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Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression

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and related Commentary: http://breast-cancer-research.com/content/6/2/89

Abstract

Introduction In order to gain a better understanding of the molecular processes that underlie apoptosis and tissue regression in mammary gland, we undertook a large-scale analysis of transcriptional changes during the mouse mammary pregnancy cycle, with emphasis on the transition from lactation to involution.

Method Affymetrix microarrays, representing 8618 genes, were used to compare mammary tissue from 12 time points (one virgin, three gestation, three lactation and five involution stages). Six animals were used for each time point. Common patterns of gene expression across all time points were identified and related to biological function.

Results The majority of significantly induced genes in involution were also differentially regulated at earlier stages in the pregnancy cycle. This included a marked increase in inflammatory mediators during involution and at parturition, which correlated with leukaemia inhibitory factor–Stat3 (signal transducer and activator of signalling-3) signalling. Before involution, expected increases in cell proliferation, biosynthesis and metabolism-related genes were observed. During involution, the first 24 hours after weaning was characterized by a transient increase in expression of components of the death receptor pathways of apoptosis, inflammatory cytokines and acute phase response genes. After 24 hours, regulators of intrinsic apoptosis were induced in conjunction with markers of phagocyte activity, matrix proteases, suppressors of neutrophils and soluble components of specific and innate immunity.

Conclusion We provide a resource of mouse mammary gene expression data for download or online analysis. Here we highlight the sequential induction of distinct apoptosis pathways in involution and the stimulation of immunomodulatory signals, which probably suppress the potentially damaging effects of a cellular inflammatory response while maintaining an appropriate antimicrobial and phagocytic environment.

Keywords: apoptosis, immunity, involution, mammary, microarray

Introduction

Mammary gland development during the pregnancy cycle is characterized by successive phases of cell growth, differentiation, high metabolic activity and apoptosis. At the ultrastructural level this includes dramatic changes in tissue architecture, involving ductal epithelial branching and morphogenesis, invasion of tissue compartments, vascularization and subsequent organized remodelling. These events are highly reproducible and strictly controlled at the transcriptional level by circulating hormones and locally derived factors [1]. Thus, many transcription factors have been shown either to directly affect this developmental program or to exhibit altered activity at specific stages in the pregnancy cycle [1].

CAM = cell adhesion molecule; C/ebp = CAAT-enhancer binding protein; IGF = insulin-like growth factor; IL = interleukin; LIF = leukaemia inhibitory factor; LPS = lipopolysaccharide; NF-κB = nuclear factor-κB; Stat = signal transducer and activator of transcription.

Our laboratory has focused on the role of transcription factors in postlactational regression of the gland after weaning. Involution can be divided into at least two phases [2–4], broadly comprising the following: an initial reversible phase whereby the gland maintains its gross morphology but undergoes a substantial increase in the rate of epithelial cell apoptosis [5]; and a secondary irreversible phase, which involves the destruction of basement membrane by matrix metalloproteinases, phagocytic clearance of milk, and apoptotic bodies and alveolar collapse [6]. Immune cells are present at all stages of mammary development, including involution [7], but the precise role of the immune system during postlactational regression has yet to be fully established. Many genes have been shown to be differentially regulated during involution [5,8,9]. However, there has been no comprehensive analysis of gene expression in mammary involution.

Microarray analysis has had a major impact on our understanding of the transcriptional basis of complex biological systems. Normal tissue development and homeostasis has been studied in a variety of mouse tissues, including retina [10], liver [11], pancreas [12], uterus [13] and mammary gland [14].

The few microarray studies of normal mouse mammary gland described in the literature have either focused on early stages in the developmental cycle [14], or have used mammary data as a tool for illustrating methods of data analysis [15,16].

In the present study we applied a microarray approach to study the transcriptional expression (mRNA levels) of 8618 mouse genes in mammary gland during the pregnancy cycle. We focused on five time points in involution but we also included earlier time points in the pregnancy cycle to establish the specificity of these involution-related genetic changes. We provide the raw microarray data files and processed genelists for online analysis or download (see the Mammary Apoptosis and Development Group Home Page: www.path.cam.ac.uk/~madgroup). Here we highlight specific aspects of the microarray data that are pertinent to involution by identifying common patterns of gene expression and relating them to specific biological functions. Thus, we demonstrate the sequential activation of death receptor genes followed by components of the mitochondrial (intrinsic) pathway after weaning, suggesting that different apoptosis mechanisms are employed at different phases of involution. We also highlight a prominent role for immune related genes in involution, equating this with phagocytic clearance of apoptotic cells and the maintenance of an antimicrobial environment during milk stasis. We provide evidence that the proapoptotic, acute phase transcription factor Stat3 (signal transducer and activator of transcription-3) [17,18] may simultaneously suppress a potentially damaging cellular infiltrate. A similar study conducted in Balb/c mice and described in the accompanying paper in this issue [19] confirms proinflammatory gene expression in involution. Together, these data provide a useful online resource for studying the activity of specific genes in mammary involution.

Method
Mouse husbandry
Six-week-old C57/Bl/6 mice were obtained from Harlan Laboratories (Bicester, UK). At 8 weeks females were either mated or culled for virgin mammary glands. Postpartum, a minimum of six pups per suckling female was ensured by cross-fostering where appropriate. Females used in involution time points were force weaned at 10 days of lactation. Lactating and involuting mammary glands were monitored daily for signs of localized inflammation or mastitis. Two out of 90 animals were excluded from the study for this reason. The study was approved by the local ethics committee and complies with the Helsinki Declaration.

Harvesting mammary gland RNA
A total of 12 stages of adult mouse mammary gland development were selected for this study: 8-week-old virgin; 5 days, 10 days and 15 days of gestation (in which day 1 was the first day postcoitum); 0 days (first day postpartum), 5 days and 10 days of lactation; and 12, 24, 48, 72 and 96 hours after forced weaning. All mammary glands were harvested between 11:00 and 13:00 to limit circadian effects. A total of six animals were used per time point (three per hybridization). In each case a single abdominal gland was removed following excision of lymph nodes and immediately frozen in liquid nitrogen. RNA was extracted from frozen tissue using Trizol reagent (Invitrogen, Paisley, UK) followed by additional column purification (Rneasy; Qiagen, Crawley, UK). Briefly, three samples of mammary tissue from the same time point were ground under liquid nitrogen, and 20 mg of each was pooled into 1.5 ml Trizol reagent. Resuspended RNA samples were then passed through purification columns according to the manufacturer's instructions and RNA integrity was monitored with Lab-on-a-chip (Agilent, West Lothian, UK) before use in microarray analysis.

Microarray hybridization and data analysis
RNA 5 μg was labelled according to manufacturers protocols (Affymetrix, High Wycombe, UK) and hybridized to Affymetrix MGU74ver2a chips representing 12 488 transcripts or 8618 genes. Computation of expression values was performed using the perfect match/mismatch model implemented in dChip [20].

Twenty-four Affymetrix hybridisations (two for each mammary time point) were selected following removal of outliers identified by dChip. All subsequent analyses were performed with Genespring analysis software (Silicon

Genetics, Redwood City, CA, USA). Two filters were applied to the complete data set of 12 488 transcripts. First, 755 transcripts (6%) were removed following elimination of genes whose mean signal intensities were below 20 normalized intensity units in at least 11 of the 12 time point conditions. Second, 5149 transcripts (44%) were removed on the basis that they exhibited no significant change in mean signal intensity across all time points (Welch ANOVA $P = 0.05$, using the Benjamini and Hochberg False Discovery Rate controlling procedure). A separate lactation/involution specific time course gene set was established by applying the same cutoff filters to all 12 488 transcripts using 5 day and 10 day lactation time points, and 12, 24, 48, 72 and 96 hour involution time points.

Both data sets were clustered (K means) according to expression pattern similarity across all 12 conditions (complete time course) or seven conditions (involution time course) using standard correlation. Nine K mean classifications were performed for each data set, starting with 10 clusters and increasing by five incrementally to 50 clusters. The optimal number of clusters was determined empirically based on highest observed variability and redundancy between similar clusters.

All genes were annotated according to known function using the Gene Ontology Consortium categories [21]: biological process, cellular component and molecular function. Onto-Express [22] was used to determine whether clusters of genes with similar expression profiles were enriched in specific GO functional categories. Based on the genes present on the GeneChip, Onto-Express calculated the expected number of occurrences of each functional category in each cluster. The probability that each functional category was over-represented in a cluster was derived using a binomial model and the *P* values were corrected for multiplicity using the Bonferroni method. Thus, Onto-Express provided information about the statistical significance of each of the pathways and categories represented by the genes in each cluster (these data are available online as Additional files 1 and 2).

Archived data

All genelists, including expression profile clusters, ontological definitions and all raw data .cel files, fulfilling MIAME criteria, were uploaded to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) and are also available through GeNet (http://genet.hgmp.mrc.ac.uk:8080/servlet/GeNet) for download and online analysis. (For information on accessing data through GeNet or from *Breast Cancer Research* see our website: www.path.cam.ac.uk/~madgroup/). Gene lists use Affymetrix probe_ID and accession number as transcript identifiers. In the body of the text and figures, gene/transcripts are named according to the Mouse Genome Database [23].

Results

We analyzed the expression of over 12 000 transcripts, or approximately one-third of the protein encoding capacity of the mouse genome, at 12 different time points in the mammary gland pregnancy cycle. We focused our analysis on the changes in gene expression that occur during the first 4 days of postlactational regression, harvesting at five time points during this period. We also included three time points in lactation, three in gestation and a single time point in nulliparous animals in order to distinguish between involution-specific genes and genes that were also highly expressed at other times in the pregnancy cycle. All raw and normalized data files were submitted to GeNet and made available for download or online analysis through our website (www.path.cam.ac.uk/~madgroup/).

Expression of 6796 transcripts (54% of the transcripts assayed) significantly changed across the 12-point time course. This suggested that approximately half of the protein encoding content of the mouse genome was regulated in mammary gland at some point during the pregnancy cycle. Among the genes that did not significantly change (nonsignificant profiles genelist in GeNet) were a number of recognized 'housekeeping' genes, including 18s ribosomal and α-actin genes. However, other genes that are ubiquitously expressed in some cell types (e.g. GAPDH and cyclophilins) exhibited differential expression in the mammary time course. This reflects a combination of factors, including changes in tissue composition and dramatic shifts in cellular metabolism and macromolecular synthesis throughout the mammary cycle, in particular during lactation.

In order to verify that the microarray time course reflected expected global expression patterns *in vivo*, we compared our results with the known mammary expression patterns of 32 genes described in the literature, all of which exhibited the expected expression profiles (see Additional file 3).

Expression profile analysis of the pregnancy cycle (12-point time course)

It was expected that a proportion of the 6797 differentially expressed transcripts would be specific to involution, whereas others may have additional roles at earlier stages in the pregnancy cycle. In order to identify these subsets of involution related genes, the expression patterns of all differentially expressed transcripts were compared and subsequently grouped according to similarity into 35 gene profile clusters. Some clusters exhibited superficially similar patterns (Fig. 1). In order to simplify the classification of expression patterns observed in the time course, these similar profiles were pooled, resulting in 11 broadly distinct expression profiles (Fig. 1a).

These 11 clusters represented maximal gene expression at different stages during the pregnancy cycle. Thus, one

Figure 1
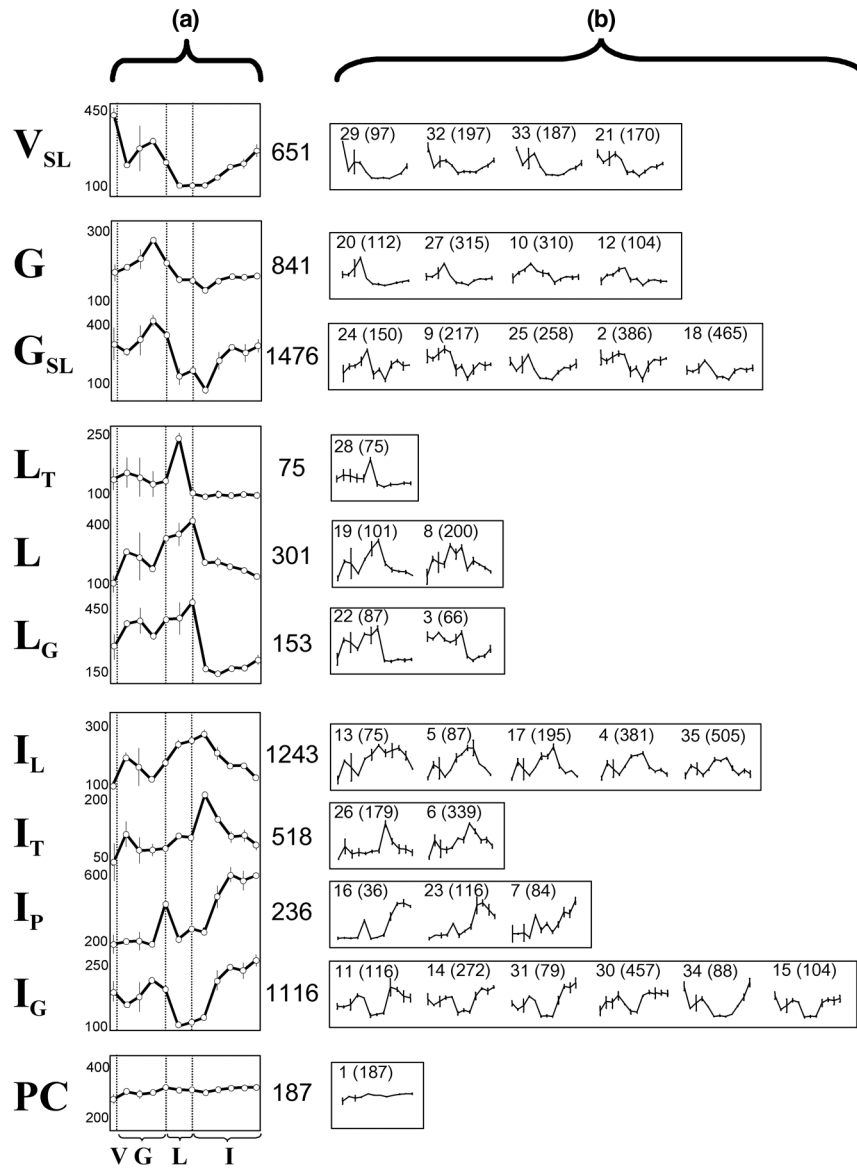
Clusters of gene expression profiles from a 12-point time course of adult mammary gland development. Thirty-five K means clusters were obtained from 6796 differentially expressed genes across a 12-point time course of the adult mammary gland pregnancy cycle. In order to identify basic trends in expression, these clusters were arbitrarily organized into groups based on the similarity between their mean profiles (b). These clusters were pooled and displayed as combined average expression profiles (a). **(a)** Combined expression profiles named according to developmental stage exhibiting maximal expression (capital) and their associated expression pattern (subscript). V_{SL}, virgin and suppressed lactation; G, gestation; G_{SL}, gestation and suppressed lactation; L_T, lactation, transient; L, lactation; L_G, lactation and gestation; I_L, involution and lactation; I_T, involution, transient; I_P, involution and parturition; I_G, involution and gestation; PC, postcoitum. Y axis is the mean normalized signal intensity. Dotted lines indicate boundaries between major phases of development: virgin to gestation; parturition; and 10-day lactation (forced weaning). Total number of genes in each combined cluster shown to the right. **(b)** Thirty-five K means clusters. Total number of genes (brackets) and the ID for each cluster is shown. Each differentially expressed transcript is represented only once in the 35 clusters. Error bars represent standard error of the mean. X axis is the 12 developmental time points used in microarray analysis: v, virgin (8 week); G, gestation (days 5, 10 and 15 postcoitum); L, lactation (days 0, 5 and 10 postpartum); I, involution (days 0.5, 1, 2, 3 and 4 after forced weaning).

cluster of 651 transcripts (5% of all transcripts on the array) were maximally expressed in virgin mammary gland (V_{SL}). Two clusters with a total of 2317 transcripts (19%) were maximally expressed in gestation (G and G_{SL}) and

three clusters (529 transcripts, 4%) were maximally induced during lactation (L_T, L and L_G). Four clusters (3113 transcripts, 25%) exhibited maximum expression during involution (I_L, I_T, I_P and I_G) whereas one cluster

included 187 transcripts (1%) that were uniformly expressed throughout the pregnancy cycle (PC). Further details on the nomenclature of these combined clusters are provided in the legend to Fig. 1. Genelists for each combined cluster and the 35 primary clusters are available online (see Method section, above).

Six clusters consisted of transcripts that were either maximally expressed during involution (I_L, I_T, I_P and I_G) or specifically suppressed following weaning (L and L_G), suggesting that a proportion of these genes may play important roles in regression of the mammary gland. These six clusters consisted of 3463 transcripts, or 28% of all transcripts on the array. Only one group of 518 genes exhibited involution-specific expression, all of which were transiently activated within 24 hours after weaning (I_T). It was expected that a proportion of these represent genes that may contribute to the initial phase of cell death characteristic of the reversible first stage of involution. All other involution related clusters exhibited elevated expression at earlier stages in the pregnancy cycle. For example, one group of 236 involution related genes (I_P) exhibited an additional peak of expression specifically at parturition. This suggested that many transcriptionally regulated processes in involution were common to gestation (I_G), parturition (I_P) or lactation (I_L).

Correlation of gene function with gene expression pattern

Of the 6747 differentially expressed transcripts, 3273 (49%) encoded proteins with known function. In order to establish what proportion of the transcripts in each cluster from Fig. 1a shared common biological functions, transcripts with known function were assigned to categories as defined by the Gene Ontology Consortium (www.geneontology.org), and statistically significant associations between gene function and each cluster were identified using Onto-Express [22]. The significant associations (corrected $P<0.05$) are listed in Table 1. Further details on the distribution of functionally related genes between these clusters are available in Additional file 4.

Unexpectedly, similar proportions of apoptosis related genes were expressed in each of the 11 clusters; the only significant correlation being with the I_L cluster, in which maximal gene expression occurred at 12 hours of involution. However, a number of other biological processes were significantly linked to particular stages of mammary development, and were consistent with our current understanding of the physiology of the mammary gland. Thus, during gestation there was a significant increase in the expression of cell cycle regulatory genes (G, G_{SL} and I_G) and a concomitant increase in nucleic acid and macromolecular synthesis ('biogenesis' and 'metabolism' in Table 1). By lactation, the proportion of cell cycle genes had diminished, to be replaced by an increase in the

Table 1

Relationship between gene expression pattern and gene function in the 12-point developmental time course

Cluster	Transcripts	Biological process	Molecular function
V_{SL}	357	Metabolism Cell surface signal Development ECM and morphogenesis	Immunity Signal transducer
G	349	Cell cycle Cell growth	Cell cycle Cytokine
G_{SL}	605	Biogenesis Cell cycle	Cancer Enzyme Nucleic acid Chemokine
L_T	35	Cell adhesion Metabolism Transport Cell surface signal Intracellular signal	Signal transducer Transport Cytokine
L	144	Biogenesis Metabolism Transport Development ECM and morphogenesis	Cancer Transport
L_G	83	Cell cycle Metabolism Development Physiological process ECM and morphogenesis	Enzyme
I_L	624	Cell death Metabolism Transport Development ECM and morphogenesis	Apoptosis Immunity Signal transducer Transport
I_T	245	Biogenesis Cell cycle Cell growth Cell surface signal Intracellular signal	Signal transducer
I_P	156	Biogenesis Homeostasis Transport Intracellular signal	Immunity Signal transducer Transport
I_G	582	Cell cycle Metabolism Proliferation Transport	Cell cycle Transport
PC	93	Transport ECM and morphogenesis	Transport

Transcripts representing genes with known function were classified by molecular function and biological process, according to the ontological definitions of the Gene Ontology Consortium. The total numbers of known transcripts classified in this way are shown for each cluster. A statistical test for nonrandom distribution of these functional groups (taking into account the total number of transcripts in each category) was performed on each cluster using Onto-Express. All statistically significant associations (corrected $P<0.05$) are listed for each cluster using broad ontological definitions. The genes making up these broad categories are provided on GeNet (see Method section). Additional information on the details of these significant associations and a graphical representation of all ontologies are available as Additional files 1 and 4. ECM, extracellular matrix.

number of genes involved in development and differenti-ated function, including fatty acid biosynthesis and other metabolic processes (L_G, L and I_L). These three clusters should therefore contain genes that are important for ter-minal differentiation and transition to a secretory pheno-type (genelists for each cluster are available online; see Method section, above).

Morphogenesis and tissue remodelling are characteristic features of the mammary gland both during pregnancy and late in postlactational regression. We observed sig-nificant correlations between morphogenesis and three clusters with broadly similar expression profiles (L_G, L and I_L), each being characterized by increased gene expres-sion during gestation and lactation followed by a marked and progressive decline at the lactation–involution boundary. A significant proportion of remodelling-related genes were also observed in the V_{SL} cluster, which exhib-ited an inverse pattern to the three profiles described above. Because the transcripts in the V_{SL} cluster were, by definition, distinct from those in the L_G, L and I_L groups, it is possible that the former may contain remodelling related genes that contribute to mammary regression whereas the latter group of three clusters contains genes that are involved in invasion and remodelling of the fat pad during pregnancy.

The major differences between these two groups of genes were the high number of cell adhesion molecules (CAMs; e.g. Ceacam-10 and -11, integrins $\alpha 5$ and $\beta 3$, Vcam1, Ncam1, Pecam and Cadherin-8) and bone morphogenic protein (Bmp5 and Bmp7) genes in clusters L_G, L and I_L compared with the relatively high proportion of matrix com-ponents (e.g. laminin-$\alpha 2$, -$\alpha 4$ and -$\beta 2$; procollagen types I$\alpha 1$, I$\alpha 2$, IV$\alpha 1$, IV$\alpha 2$, V$\alpha 1$, VI$\alpha 1$, VI$\alpha 2$, VI$\alpha 3$, XIV$\alpha 1$ and XV; fibronectin-1 and decorin), matrix metalloproteinases and inhibitors (Mmp9, Mmp14, Timp3) and fibroblast growth factors (Fgf1, Fgf2) in the V_{SL} cluster.

A strong statistical relationship existed between involu-tion and immune-related genes (Table 1 and Fig. 2). Of the four clusters exhibiting maximal gene expression during involution, three were significantly associated with inflammation (I_T), the acute phase response (I_P and I_L), or humoral immunity (I_P). Of the remaining eight clus-ters, V_{SL}, whose transcripts exhibited a modest increase during involution (and were linked with remodelling), was associated with innate cellular defence (Fig. 2). Immune genes were most prevalent in the I_P cluster, in which transcripts were transiently expressed at parturi-tion and maximally expressed by 48 hours of involution (Fig. 2). Of the 57 immune-related genes present in the I_P cluster (24% of known genes in the cluster), 43 (18%) were immunoglobulin genes, seven (3%) were acute phase protein genes and seven (3%) were inflam-matory mediators.

Figure 2
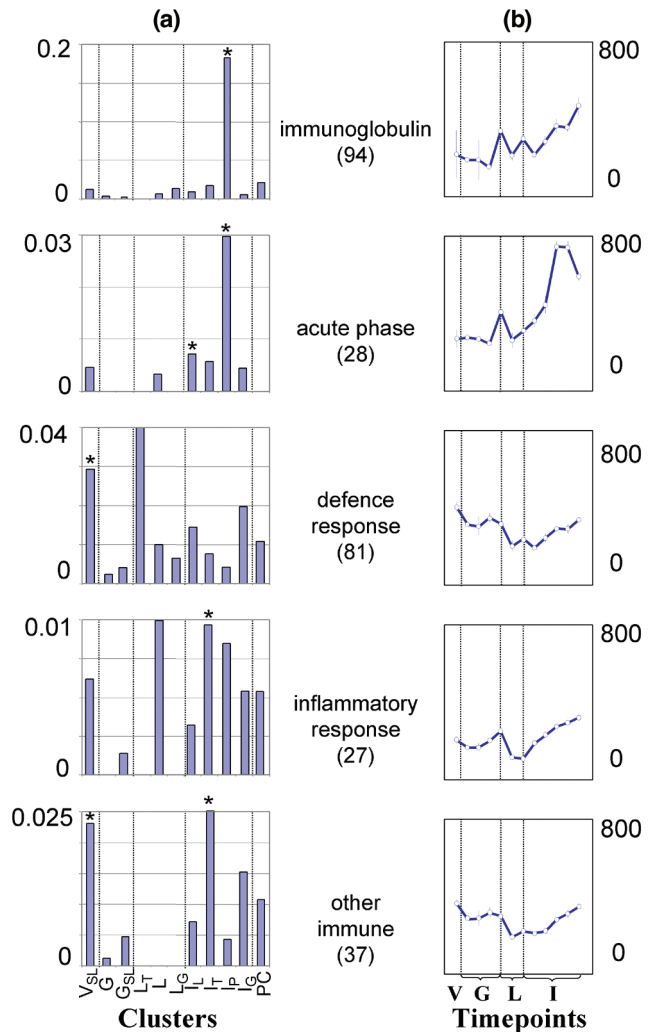
Relative expression of immune related genes in the 12-point timecourse of mammary development. Immune-related transcripts differentially expressed in the 12-point developmental time course were grouped into five categories that represent different immunological functions. The number of transcripts in each category is shown in brackets. Genelists of all transcripts assigned to these immunological categories are available online. **(a)** The frequency of transcripts in each expression profile cluster for each immunological category. Statistically significant associations, as determined by Onto-Express, are indicated with asterisks. Y axes indicate the percentage for each cluster. X axis indicates the combined expression profile clusters. **(b)** The mean expression profiles for all transcripts in each immunological category. Y axis indicates the mean normalized signal intensity, and the X axis indicates the 12 developmental time points (see Fig. 1).

Acute phase gene expression correlates with Stat3 activity
We have previously shown that the IL-6 family cytokine leukaemia inhibitory factor (LIF) induced phosphorylation (and activation) of Stat3 in mammary gland during involu-tion, and that this transcriptional signalling pathway was important for the induction of apoptosis in the early involut-ing gland [17,24]. Stat3 is also a recognised mediator of

Figure 3
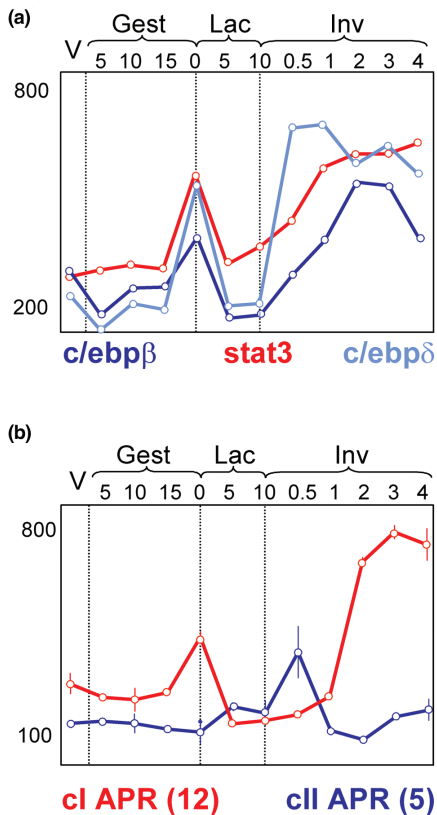
Mean expression profiles of acute phase genes. **(a)** Expression profile of the signal transducer and activator of transcription (Stat)3 transcript (red), as compared with CAAT-enhancer binding protein (c/ebp)β (dark blue) and c/ebpδ (light blue) from the microarray. **(b)** The mean expression profiles of 12 class I acute phase response genes (cI APR; red) and five class II acute phase response genes (cII APR; blue). Y axes indicate mean normalized intensity, and X axes indicate the developmental time points in days. Error bars represent standard error of the mean. Gest, gestation; Lac, Lactation; Inv, involution.

the acute phase response, and because acute phase related genes exhibited a parturition/involution expression profile (Fig. 2) we assessed the relationship between Stat3 activity and acute phase gene expression.

Acute phase proteins are classified according to the transcriptional signalling pathways that regulate their expression [25]. Thus, class I acute phase genes are activated by cooperative binding of CAAT-enhancer binding protein (C/ebp)β, C/ebpδ and/or nuclear factor (NF)-κB (and in some cases also Stat3) to specific sites in their promoters. Class II genes are activated by Stat3 alone. Class I genes were induced at parturition and involution (similar to the I_P profile). Class II genes were transiently, and specifically, expressed at the onset of involution (similar to the I_T profile). We previously described Stat3 and NF-κB activities in the mammary pregnancy cycle [24,26], whereas *Cebp* gene expression in mammary gland has also been

described [27]. We compared the expression of these transcription factors and found that *Stat3* and both *Cebp* genes in addition to phosphorylated Stat3 DNA binding activity [17] correlated with class I acute phase gene expression (Fig. 3).
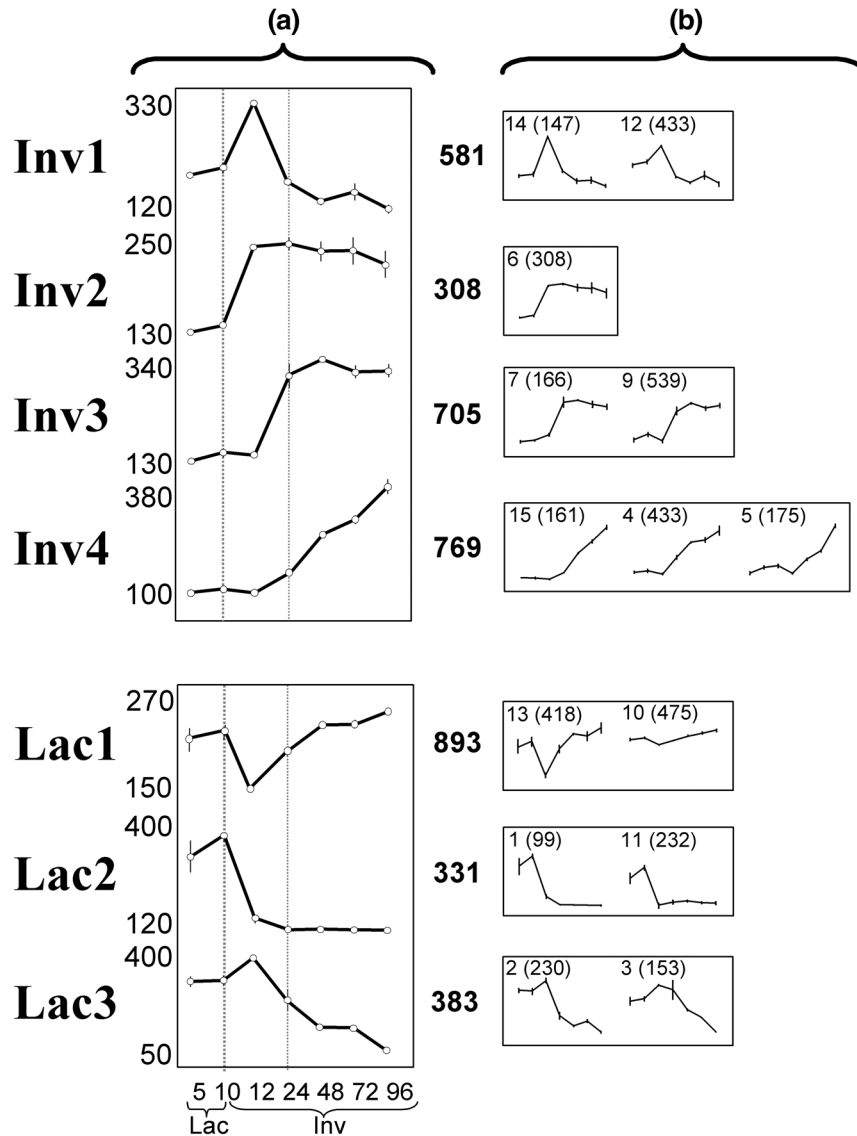
Expression profile analysis of lactation/involution (seven-point time course)
In order to define patterns of gene expression pertinent to the lactation–involution transition, irrespective of expression in pregnancy, differentially expressed genes were reclustered using a truncated time course involving lactation and involution time points only (Fig. 4). A total of 4103 transcripts (33% of all transcripts) exhibited significant changes in expression across the seven time points, namely 5 days and 10 days of lactation, and 12, 24, 48, 72 and 96 hours of involution. These profiles were sorted according to similarity into 15 clusters (Fig. 4b). Similar clusters were then combined to produce seven basic profiles that differed according to the kinetics of gene induction or suppression during lactation and involution (Fig. 4a). Thus, four patterns of activation were observed: a rapid but transient increase in gene expression 12 hours after weaning (Inv1); a rapid activation, which was maximal by 12 hours and sustained for up to 4 days (Inv2); a slightly delayed induction, maximal by 24/48 hours, and sustained for at least 96 hours after weaning; and a gradual increase in expression, which peaked at or beyond 96 hours (Inv4). Similarly, three patterns of suppression were observed: a transient decrease in transcript levels that mirrored the pattern observed in Inv1 (Lac1); a rapid loss of expression, reaching baseline levels at 12/24 hours and remaining low for up to 96 hours (Lac2); and a delayed reduction in expression, originating from a peak in expression at 12 hours involution and reaching a minimum beyond 96 hours (Lac3).

These patterns inferred three phases of gene expression during involution: rapid changes within the first 12 hours (Inv1, Inv2, Lac1, Lac2), delayed expression by 24–48 hours (Inv3), and prolonged changes extending beyond 4 days (Inv4, Lac3).

Consistent with our analysis of the complete 12-point time course, we found that involution related gene expression was associated with an overall increase in immune gene expression (Table 2 and Fig. 5; also see Additional file 5). There was an initial transient increase in proinflammatory genes (Inv1). However, the association was most pronounced with transcripts exhibiting delayed kinetics (Inv4), in which known genes involved in innate immunity, antimicrobial defence and inflammation were all preferentially expressed. The delayed immunological changes also coincided with a significant increase in apoptosis markers (see below) and factors that affect tissue architecture, including genes encoding structural proteins (decorin, laminins,

Figure 4

Clusters of gene expression profiles from a seven-point time course of mammary lactation and involution. Fifteen K means clusters of 4103 differentially expressed transcripts from seven stages of lactation and involution. Fourteen primary clusters **(b)** were grouped according to similarity into **(a)** seven basic profiles: Inv1, Inv2, Inv3, Inv4, Lac1, Lac2 and Lac3. These similar clusters were pooled and plotted as seven combined expression profiles. The 15th cluster represented differential expression between 5-day and 10-day lactation only (not shown). The number of genes for each pooled cluster is indicated to the right of panel A. The number of genes (brackets) and the cluster ID is indicated for each primary cluster in panel B. Each differentially expressed transcript is represented only once in the 15 primary clusters. The number of genes in each combined profile (panel A) is the sum of its primary profiles (panel B). Y axis is the mean of normalized intensities. Error bars represent the standard error of the mean for all transcripts in each cluster. X axis = 7 point timecourse across lactation (Lac) and involution (Inv). Dotted lines highlight 10 day lactation and 24 hour involution timepoints.

Nid1, collagen $1\alpha1$, $1\alpha2$, $3\alpha1$ and $4\alpha2$) and matrix associated proteins (Mmp3, Mmp12, Gelsolin, uPa and Adamts1), thus corroborating previous reports of the role of matrix proteases in remodelling of the regressing mammary gland [2]. Lactation specific expression, as expected, was associated with downregulation of differentiation and metabolic processes after weaning (Lac2 and Lac3; Table 2).

Cytokine expression highlights two immune phases during involution

In order to gain further insight into the immune responses associated with mammary involution, we listed the cytokines, chemokines and immune-related genes associated with each involution profile from Fig. 4 (Table 3). This confirmed the two phases of immune-related gene expression illustrated by Fig. 5: an initial transient expression of

Table 2

The relationship between gene expression pattern and gene function in the seven-point lactation/involution time course

Cluster	Transcripts	Biological process	Molecular function
Inv1	292	Transport Cell surface signal Intracellular signal	Immunity Enzyme Structural Growth factors
Inv2	153	Transport	Enzyme
Inv3	336	Biogenesis Metabolism Development	Apoptosis Enzyme Nucleic acid Signal transducer Structural
Inv4	420	Cell adhesion Cell surface signal ECM and morphogenesis	Apoptosis Immuntiy Enzyme Signal transducer Structural Cytokine
Lac1	374	Intracellular signal	
Lac2	163	Biogenesis Metabolism Transport ECM and morphogenesis	Enzyme Nucleic acid Transport
Lac3	188	Cell adhesion Transport Development	Signal transducer Transport Cytokine Growth factors

Transcripts representing genes with known function were classified by molecular function and biological process, according to the ontological definitions of the Gene Ontology Consortium. The total numbers of known transcripts classified in this way are shown for each cluster. A statistical test for nonrandom distribution of these functional groups (taking into account the total number of transcripts in each category) was performed on each cluster using Onto-Express. All statistically significant associations (corrected $P<0.05$) are listed for each cluster using the same ontological definitions used in Table 1 and available on GeNet. Additional information on the details of these significant associations and a graphical representation of all ontologies are available as Additional files 2 and 5. ECM, extracellular matrix.

proinflammatory cytokines and TNF superfamily genes followed by delayed expression of monocyte and lymphoid chemokines and immunoglobulin genes.

Thus, an initial burst of cytokine and cytokine receptor expression was observed within 12 hours of forced weaning (Inv1; Table 3), which included the acute-phase mediators LIF receptor (*Lifr*), IL-11 (*Il11*) and the neutrophil chemoattractant Gro1 (*Cxcl1*), and the proinflammatory mediators IL-1a, IL-1b and IL-13 (*Il1a, Il1b* and *Il13*). This was accompanied by a transient decrease in other cytokine receptors (including *Il11r*) and an inhibitor of IL-1 receptor signalling (*Il1rn*), suggesting that specific proinflammatory signals may be suppressed at the transcriptional level.
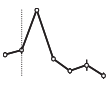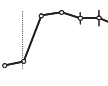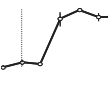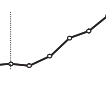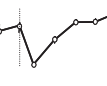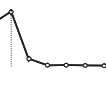
Figure 5
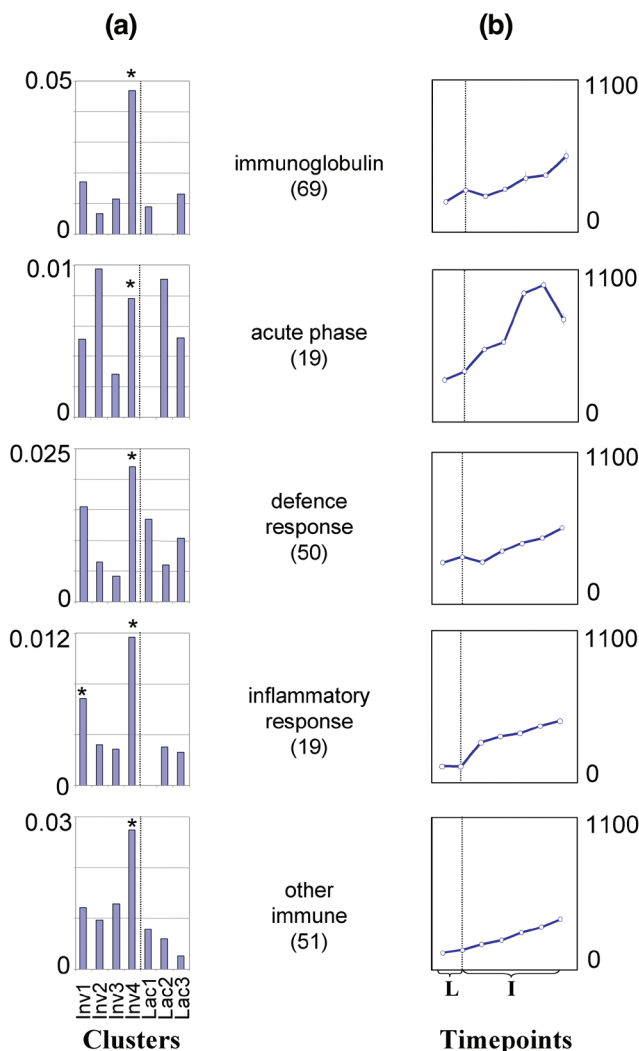
Relative expression of immune related genes in the seven-point time course of mammary lactation and involution. Immune-related transcripts that were differentially expressed in the seven-point lactation/involution time course were grouped into five categories representing different immunological functions. The number of transcripts in each category is shown in brackets. Genelists of all transcripts assigned to these immunological categories are available online. **(a)** For each category, the frequency of transcripts in each expression profile cluster was plotted. Statistically significant associations, as determined by Onto-Express, are indicated with asterisks. The Y axis indicates the percentage of each expression profile cluster, and the X axis indicates the expression profile cluster. **(b)** The mean combined expression profiles of transcripts in each immunological category are also shown (right). The Y axis indicates the mean normalized signal intensity, and the X axis indicates the seven developmental timepoints from lactation (L) and involution (I; see Fig. 2).

One expected outcome of the transient cytokine expression was an infiltration of inflammatory cells, in particular, neutrophils due to the expression of *Cxcl1*. Analysis of immune cell specific antigen expression demonstrated a

Table 3

Identity of immune-related genes in lactation and Involution clusters

	Inv1	Inv2	Inv3	Inv4	Lac 1	Lac2	Lac3
Cytokines	Lifr Il1a Il1b Il1r2 Il11 Il13	Il11ra2 Ltbr	Il12rb1	Osmr Il1r1 Il10rb Csf1r	Il1rn Il4ra Il7r Il11r Csf1	Il1rl1l	Il1r2
Chemokines	Cxcl1 (Gro1)		Ccl9	Ccl6 Ccl7 Ccl8 Ccl21a Cxcl9 Cxcl14			
Immune cell antigens	Lsp1 Cd19 H2-M1 Cd83 Tnfsf7 (Cd27l) Tnfrsf9 (Cd137)	Cd80 Tnfrsf7 (Cd27)	Cd14	Cd79a Ly86 Cd86 Tnfrsf4 (Ox40) Lrp1 (Cd91)			
Acute phase response	A2m Serpina1c	Hpxn Orm2 Cebpd	Cebpb Lcn2	Hp Saa1 Saa2 Saa3 Saa-ps		Hpxn Nr3c1 Serpina3g	Orm1
Immunoglobulin	IgG-3 h-ch IgA h-ch	Ig variable regn.	IgK l-ch (×2) IgL l-ch IgA h-ch	IgM h-ch (×1) IgM l-ch (×2) IgA h-ch (×4) Ig K l-ch (×14) Ig L l-ch (×3) IgG l-ch (×4) Ig H18	Ig l-ch		
Immunoglobulin-related	Fcer1g (Fc rec) Pigr Sema4b Sema7a	Axcam	Necl1-pending	Sema4a Ltf	Sema4a Sema4d Igsf8		
Complement	C1qa C3ar1 C9		C2	H2-Bf C3 C4 C2 C1qa C1qc	C1qr C1qbp C2		
Immune related	Tnfsf4 (OX40L) Tnfsf6 (FasL) Tnfsf7 (Cd70) Tnfsf10 Tnfsf12 (Tweak) Tnfrsf9 (Cd137) Il4i1 Gp5	E4bp4 Inhbb Pafah1b2	Tgfb1 Tgfb3 Tnfrsf6 (Fas) Myd88 Emr1 Ecgf1 Pla2g2a Fadd	Casp1	Lig1	Irak1 H2-Aa	Tnfaip2 Traf6 Tnfrsf11a

The identity of immune-related transcripts was determined for each of the combined profile clusters illustrated in Fig. 4. Thus, transcripts belonging to the ontological category Immunity from each cluster were listed according to the immunological subcategories shown in the first column of the table. All gene names conform to the mouse genome informatics database [23], synonyms are included in brackets where appropriate. The number of transcripts of the same immunoglobulin chain genes are indicated. Complete genelists for these immunological categories are available on GeNet (see Method section).

Figure 6
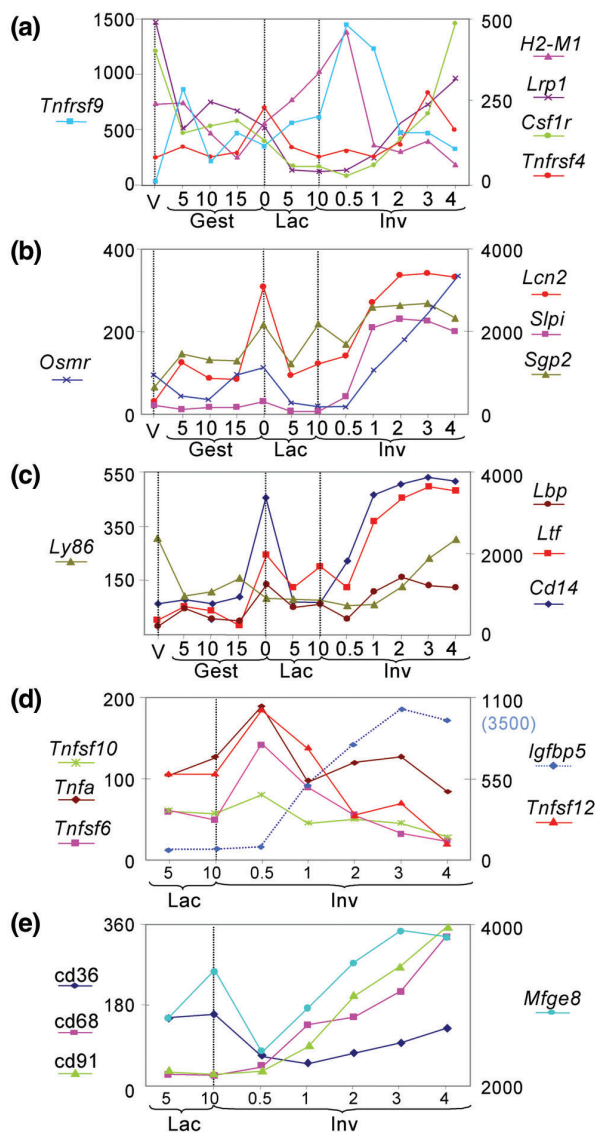
Involution related expression profiles: apoptosis and immune related genes. **(a)** Expression profiles of differentially expressed immune cell antigens, illustrating the relative proportions of immune cell subtypes in mammary gland during the complete pregnancy cycle. Monocyte/macrophage markers: *Lrp1* = Cd91; *Csf1r* = Cd115. Lymphocyte markers: *Tnfrsf9* = Cd137; *H2-M1* = Mb1; *Tnfrsf4* = OX40. **(b)** Profiles of differentially expressed anti-inflammatory factors in the complete mammary time course. **(c)** Profiles of differentially expressed soluble defence factors, including the innate components lactoferrin (*Ltf*), Cd14, Ly86 and Lbp. Also shown is the mean expression profile for all differentially expressed immunoglobulin transcripts. **(d)** Differentially expressed death receptor ligands (*Tnfsf6* = FasL, *Tnfsf12* = Tweak, *TNFa* and *Tnfsf10* = Trail) and the insulin-like growth factor (IGF) inhibitor *Igfbp5*. **(e)** Differentially expressed receptors and ligands involved in recognition and engulfment of apoptotic cells. Y axes indicate the normalized intensity. (Note that the brackets in panel d represent scale for *Igfbp5* only.) X axes indicate developmental time points for the complete 12-point time course (panels a, b and c) and 7-point lactation/involution time course (d, e).

marked increase in the monocyte/macrophage markers *Lrp*, *Csf1r* and *Cd14* (Table 3 and Fig. 6a), but no evidence for an accompanying increase in neutrophils or eosinophils (polymorphonuclear leucocyte markers *Fut4*, *Fcgr3, Caecam1, Il8rb* and *Tnfrsf5* were not induced during involution; data not shown). This is consistent with previous observations of the immune cell complement in regressing murine mammary gland [28] and supports the hypothesis that neutrophil numbers are suppressed in involuting tissues in order to prevent subsequent tissue damage [28,29]. Interestingly, the gene for oncostatin M receptor (*Osmr*) was upregulated in involution (Fig. 6b). In models of inflammation oncostatin M is produced locally to prevent proinflammatory cytokines such as IL-8 from recruiting neutrophils, thus attenuating the inflammatory response [30]. We also confirm a previous report that uterocalin (*Lcn2*) and *Sgp2*, a subunit of clusterin, are upregulated during involution and parturition [19], because their expression correlates with the I_P profile (Fig. 6b). Uterocalin, an acute-phase response gene that is regulated by Stat3 [31,32], prevents neutrophil accumulation possibly through targeted destruction of neutrophils [29], whereas clusterin protects cells from the damaging effects of neutrophils [33,34]. Furthermore, secretory leukocyte protease inhibitor (*Slpi*), a potent inhibitor of neutrophil proteases [35], and a Stat3-induced gene (Boland M and coworkers, unpublished data) was also markedly upregulated during involution (Fig. 6b). Thus, although the transcriptional effects of the acute-phase mediators LIF–Stat3 and C/ebpβ/δ are clearly evident (Fig. 3), the immunological outcome at the cellular level is likely to be minimised by coexpression of anti-inflammatory factors such as these.
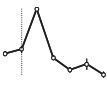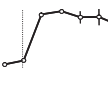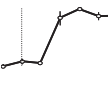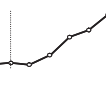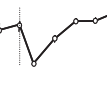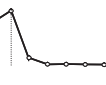
Following the initial 'spike' in inflammatory cytokine expression, six different chemokine genes were slowly induced over the 4-day period of involution (Inv4; Table 3). These included chemoattractants for monocytes and macrophages (C*cl6*, C*cl7*, C*cl8* and C*xcl14*) and lymphocytes (C*cl6*, C*cl21a*, C*xcl9* and C*xcl14*). This chemokine induction was accompanied by a marked increase in immunoglobulin gene expression (Fig. 5, Table 3). Consistent with these observations, the monocyte/macrophage markers *Lrp1*, *Csf1r* and *Cd14*, and the lymphocyte antigens *Tnfrsf9*, *H2-M1* and *Tnfrsf4* were increased during involution (Table 3 and Fig. 6a).

Soluble innate defence factors are induced in involution
In addition to the immunoglobulins, genes that encode a number of innate soluble defence factors were highly expressed in involution (Table 3 and Fig. 6c). These soluble factors possess antimicrobial properties that are secreted to protect against mastitis during the vulnerable period of milk stasis [36]. Thus, the iron chelator lactoferrin (*Ltf*), which is found at high levels in the milk of humans and cattle [37,38], is induced with kinetics similar to those of the antibody transcripts described above (Fig. 5). Comple-

Table 4

Cell death-related genes in lactation and involution clusters

	Inv1	Inv2	Inv3	Inv4	Lac 1	Lac2	Lac3
Cell survival	Prdx2 Bcl10	Nfkb2	Api5 Ank3	Bcl2l (BclX) Mcl1 Psap	Psap Birc4(mXIAP)	Akt1 Dad1 Gfi1	
Cell death	Hrk Gzmb Nkx3-1 Tnfsf6(FasL) Lsp1 Tnfsf4(OX40L) Tnfsf7 (Cd27l) Tnfsf10 (Trail) Tnfsf12 (Tweak) Tnfrsf9 (Cd137) Tnf (Tnfa)	Bcl2l10 (Diva) Casp4 (casp11) Casp12 Gadd45b Pdcd6 Cdkn1a (p21) Unc5 Foxb2 Mycs (s-myc) E4bp4 Ngfa Ltbr	Fsp27 Bax Casp7 Serpinb5 Pdcd6 Pdcd5 Tnfrsf6 (Fas) Fadd Myd88 Tnfrsf1a	Igfbp5 Casp1 Foxm1 Apaf1 Gzma Tia1 Phlda1 Tnfrsf4 (Ox40) Tgfb1	Tnip1 (ABIN1) Eya2 Tial1 Dapk3 Foxb1 Rbl2 Tnfsf5 (TRAP)		Tnfrsf11a(Rank) Cidea Dffb Perp-pending Traf6 Tnfsf9

The identity of cell death-related transcripts was determined for each of the profile clusters illustrated in Fig. 4. Thus, transcripts belonging to the ontological category apoptosis or cell death from each cluster were listed according to their expected role as proapoptotic or survival factors. All gene names conform to the mouse genome informatics database [23], and synonyms are included in brackets where appropriate. A complete genelists for this apoptosis category is available on GeNet (see Method section).

ment components are prevalent at all stages during involution (Table 3). These factors are found at high levels in mastitic milk and in normal bovine mammary glands during involution, and are thought to play a dual role in opsonization and lysis of bacteria and as a proinflammatory mediator [39,40]. *Cd14* was significantly upregulated in involution and was also highly expressed during parturition (Fig. 6c). This antigen is expressed on bovine mammary polymorphonuclear neutrophils and macrophages [41] and plays an integral role in suppressing bacterial infections in response to endotoxin. Furthermore, *Lbp* [42] and *Ly86* [43], which in conjunction with *Cd14* augment responses to lipopolysaccharide (LPS), were also upregulated. The significant regulation of these genes in uninfected mammary gland confirms a role for soluble defence factors in normal development of mouse mammary gland.

Involution is associated with a transient induction of death receptor genes

Involution is characterized by extensive apoptosis of epithelial cells, which occurs both before and during the tissue remodelling process. By subdividing the first 4 days of involution into three discrete phases of gene expression, we demonstrated a link between the expression of apoptosis-related genes and the delayed onset of extracellular matrix degradation and alveolar collapse at 48 hours (Inv3 and Inv4; Table 2). However, we were unable to identify a significant increase in the number of apoptosis genes expressed at the onset of involution. One explanation for this was that specific combinations of cell

death genes, rather than an increase in the number of genes activated, influenced apoptosis at the onset of involution. In order to address this, all cell death related genes found to be regulated during involution were grouped according to their lactation/involution expression pattern (Table 4). Regulators (Bax, BclX, Mcl1, mXIAP and Apaf-1) and effectors (caspases) of classical apoptosis and immune cell-mediated killing (granzyme A, granzyme B, Fadd, Fas, Fas ligand) were regulated with different kinetics during involution and were relatively evenly distributed across the four involution clusters (Table 4).

However, we observed a strong bias in the expression of extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis genes, in which extrinsic-related components were generally induced within the first 24 hours of involution whereas intrinsic genes, with the exception of Hrk and Diva, were not maximally transcribed until at least 96 hours.

Thus, six ligands of the TNF superfamily (*Tnf, Tnfsf4, Tnfsf6, Tnfsf7, Tnfsf10* and *Tnfsf12*) were specifically induced in involution and all were transiently activated at 12 hours after weaning (Inv1; Fig. 6d). Four of these ligands (death receptor ligands: Fas ligand, TNF-α, TWEAK and TRAIL) are responsible for activating the five principal mammalian death receptor (extrinsic) apoptosis pathways through their cognate receptors Fas, TNFR-1, TNFR-2, DR3 and DR4. Two of these receptors (*Fas* and *Tnfrsf1a*) were also induced, and maximally coexpressed, within 24 hours of weaning (Inv3; Table 4). This correlates

with previous studies in mammary gland of two of these ligand–receptor pairs [44,45]. NF-κB activity also correlated with the rapid activation of these TNF superfamily ligands. DNA binding activity of this transcription factor is markedly upregulated within 3 hours of forced involution and is suggested to promote survival of a subpopulation of mammary epithelial cells [26]. Thus *Nfkb2* and activators of NF-κB (*Tnfsf12*, *Bcl10*, *Tnfrsf11a*, *Traf6* and *Tnfsf10*) were upregulated, whereas an inhibitor of NF-κB function (*Tnip1*) was inhibited during the initial 24 hours following forced weaning, when NF-κB activity was induced [26]. Previous studies demonstrating the direct activation of NF-κB by TNF superfamily ligands in mammary epithelial cells [26,46,47] therefore suggests that the observed transient increase in these ligands may be functionally relevant.

In contrast to the rapid response of extrinsic factors, intrinsic apoptosis genes were predominantly (but not exclusively) expressed at least 24 hours after forced weaning. Proapoptotic and antiapoptotic members of the Bcl-2 family (*Bax, Mcl1* and *Bcl2l*) were induced together with the downstream effector of the intrinsic pathway *Apaf1*. Although the precise mechanism(s) of cell death in mammary gland have yet to be established, the trends in apoptosis gene expression identified here hint that extrinsic (death receptor) and intrinsic death programmes may predominate at different times. A marked induction of *Igfbp5* complemented the delayed induction of intrinsic apoptosis genes (Fig. 6d). The kinetics of this potent inducer of mammary apoptosis and involution [48] suggests that it probably contributes to the stimulation of intrinsic cell death at this time.

Apoptotic bodies initially shed into the lumen and the residual dying cells left in the epithelium are believed to be cleared from the mammary gland by nonprofessional phagocytes and interstitial macrophages, respectively [28]. Receptors and ligands that mediate recognition and engulfment of apoptotic cells include members that are common to both types of phagocytic cell (e.g. *Cd36*, *Lrp1* and *Mfge8*) and those specific to professional macrophages (e.g. *Cd14* and *Cd68*). The genes for each of these factors (except *Cd36*, which exhibits an initial downregulation before a modest increase by 96 hours) are induced with similar kinetics (Fig. 6e). This is indicative of the increasing demands placed on the mammary gland to clear dead/dying cells during regression, and also probably reflects the increasing numbers of macrophages occupying the gland during the first 4 days of involution.

Discussion

Microarray analysis of large sequence verified cDNA probe sets provides a powerful tool for analyzing complex biological systems because it can extract patterns of gene expression from a significant proportion of the total genomic content of an organism. In this study we simulta-

neously analyzed approximately 12 000 transcripts, representing 8618 unigene clusters, or about one-third of the protein encoding capacity of the mouse, to determine basic expression patterns in adult mammary gland during the pregnancy cycle.

Although a previous study of approximately 5000 transcripts described gene expression in early phases of the pregnancy cycle [14], we focused our analysis on the first 4 days after forced weaning, using time points from earlier stages in development to establish the specificity of these involution-related genetic changes. In the accompanying paper, Stein and coworkers [19] describe a similar study in Balb/c animals. Despite differences in the methods of analysis, their findings on the activation of inflammatory mediators and monocyte/lymphocyte markers in involution correlate with the results described here, further verifying our microarray data. Thus, strain differences did not appreciably influence the immune responses associated with involution.

The principal objective of the present study was to provide a comprehensive set of gene expression data from late stages of mouse mammary development to be used as an online resource for further analysis and/or download. The raw microarray data files and processed genelists from the study are therefore available for download or online analysis (see www.path.cam.ac.uk/~madgroup/).

Here we have outlined the common patterns of gene expression across the developmental time course and have correlated these patterns with biological function. Thus, as expected, pregnancy was associated with the induction of genes that are involved in cell proliferation, differentiation and tissue morphogenesis. This was superceded in lactation by genes involved in biosynthetic (fatty acid synthesis) and metabolic pathways, correlating with the findings of an earlier microarray study of the pregnancy cycle [14]. We have not attempted to provide a detailed account of the genetic changes that occur early in the pregnancy cycle; however, the data are available online for further scrutiny.

By concentrating six of our 12 time points around the first 96 hours of involution, we were able to determine the general kinetics of gene expression during this period. Thus, according to the cluster analysis presented in Fig. 4, most differentially expressed transcripts in involution were initially induced (or suppressed) within 24 hours of weaning, whereas maximum expression generally occurred at 12 (Inv1, Inv2), 48 (Inv3) or 96 (Inv4) hours. This implies three phases of gene expression in the first 4 days of involution – phases that are characterized both by their time of initiation and their rate of induction. At the cellular level this period of involution has previously been divided into two stages [2]: an initial reversible period of

approximately 48 hours characterized by increased apoptosis and loss of differentiated function, followed by matrix degradation and alveolar collapse. The pattern of gene expression appeared to correlate with this cellular model. We observed a swift increase in specific apoptosis transcripts (Inv1; Table 4) and a subsequent decline in differentiated function by 48 hours (Inv3; Table 2), followed by an increase in factors affecting tissue architecture (Inv4; Table 2) and phagocyte function (Fig. 6). Further detailed analysis of the Inv3 cluster, which exhibits maximal expression at 48 hours, may elucidate underlying mechanisms that are responsible for the transition to phase two involution.

Transcriptional regulation of apoptosis in involution: two phases of death?
Our data also provide provisional evidence for the existence of a two-phase apoptotic process in involution and which also coincides with the two phases previously described [2]. While the number of apoptosis genes was similar in each involution cluster, there was a pronounced bias in the distribution of extrinsic and intrinsic apoptosis genes. The initial spike of activity consisted of four death receptor ligands, namely *Tnf* (TNF-α) *Tnfsf6* (Fas ligand), *Tnfsf10* (TRAIL) and *Tnfsf12* (TWEAK). These ligands bind to the principal death receptors Fas, TNFR-1, TNFR-2, DR3 and DR4, which initiate apoptosis in cell mediated immune cell death and in a variety of physiological and pathological situations. These data confirm previous observations that Fas and Fas ligand are upregulated within a day of involution in mouse mammary gland, and that the deletion of these genes results in delayed regression and loss of apoptosis [44]. Deregulation of Fas signalling through aberrant Akt and NF-κB activity is believed to play a role in the immune escape of breast cancers and in resistance to chemotherapy [49]. TNF-α also induces NF-κB signalling to prevent apoptosis in a subpopulation of cultured mammary epithelial cells [26,47], and has been shown to be downregulated late in involution (day 7); however, it has not been studied at earlier time points [50]. Furthermore, elevated IL-10 dependent TRAIL expression (and its receptor DR4) has previously been demonstrated in involution [45].

In contrast to the transient activation of death receptor ligands, components of mitochondrial (or intrinsic) apoptosis exhibit sustained, often delayed induction. Thus the caspase 9 effector, *Apaf1*, and *Bax* were induced with delayed kinetics. Costimulation of the survival factors *Mcl1* and *Bcl2l* (*BclXl*) suggests that the transcriptional regulation of Bax may be counterbalanced, and therefore controlled, by expression of Bcl-2 homologues. Suppression of BclX, for example, promotes death during mammary involution [51], whereas delayed induction of Bax has previously been demonstrated [52]. Regulation of Bcl homologues was not exclusively restricted to late stage involution. *Hrk* and *Boo/Diva* were differentially expressed

within 24 hours. Hrk is a proapoptotic factor that is transcriptionally suppressed by progesterone signals in breast cancer cell lines [53]. The function of Boo/Diva, which has only previously been described in mouse ovary, is poorly defined [54]. Cross-talk between death receptor pathways and Bcl-2 regulatory factors is well established [55] and may explain the early activation of these two Bcl homologues. Probable upstream initiators of a putative delayed apoptotic mechanism include the loss of survival signals, such as insulin-like growth factor (IGF)-1 [48,56], and the destruction of extracellular matrix–integrin contacts leading to an anoikis-type death [6,57]. The dramatic induction of the proapoptotic mammary protein IGF-binding protein 5 proposed previously [48] coincides with the onset of second phase involution (Fig. 6d, Table 4) and the upregulation of metalloproteinases [2,6]. Both IGF-1 and integrins signal through Akt, which was also downregulated in involution (Table 4). Loss of this signal could then induce Bax [57]. Our data suggest that apoptosis is induced in involution following the specific transcriptional activation of a subset of proapoptotic genes encoding death receptors, and that delayed expression of inhibitors of cell survival (e.g. IGF-binding protein 5) induce alternate apoptosis pathways during the second phase of involution. This hypothesis warrants further investigation.

Integral to the apoptotic process is the phagocytic clearance of apoptotic bodies by macrophages. It has been suggested that monocytic macrophages are recruited to the mammary gland to supercede phagocytic epithelial cells in the clearance of milk and dead cells [28]. We have demonstrated monocyte specific (*Cd14* and *Cd68*) and nonspecific phagocyte receptors during involution, supporting a role for monocytic phagocytes in mammary involution.

Multiple roles for immune cells in mammary involution
Measurements of the cellular content in milk from involuting glands of sheep, pigs and cattle indicate an early rise in polymorphonuclear neutrophils, a subsequent increase in macrophages and low numbers of eosinophils, B cells and T cells [7,28]. However, the immune cell complement of human and rodent mammary gland is poorly defined. It is believed that these phagocytes are recruited to the gland to perform several functions in phagocytic clearance and cellular immunity.

We have described the sequential activation of cytokine and chemokine genes in involution. The effect of these would be to attract monocytes and lymphocytes to the gland, and we identified an increase in a number of immune cell markers, supporting this hypothesis. For example, increased expression of the Csf-1 receptor, c-fms (*Csf1r*; Fig. 6a), which is restricted to macrophages of the monocyte lineage in mammary gland [58] and is required for appropriate branching morphogenesis in pregnancy [59], confirmed that macrophages are critical

for normal development [58]. A role for these cells in phagocytic clearance during involution is emphasized by the concomitant increase in two receptors (*Cd14* and *Cd91*) and the milk factor *Mgfe8*, which mediate recognition of apoptotic cells by macrophages (Fig. 6c,e).

Recruitment of cells of the monocytic lineage comprises an inflammatory response. However, in the absence of a persistent bacterial infection, this does not occur in the regressing mammary gland, even though inflammatory cytokines and downstream mediators are activated, presumably to perform additional roles during involution. During chronic inflammatory diseases such as rheumatoid arthritis or inflammatory bowel disease, uncontrolled accumulation of neutrophils yield aberrantly elevated levels of inflammatory compounds, which become major contributors to tissue damage [60,61] Thus, regulation of neutrophil recruitment into inflammatory sites and their clearance are critical processes that ensure effective host defence without tissue injury. In mammary gland, the LIF–Stat3 signaling pathway is known to be crucial for appropriate regression during early involution by initiating epithelial apoptosis [17,24]. With the concomitant downregulation of IL-11 receptor (Table 3) and lack of a significant increase in IL-6 or its receptor (data not shown), it is also likely to be the predominant protagonist of acute phase signalling. This is confirmed by previous observations that C/ebpβ and C/ebpδ are both transcriptional targets of Stat3 *in vivo* [24] (Boland M, unpublished data) and that the profiles of Class I acute phase genes correlated precisely with phosphorylated Stat3 activity [24] and *Stat3*, *C/ebpβ* and *C/ebpδ* expression (Fig. 3). Class II acute-phase response genes exhibit specific and transient expression in involution. It is thought that these genes respond transiently to proinflammatory signals [25] and therefore may respond in a similar manner to the rapid activation of Stat3 at the onset of involution. Why they are not induced at parturition is unclear, although a molecular switch that determines the specificity of signalling pathways downstream of LIF has been proposed [24].

Thus, inhibitory mechanism(s) must exist to prevent the accumulation of potentially damaging cellular infiltrates in response to LIF. In a previous study of involution in uterus and mammary gland, the acute phase protein uterocalin (*Lcn2*) was shown to be induced [29]. It was proposed that one role of this protein in involuting mammary gland and uterus was to eliminate infiltrating neutrophils by inducing apoptosis [62] and therefore to prevent subsequent tissue damage [63]. We are able to confirm significant activation of uterocalin expression in mammary involution (Fig. 6b) and the concomitant absence of neutrophil antigens (Table 3). In conjunction with the induction of uterocalin, clusterin (*Sgp2*), oncostatin M receptor (*Osmr*) and secretory leukocyte inhibitory protein (*Slpi*) were also increased (Fig. 6b). Clusterin protects cells

against oxidative and chemical damage [33,34] and has been suggested to be coexpressed with uterocalin as part of a localized acute-phase response in order to limit the damaging effects of residual neutrophils in involuting mammary gland [29,63]. SLPi, an antichymotrypsin, also contributes to the elimination of neutrophil function, whereas Osmr may possess similar anti-inflammatory effects through the sequestration of proinflammatory cytokines. Osmr, Slpi and Sgp2 are all directly induced by Stat3 in mammary epithelial cells (Boland M, unpublished data) [64], whereas uterocalin expression conformed with Stat3 activity (Fig. 3 and 6b). A putative anti-inflammatory role for Stat3 was suggested in our previous study of Stat3-deficient mammary glands [17], in which we observed an increase in mastitis in the absence of Stat3. A similar protective mechanism may exist during parturition, when the LIF–Stat3 signal is also active [24,65]. We are currently investigating the possibility that LIF–Stat3 signalling is responsible for the induction of these four genes in mammary gland, therefore confirming a dual role for LIF in involuting mammary gland. In addition to the direct effects of these anti-inflammatory acute phase genes, immunosuppressive factors including transforming growth factor-β (Inv3; Table 3) expressed by phagocytic macrophages are likely also to contribute to the suppression of an inflammatory response [64,66].

Soluble defence factors are induced during involution
Soluble defence factors and cells that are involved in humoral immunity are thought to be induced to prevent mastitis during this extended period of milk stasis. For example, the iron chelator lactoferrin is induced in mammary involution (Fig. 6c), which plays a role in mammary defence by protecting against coliform infection [36]. Indeed, low levels of lactoferrin is a predisposing factor for mastitis in humans [38]. Immunoglobulins not only have an important role in supplementing milk but are also required to facilitate opsonization by macrophages and neutrophils (IgM and IgG$_2$) [67] and the direct sequestration of bacteria (IgA).

Cd14 exhibited a dramatic pattern of expression throughout the complete time course (Fig. 6c). Cd14 is a core component of innate immunity, which binds LPS and augments LPS responses [42]. It is found bound to intramammary monocytes or neutrophils [41] in cattle subjected to endotoxin, and its soluble form is a known constituent of milk, in which it helps Cd14-negative cells respond to infection [68] and contributes to inhibition of gastrointestinal infections in human infants [69]. It may also play a role in recognition and phagocytosis of apoptotic cells by macrophages expressing cell surface Cd14 [70]. Thus, the role of elevated Cd14 may be both to prevent infection and potentiate apoptotic cell clearance. Binding of soluble Cd14 to LPS is augmented by the acute phase protein Lbp, which also exhibited an I$_P$ expression profile (Fig. 6c).

Conclusion

We highlighted two general trends in gene expression during mouse mammary involution: an increase in immune-related genes and a shift in the identity of apoptosis components. The increase in immune genes correlated with previously described roles for immune cells in involuting mammary gland, namely antimicrobial defence, cytokine signalling and phagocytic clearance. The proposed change in the cell death machinery based on extrinsic and intrinsic apoptosis gene expression has not previously been observed. This putative mechanism clearly needs further investigation. One common link between apoptosis and immune responses in mammary gland is the previously defined role for LIF and Stat3. We are currently investigating the possibility that Stat3 mediates the anti-inflammatory effects vital for the protection of the mammary gland in addition to its recognized role as a proapoptotic factor in mammary gland.

Additional files

The following Additional files are available online:

Additional file 1
Table showing all significant ontological associations (corrected $P < 0.05$) with combined clusters and primary clusters from the 12-point timecourse from Fig. 1.
See http://breast-cancer-research.com/content/supplementary/bcr754-S1.doc

Additional file 2
Table showing all significant ontological associations (corrected $P < 0.05$) with combined clusters and primary clusters from the 7-point timecourse from Fig. 4.
See http://breast-cancer-research.com/content/supplementary/bcr754-S2.doc

Additional file 3
Table showing the relative expression of genes previously identified as differentially regulated during the pregnancy cycle are compared to the relative expression of the same genes in our microarray study.
See http://breast-cancer-research.com/content/supplementary/bcr754-S3.xls

Additional file 4
A graphical representation of the relationship between gene expression pattern and gene function in the 12-point developmental time course.
See http://breast-cancer-research.com/content/supplementary/bcr754-S4.tiff

Additional file 5
A graphical representation of the relationship between gene expression pattern and gene function in the seven-point lactation/involution time course.
See http://breast-cancer-research.com/content/supplementary/bcr754-S5.tiff

References

1. Visvader JE, Lindeman GJ: **Transcriptional regulators in mammary gland development and cancer.** *Int J Biochem Cell Biol* 2003, **35**:1034-1051.
2. Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, Dano K, Werb Z: **Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways.** *Development* 1996, **122**:181-193.
3. Alexander CM, Selvarajan S, Mudgett J, Werb Z: **Stromelysin-1 regulates adipogenesis during mammary gland involution.** *J Cell Biol* 2001, **152**:693-703.
4. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, Hennighausen L, Furth PA: **Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution.** *Proc Natl Acad Sci USA* 1997, **94**:3425-3430.
5. Strange R, Li F, Saurer S, Burkhardt A, Friis RR: **Apoptotic cell death and tissue remodelling during mouse mammary gland involution.** *Development* 1992, **115**:49-58.
6. Streuli CH, Gilmore AP: **Adhesion-mediated signaling in the regulation of mammary epithelial cell survival.** *J Mammary Gland Biol Neoplasia* 1999, **4**:183-191.
7. Paape MJ, Shafer-Weaver K, Capuco AV, Van Oostveldt K, Burvenich C: **Immune surveillance of mammary tissue by phagocytic cells.** *Adv Exp Med Biol* 2000, **480**:259-277.
8. Clarkson RW, Watson CJ: **NF-kappaB and apoptosis in mammary epithelial cells.** *J Mammary Gland Biol Neoplasia* 1999, **4**:165-175.
9. Wilde CJ, Knight CH, Flint DJ: **Control of milk secretion and apoptosis during mammary involution.** *J Mammary Gland Biol Neoplasia* 1999, **4**:129-136.
10. Diaz E, Yang YH, Ferreira T, Loh KC, Okazaki Y, Hayashizaki Y, Tessier-Lavigne M, Speed TP, Ngai J: **Analysis of gene expression in the developing mouse retina.** *Proc Natl Acad Sci USA* 2003, **100**:5491-5496.
11. Arai M, Yokosuka O, Chiba T, Imazeki F, Kato M, Hashida J, Ueda Y, Sugano S, Hashimoto K, Saisho H, Takiguchi M, Seki N: **Gene expression profiling reveals the mechanism and pathophysiology of mouse liver regeneration.** *J Biol Chem* 2003, **278**:29813-29818.
12. Chiang MK, Melton DA: **Single-cell transcript analysis of pancreas development.** *Dev Cell* 2003, **4**:383-393.
13. Tan Y, Li X, Piao Y, Sun X, Wang Y: **Global gene profiling analysis of mouse uterus during the oestrus cycle.** *Reproduction* 2003, **126**:171-182.
14. Master SR, Hartman JL, D'Cruz CM, Moody SE, Keiper EA, Ha SI, Cox JD, Belka GK, Chodosh LA: **Functional microarray analysis of mammary organogenesis reveals a developmental role in adaptive thermogenesis.** *Mol Endocrinol* 2002, **16**:1185-1203.
15. Lemkin PF, Thornwall GC, Walton KD, Hennighausen L: **The microarray explorer tool for data mining of cDNA microarrays: application for the mammary gland.** *Nucleic Acids Res* 2000, **28**:4452-4459.
16. Phang TL, Neville MC, Rudolph M, Hunter L: **Trajectory clustering: a non-parametric method for grouping gene expression time courses, with applications to mammary development.** *Pac Symp Biocomput* 2003, **5**:351-362.
17. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, Takeda K, Akira S, Clarke AR, Watson CJ: **Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3.** *Genes Dev* 1999, **13**:2604-2616.
18. Alonzi T, Maritano D, Gorgoni B, Rizzuto G, Libert C, Poli V: **Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation (correction of activation) in the liver.** *Mol Cell Biol* 2001, **21**:1621-1632.

19. Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA: **Involution of the mouse mammary gland is associated with an immune cascade and an acute phase response, involving LBP, CD14 and STAT3.** *Breast Cancer Res* 2004, **6:**R75-R91.

20. Li C, Wong WH: **Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection.** *Proc Natl Acad Sci USA* 2001, **98:**31-36.

21. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: **Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.** *Nat Genet* 2000, **25:**25-29.

22. Khatri P, Draghici S, Ostermeier GC, Krawetz SA: **Profiling gene expression using onto-express.** *Genomics* 2002, **79:**266-270.

23. Blake JA, Richardson JE, Bult CJ, Kadin JA, Eppig JT: **MGD: the Mouse Genome Database.** *Nucleic Acids Res* 2003, **31:**193-195.

24. Kritikou EA, Sharkey A, Abell K, Came PJ, Anderson E, Clarkson RW, Watson CJ: **A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland.** *Development* 2003, **130:**3459-3468.

25. Poli V: **The role of C/EBP isoforms in the control of inflammatory and native immunity functions.** *J Biol Chem* 1998, **273:** 29279-29282.

26. Clarkson RW, Heeley JL, Chapman R, Aillet F, Hay RT, Wyllie A, Watson CJ: **NF-kappaB inhibits apoptosis in murine mammary epithelia.** *J Biol Chem* 2000, **275:**12737-12742.

27. Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM: **C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland.** *Genes Dev* 1998, **12:**1917-1928.

28. Monks J, Geske FJ, Lehman L, Fadok VA: **Do inflammatory cells participate in mammary gland involution?** *J Mammary Gland Biol Neoplasia* 2002, **7:**163-176.

29. Ryon J, Bendickson L, Nilsen-Hamilton M: **High expression in involuting reproductive tissues of uterocalin/24p3, a lipocalin and acute phase protein.** *Biochem J* 2002, **367:**271-277.

30. Wahl AF, Wallace PM: **Oncostatin M in the anti-inflammatory response.** *Ann Rheum Dis* 2001, **Suppl 3:**iii75-iii80.

31. Orabona C, Dumoutier L, Renauld JC: **Interleukin-9 induces 24P3 lipocalin gene expression in murine T cell lymphomas.** *Eur Cytokine Netw* 2001, **12:**154-161.

32. Liu Q, Ryon J, Nilsen-Hamilton M: **Uterocalin: a mouse acute phase protein expressed in the uterus around birth.** *Mol Reprod Dev* 1997, **46:**507-514.

33. Miyake H, Nelson C, Rennie PS, Gleave ME: **Acquisition of chemoresistant phenotype by overexpression of the anti-apoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models.** *Cancer Res* 2000, **60:** 2547-2554.

34. Viard I, Wehrli P, Jornot L, Bullani R, Vechietti JL, Schifferli JA, Tschopp J, French LE: **Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress.** *J Invest Dermatol* 1999, **112:**290-296.

35. Smith CE, Johnson DA: **Human bronchial leucocyte proteinase inhibitor. Rapid isolation and kinetic analysis with human leucocyte proteinases.** *Biochem J* 1985, **225:**463-472.

36. Sordillo LM, Streicher KL: **Mammary gland immunity and mastitis susceptibility.** *J Mammary Gland Biol Neoplasia* 2002, **7:** 135-146.

37. Goodman RE, Schanbacher FL: **Bovine lactoferrin mRNA: sequence, analysis, and expression in the mammary gland.** *Biochem Biophys Res Commun* 1991, **180:**75-84.

38. Breton-Gorius J, Mason DY, Buriot D, Vilde JL, Griscelli C: **Lactoferrin deficiency as a consequence of a lack of specific granules in neutrophils from a patient with recurrent infections. Detection by immunoperoxidase staining for lactoferrin and cytochemical electron microscopy.** *Am J Pathol* 1980, **99:**413-428.

39. Rainard P, Poutrel B: **Deposition of complement components on Streptococcus agalactiae in bovine milk in the absence of inflammation.** *Infect Immun* 1995, **63:**3422-3427.

40. Riollet C, Rainard P, Poutrel B: **Differential induction of complement fragment C5a and inflammatory cytokines during intra-mammary infections with *Escherichia coli* and *Staphylococcus aureus*.** *Clin Diagn Lab Immunol* 2000, **7:**161-167.

41. Paape MJ, Lilius EM, Wiitanen PA, Kontio MP, Miller RH: **Intramammary defense against infections induced by *Escherichia coli* in cows.** *Am J Vet Res* 1996, **57:**477-482.

42. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC: **CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein.** *Science* 1990, **249:**1431-1433.

43. Miyake K: **Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2.** *Int Immunopharmacol* 2003, **3:**119-128.

44. Song J, Sapi E, Brown W, Nilsen J, Tartaro K, Kacinski BM, Craft J, Naftolin F, Mor G: **Roles of Fas and Fas ligand during mammary gland remodeling.** *J Clin Invest* 2000, **106:**1209-1220.

45. Sohn BH, Moon HB, Kim TY, Kang HS, Bae YS, Lee KK, Kim SJ: **Interleukin-10 up-regulates tumour-necrosis-factor-alpha-related apoptosis-inducing ligand (TRAIL) gene expression in mammary epithelial cells at the involution stage.** *Biochem J* 2001, **360:**31-38.

46. Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, Karin M: **IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development.** *Cell* 2001, **107:**763-775.

47. Varela LM, Stangle-Castor NC, Shoemaker SF, Shea-Eaton WK, Ip MM: **TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells.** *J Cell Physiol* 2001, **188:**120-131.

48. Marshman E, Green KA, Flint DJ, White A, Streuli CH, Westwood M: **Insulin-like growth factor binding protein 5 and apoptosis in mammary epithelial cells.** *J Cell Sci* 2003, **116:**675-682.

49. Toillon RA, Descamps S, Adriaenssens E, Ricort JM, Bernard D, Boilly B, Le Bourhis X: **Normal breast epithelial cells induce apoptosis of breast cancer cells via Fas signaling.** *Exp Cell Res* 2002, **275:**31-43.

50. Varela LM, Ip MM: **Tumor necrosis factor-alpha: a multifunctional regulator of mammary gland development.** *Endocrinology* 1996, **137:**4915-4924.

51. Walton KD, Wagner KU, Rucker EB III, Shillingford JM, Miyoshi K, Hennighausen L: **Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation.** *Mech Dev* 2001, **109:**281-293.

52. Metcalfe AD, Gilmore A, Klinowska T, Oliver J, Valentijn AJ, Brown R, Ross A, MacGregor G, Hickman JA, Streuli CH: **Developmental regulation of Bcl-2 family protein expression in the involuting mammary gland.** *J Cell Sci* 1999, **112:**1771-1783.

53. Ory K, Lebeau J, Levalois C, Bishay K, Fouchet P, Allemand I, Therwath A, Chevillard S: **Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines.** *Breast Cancer Res Treat* 2001, **68:**187-198.

54. Russell HR, Lee Y, Miller HL, Zhao J, McKinnon PJ: **Murine ovarian development is not affected by inactivation of the bcl-2 family member diva.** *Mol Cell Biol* 2002, **22:**6866-6870.

55. Cory S, Adams JM: **The Bcl2 family: regulators of the cellular life-or-death switch.** *Nat Rev Cancer* 2002, **2:**647-656.

56. Campana WM, Darin SJ, O'Brien JS: **Phosphatidylinositol 3-kinase and Akt protein kinase mediate IGF-I- and prosaptide-induced survival in Schwann cells.** *J Neurosci Res* 1999, **57:** 332-341.

57. Wang P, Valentijn AJ, Gilmore AP, Streuli CH: **Early events in the anoikis program occur in the absence of caspase activation.** *J Biol Chem* 2003, **278:**19917-19925.

58. Gouon-Evans V, Rothenberg ME, Pollard PW: **Postnatal mammary gland development requires macrophages and eosinophils.** *Development* 2000, **7:**2269-2282.

59. Pollard JW, Hennighausen L: **Colony stimulating factor 1 is required for mammary gland development during pregnancy.** *Proc Natl Acad Sci USA* 1994, **91:**9312-9316.

60. Dallegri F, Ottonello L: **Tissue injury in neutrophilic inflammation.** *Inflamm Res* 1997, **46:**382-391.

61. Pillinger MH, Abramson SB: **The neutrophil in rheumatoid arthritis.** *Rheum Dis Clin North Am* 1995, **21:**691-714.

62. Devireddy LR, Teodoro JG, Richard FA, Green MR: **Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation.** *Science* 2001, **293:**829-834.

63. Nilsen-Hamilton M, Liu Q, Ryon J, Bendickson L, Lepont P, Chang Q: **Tissue involution and the acute phase response.** *Ann N Y Acad Sci* 2003, **995:**94-108.
64. Odaka C, Mizuochi T, Yang J, Ding A: **Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response.** *J Immunol* 2003, **171:**1507-1514.
65. Chapman RS, Lourenco P, Tonner E, Flint D, Selbert S, Takeda K, Akira S, Clarke AR, Watson CJ: **The role of Stat3 in apoptosis and mammary gland involution. Conditional deletion of Stat3.** *Adv Exp Med Biol* 2000, **480:**129-138.
66. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, Ridley AJ, Fadok VA, Henson PM: **Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells.** *J Cell Biol* 2001, **155:**649-659.
67. Waller KP: **Mammary gland immunology around parturition. Influence of stress, nutrition and genetics.** *Adv Exp Med Biol* 2000, **480:**231-245.
68. Lee JW, Paape MJ, Elsasser TH, Zhao X: **Elevated milk soluble CD14 in bovine mammary glands challenged with *Escherichia coli* lipopolysaccharide.** *J Dairy Sci* 2003, **86:**2382-2389.
69. Labeta MO, Vidal K, Nores JE, Arias M, Vita N, Morgan BP, Guillemot JC, Loyaux D, Ferrara P, Schmid D, Affolter M, Borysiewicz LK, Donnet-Hughes A, Schiffrin EJ: **Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14.** *J Exp Med* 2000, **191:**1807-1812.
70. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD: **Human CD14 mediates recognition and phagocytosis of apoptotic cells.** *Nature* 1998, **392:**505-509.

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