue (Nguyen et al. 2000). MMP-9 expression is upregulated in breast cancer cells by TGFbeta1 (Kalembeyi et al. 2003; Peters et al. 2003). Expression of members of the transforming growth factor family (TGFbeta3 and TGFbeta1) is correlated with MMP gene expression during pregnancy and involution. TGFbeta expression, however, precedes MMP gene activation during involution by 48 h (Fig. 4b), which suggests that TGF may affect MMP gene expression indirectly.

Hepatocyte growth factor/scatter factor (HGF) another potent inducer of MMP genes shows no detectable fluctuations of expression during mammary development in the micro-array analysis. Published data, however, suggest that HGF and its receptor c-met are differentially regulated in rat mammary gland development, with peaks of expression occurring during pregnancy and involution (Pepper et al. 1995). HGF stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells *in vitro* (Soriano et al. 1995). Over-expression of HGF in the mammary gland of transgenic mice results in an increased size and number of terminal end buds and hyperplastic branching morphogenesis in the virgin gland (Yant et al. 1998). These results suggest that HGF plays an important role in mammary development, which is consistent with an induction of MMP gene expression. The absence of any differential regulation of HGF transcription in the micro-array format may suggest that HGF expression is below the level of detection.

Leukaemia inhibitory factor (LIF) has recently been implicated as an inducer of mammary gland apoptosis (Kritikou et al. 2003; Schere-Levy et al. 2003). LIF expression is induced rapidly, but transiently, after litter removal (data not shown). However, LIF expression decreases again before induction of MMP genes occurs at around 48 h post litter removal. This suggests that the influence of LIF on the expression of proteolytic enzymes during mammary involution is not direct.

Conclusions

The expression of proteolytic enzymes is essential for the tissue remodelling processes that take place during mammary gland development. The available micro-array data suggest that a number of MMP genes (but not all) are activated co-ordinately. Similarly, not all aminopeptidase and carboxypetidase genes are differentially regulated during mammary development. Comparison of data obtained by micro-array and quantitative PCR shows that the two experimental approaches are in agreement for highly expressed genes. Genes that are expressed at a low level (e.g., MMP7), however, may escape detection as differentially regulated by the micro-array approach.

The available micro-array data (e.g., in the case of EGF) suggest that different molecules are involved in the induction of genes encoding proteolytic enzymes during pregnancy and involution. This is in accordance with the underlying biology. During pregnancy the epithelium requires tissue-remodelling enzymes in order to migrate, proliferate and differentiate in the mammary fat pad, whereas during involution proteolytic activity is required to remove a regressing epithelium and support the differentiation of adipocytes.

It is difficult, at present, to predict which regulators induce the expression of MMP and other proteolytic enzymes during involution. A number of molecules have been identified as being critical for the induction of apoptosis in the mammary gland, including LIF, STAT3, TGFbeta3 and IGFBP5 (Chapman et al. 1999; Nguyen et al. 2000; Tonner et al. 2002; Kritikou et al. 2003). However these signals may not directly induce expression of proteolytic enzymes. Other molecules may therefore be required to mediate the induction of MMP genes after the initiation of epithelial apoptosis. These signals are likely to induce MMP expression in the stromal compartment (Lund et al. 1996), *via* the fos/jun and ets families of transcription

factors. Some signals, like glucocorticoid hormones (Lund et al. 1996) and prolactin (Lee et al. 2001) (A Kolb, unpublished observations), which suppress MMP gene expression in the lactating mammary gland, are switched off at the onset of involution. In addition, the activity of some MMP proteins is enhanced by the down-regulation of TIMP1 (whose expression is correlated with glucocorticoid hormones).

Candidate molecules, which induce MMP genes during pregnancy, can be studied meaningfully in a number of cell culture models and tissue recombination experiments (Soriano et al. 1995; Wiesen et al. 1999). The induction of MMP gene expression during involution is more difficult to assess experimentally. Presently no suitable cell culture systems that mimic the interaction of all cell types in the involuting mammary gland (epithelial cells undergoing apoptosis, stromal fibroblasts, myeloid cells, adipocytes), have been established. The availability of such a system would greatly facilitate the analysis of the involution-specific induction of MMP genes.

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