A population of mammary epithelial cells do not require hormones or growth factors to survive

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Abstract

Hormonal stimulation of mammary explants mimics many of the biochemical changes observed during lactogenesis. Previous studies using eutherian species conclude that mammary explants require addition of exogenous macromolecules to remain hormone responsive in culture. The present study examines the survival of mammary explants from the wallaby and mouse using milk protein gene expression as a functional marker of lactation and cell viability. Mammary explants from pregnant tammars and mice showed that milk protein gene expression was significantly elevated after 3 days of culture with lactogenic hormones. The subsequent removal of exogenous hormones from the media for 10 days resulted in the down-regulation of milk protein genes. Surprisingly, mammary explants remained hormone responsive and expression of milk protein genes was re-induced after a second challenge with lactogenic hormones. Furthermore, the alveolar architecture was maintained. Global functional microarray analysis showed that classic involution markers were not differentially expressed, although two stress-induced survival genes were significantly up-regulated. We report that a population of mammary epithelial cells have an intrinsic capacity to remain viable and hormone responsive for extended periods in chemically defined media without any exogenous macromolecules. We propose that the mammary explant culture model uncouples the first phase of involution, as milk accumulation that normally provides involution stimuli is absent in this culture model allowing a population of cells to survive.

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Introduction

The nature of the lactation cycle is common to all mammals although marsupials and eutherians have evolved appreciably divergent reproductive strategies. Eutherians have a long gestation relative to their lactation period, whereas marsupial reproduction is characterised by a short gestation followed by a long lactation (Tyndale-Biscoe & Janssens 1988). Mammary gland development during the lactation cycle has been extensively studied in eutherian species. Mammary epithelial cell proliferation occurs at pregnancy to form additional ductal branching and lobulo-alveolar growth from the ductal skeleton (Humphreys & Hennighausen 1999). The subsequent overt differentiation of mammary epithelial cells through lactogenesis predisposes the gland for copious milk secretion *post partum* (Lund *et al*. 1996). Involution is initiated by weaning and reduces the tissue to a state that resembles the mature virgin gland (Wilde *et al*. 1999, Prince *et al*. 2002). In the rodent, involution comprises two phases: early limited apoptosis of secretory cells followed by widespread apoptosis and tissue remodelling (Stein *et al*. 2004). Teat sealing in mice has demonstrated that phase 1 of involution is regulated by local factors and not by systemic hormones (Li *et al*. 1997, Marti *et al*. 1997), whereas phase 2 of involution is associated with a decline in circulating galactopoietic factors (Lund *et al*. 1996).

Several mechanisms have been postulated as the trigger of phase 1 mammary gland involution (Li *et al*. 1997, Green & Streuli 2004). Milk-borne factors involved in the control of mammary gland function have been identified in a number of species (Hendry *et al*. 1998, Marti *et al*. 1999). A protein known as the feedback inhibitor of lactation is synthesised and secreted in milk, and acts specifically through interaction with the apical surface of the mammary epithelial cell to reduce secretory efficiency by inhibiting the movement of proteins (Wilde *et al*. 1995, Hendry *et al*. 1998). It has been suggested that this autocrine mechanism is sensitive to the frequency and completeness of milk removal (Hendry *et al*. 1998, Simpson *et al*. 1998). There are several reports showing that the whey protein α-lactalbumin can induce apoptosis of epithelial cells (Hakansson *et al*. 1999, Permyakov *et al*. 2004). Multimeric human α-lactalbumin isolated from the casein fraction of milk interacts directly with mitochondria to initiate release of cytochrome *c* and subsequent activation of the caspase cascade (Hakansson *et al*. 1999, Kohler *et al*. 1999, Permyakov *et al*. 2004). Human α-lactalbumin made lethal to

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tumour cells is able to interact with histones and chromatin in cell nuclei, locking the cells into the death pathway through irreversible disruption of chromatin organisation (Permyakov *et al.* 2004).

Another possible trigger of involution is the change in cell shape as the gland becomes engorged with milk and stretching of the mammary epithelial cells occurs. Two systems may be affected by the altered cell shape. First, tight junctions become leaky and pro-apoptotic factors may diffuse from the apical to the basolateral side of the mammary epithelial cells to induce involution and apoptosis (Stelwagen *et al.* 1994, 1997). The second possibility is the altered interaction of the cell with the extracellular matrix, through focal adhesion complexes (Singh *et al.* 2005). However, these suggestions are confounded since the physical distension of goat mammary glands following injection of sucrose up to the galactophore of the teat does not result in involution (Henderson & Peaker 1987).

The mammary explant culture model is an *in vitro* system that can be stimulated hormonally to mimic the biochemical changes during lactogenesis (Recklies *et al.* 1985), although milk constituents secreted by mouse and tammar mammary explants do not accumulate to elevated concentrations such as those at weaning (Nagamatsu & Oka 1980, Nicholas & Tyndale-Biscoe 1985). The induction of milk protein synthesis in mammary gland explants requires that the tissue is maintained in a viable state and stimulated by the appropriate complex of hormones (Topper & Freeman 1980). Previous studies in eutherian species have concluded that mammary explants require addition of either serum, a growth factor or insulin to retain viability of mammary tissue in culture (Topper & Freeman 1980, Kulski *et al.* 1983, Topper *et al.* 1984). It has been suggested that the withdrawal of these hormones is responsible for epithelial cell death in mammary gland explants (Atwood *et al.* 1995, Accorsi *et al.* 2002). In a study reported by Nicholas *et al.* (1991), however, it was demonstrated that mammary explants from pregnant tammar wallabies could be induced to express milk protein genes but then remained viable and hormone responsive for 4 days in the absence of exogenous macromolecules.

This study re-examines the survival mechanisms of mammary explants from both the wallaby and the mouse using milk protein gene expression as functional markers of lactation and cell viability. We found that the tammar wallaby has a similar pattern of involution to the mouse. Furthermore, mammary explants from both pregnant tammars and mice showed that milk protein gene expression was significantly elevated after 3 days of culture with lactogenic hormones. The subsequent removal of exogenous hormones from the media for 10 days resulted in the down-regulation of milk protein genes. We show for the first time that a population of surviving cells remain hormone responsive, are capable of milk protein gene expression and maintain alveolar architecture in the absence of exogenous hormones and growth factors for at least 10 days.

Materials and Methods

Animals

Tammar wallabies (*Macropus eugenii*) were maintained in open enclosures at The University of Melbourne Macropod Research Facility (Wantirna, VIC, Australia), with stock originally from Kangaroo Island, South Australia. Mammary glands from late pregnant (days 24–25), lactating (days 70 and 220) and involuting (days 1, 2, 5 and 10) tammar wallabies were excised under sterile conditions after animals were euthanised. The day of gestation was scheduled as described previously (Shaw *et al.* 1996). The day of lactation was determined using head length measurements of the pouch young to estimate their age (Poole *et al.* 1991). Swiss mice were obtained from The University of Melbourne animal house, Department of Zoology, Melbourne, Australia. Mammary glands from day 13 pregnant, lactating (days 4 and 10) and involuting (6 h, days 1 and 6) mice were excised under sterile conditions after animals were euthanised. Mammary glands not used in tissue culture were collected and frozen at −80 °C for northern analysis. For histological analysis, mammary tissue was fixed in 10% formaldehyde solution. All experiments were approved by the Institutional Animal Care and Ethics Committee.

Tissue culture

Mammary gland explants were prepared and cultured in Medium 199 with Earle's salts (Gibco BRL Life Technologies) as described previously (Nicholas & Tyndale-Biscoe 1985). Briefly, tissues were cut to obtain 10–20 mg pieces and explants were incubated at 37 °C (5% CO_2) in Medium 199 without the addition of growth factors or hormones (NH), or with the combination of bovine insulin (I; 1 μg/ml, Sigma #I-5500), hydrocortisone (F; 50 ng/ml, Sigma #H-4001) and ovine prolactin (P; 1 μg/ml, National Hormone and Pituitary Program USA-oPRL-21). All experiments were performed in triplicate. Mammary explants were fixed in 4% formaldehyde solution for histological analysis.

Light microscopy

The mammary tissue and explants were fixed in 10% formalin for 24 h. Samples were processed (Citadel; Shandon Scientific Ltd, Cheshire, England) and embedded in paraffin using routine procedures. Paraffin-embedded sections of 8 μm thickness were cut, mounted on 3-aminopropyl-triethoxy-silane-coated slides and submerged in histolene to remove the paraffin. After rehydration, transverse sections were stained with haematoxylin and eosin (Kiernan 2000). Adjacent paraffin sections were immunolabelled for *in situ* apoptosis using the TUNEL assay. Finally, sections were coverslipped and examined using a Coolscope digital (Nikon) microscope for cyto-architectural evaluation.

Immunohistochemistry: in situ *end-labelling of fragmented DNA*

For the detection of DNA fragmentation as a biochemical marker of apoptosis, transverse sections were subjected to TUNEL using the ApopTag Peroxidase assay kit (Chemicon #S7100). Negative control sections processed without *T*dT enzyme were used to ensure that the staining was not due to non-specific incorporation of nucleotides. Sections were counterstained with 0·5% methyl green, coverslipped and examined using a Coolscope digital (Nikon) for light microscopy. ApopTag-positive cells were brown and considered apoptotic cells. ApopTag-negative cells were green and considered live cells.

Morphometric analysis

The total number of non-apoptotic and apoptotic epithelial cells was counted in ten random fields using a Coolscope digital (Nikon) light microscope with a 40× objective. The apoptotic index was calculated as the average number of immunoreactive nuclei per total number of epithelial cells, and expressed as a percentage. Significant differences in the mean number of apoptotic cells between control and experimental groups were determined by one-way ANOVA (ANOVA) followed by Tukey's multiple comparison tests. In all analyses, P values $<0{\cdot}05$ were considered statistically significant.

Northern blot analyses

Total RNA was isolated from the tissue using a Qiagen RNA Extraction kit (Qiagen #75144) following the manufacturer's instructions. For analysis, 10 μg total RNA was electrophoresed in MOPS 1% agarose gels at 100 V/cm using 10% MOPS buffer, transferred to Zeta-probe GT membranes and pre-hybridised for 4 h at 42 °C in 30% formamide hybridisation buffer. Membranes were hybridised at 42 °C with β-lactoglobulin (BLG) and β-casein cDNA labelled with [$\propto$-^{32}P]dCTP (10 mCi/ml, Perkin–Elmer, Melbourne, Victoria, Australia) using a DECAprime II random priming DNA labelling kit (Ambion #1455, Scoresby, Victoria, Australia). Membranes were washed once at room temperature with 2% SSC, 1% SDS, twice at room temperature with 1% SSC, 0·1% SDS, and twice at 42 °C in 0·1% SDS, 0·1% SSC. The ^{32}P-labelled cDNA was detected by the use of a phosphor screen at room temperature for 24–48 h (Bio-Rad, Molecular Dynamics Typhoon Scanner).

Reverse transcription PCR

Reverse transcription of RNA was carried out at 37 °C for 1 h using the Superscript II reverse transcriptase (Promega) kit with 1 μg total RNA from the mammary tissue of a pregnant tammar and mammary explants from a pregnant tammar cultured for 4 days in the absence of hormones. For PCR, 0·5 μl of the reverse transcription was used as a template with 0·4 mM primer (Table 1), 2·5 U Taq DNA polymerase (Promega), 0·8 mM dNTP and 80 μM $MgCl_2$. Amplification was performed using 25 cycles of 95 °C for 40 s, 62 °C for 40 s and 72 °C for 30 s, and a final extension of 72 °C for 5 min. PCR products were electrophoresed in 1% agarose gels and viewed following SybrGreen staining. The levels of gene expression relative to GAPDH were quantitated using ImageJ software (http://rsb.info.nih.gov/ij/). Significant differences between gene expression in the mammary tissue from pregnant tammars and mammary explants cultured in no hormone for 4 days were determined by paired t-tests. In all analyses, P value $<0{\cdot}05$ were interpreted as statistically significant.

Analysis of gene expression by micro-array

Total RNA was extracted using an RNeasy Lipid Tissue Mini-kit (Qiagen) following the manufacturer's instructions. RNA from each treatment group was labelled using amino-allyl reverse transcription followed by Cy_3 and Cy_5 coupling. Samples of total RNA (50 μg) were annealed at 70 °C for 10 min with 0·2 μg/ml T7 Anchored PolyT Primer (Geneworks), and reverse transcribed using amino-allyl dNTP mix (Sigma) and RNAse H enzyme for reverse transcription (Promega) at 42 °C for 2·5 h. The reaction mix was hydrolyzed by incubation at 65 °C for 15 min in the presence of 33 mM NaOH, 33 mM EDTA and 40 mM acetic acid. The cDNA was then adsorbed to a Qiagen QIAquick PCR Purification column. Coupling of either Cy_3 or Cy_5 dye was performed using incubation with adsorbed cDNA in 0·1 M sodium bicarbonate for 1 h at room temperature in darkness, followed by elution in 80 μl water. Labelled cDNA was further purified using a second Qiagen QIAquick PCR Purification column. Cy_3- and Cy_5-labelled probes in a final concentration of 400 μg/ml yeast tRNA, 1 mg/ml human Cot 1 DNA, 200 μg/ml polydT50, 1·2×Denharts, 1 mg/ml herring sperm DNA, 3·2×SSC, 50% formamide and 0·1% SDS were heated at 100 °C for 3 min. Probes were hybridised

Table 1 PCR primers

	5′-primer	**3′-primer**	**Size** (bp)
Gene			
Heme oxygenase-1	ctgtggctgtggggttctat	tgaggaccacccctaaaatg	240
Testicular enhanced gene transcript	gagactgggccttctgactg	gctcatggctgacatcaaga	208
GAPDH	gactcatgactacagtccatgccat	ggacatgtagaccatgaggtccac	500

overnight at 42 °C in a humidified chamber and transferred to a custom tammar wallaby EST microarray, printed with 10 000 ESTs from tammar mammary gland cDNA libraries generated from the tissue collected across the lactation cycle (Lefèvre, manuscript in preparation). Microarrays were washed in 0·5×SSC, 0·01% SDS for 1 min, 0·5×SSC for 3 min then 0·006×SSC for 3 min at room temperature in the darkness. Microarray slides were scanned with an Agilent Scanner and images analysed using Versarray Software (Bio-Rad). Gene expression data were analyzed using the limma package of Bioconductor ($P<0·01$; Smyth 2004). Data were analyzed for function using the Ingenuity Pathways Analysis software (Ingenuity Systems, http://www.ingenuity.com).

Results

Characterisation of the temporal expression of β-casein and β-lactoglobulin genes during lactation and involution in the tammar wallaby and mouse respectively

The RNA isolated from mammary glands of pregnant (day 13), lactating (day 10) and involuting (6 h, 24 h and 6 days) mice was analysed for milk protein gene expression by northern analysis (Fig. 1b). In pregnant animals, the level of β-casein gene expression was undetectable and increased dramatically after parturition and at the onset of lactation. Forced weaning by the removal of pups at day 10 of lactation resulted in a progressive decline of β-casein mRNA from 6 h to day 1 of involution, and β-casein transcripts were almost undetectable at day 6. The wallaby and mouse have a similar milk protein gene expression pattern during the lactation cycle. The RNA isolated from mammary glands of late pregnant (day 24), lactating (days 70 and 200) and involuting (1, 2, 5 and 10 days) tammar wallabies was analysed for milk protein gene expression by northern analysis (Fig. 1a). The level of BLG gene expression was barely detectable in pregnant animals, increased dramatically in early lactation, and was maximal during late lactation. The two BLG gene transcripts (880 and 1000 bp) are the result of differential utilisation of two polyadenylation signals (Collet *et al.* 1991). Forced weaning by the removal of pouch young at day 160 of lactation resulted in a decline of BLG mRNA from day 1 to day 2 of involution, and BLG transcripts were almost undetectable at days 5 and 10. Other milk protein genes in the same mammary tissue from the mouse and the wallaby showed similar expression patterns (data not shown).

Endocrine regulation of milk protein gene expression in mammary explants from tammar wallabies and mice

Northern analysis of RNA isolated from the mammary tissue of pregnant tammars (Fig. 2a) and pregnant mice (Fig. 2b) showed low levels of BLG and β-casein mRNA expression respectively, which were significantly elevated in mammary explants after 3 days of culture in medium 199 containing

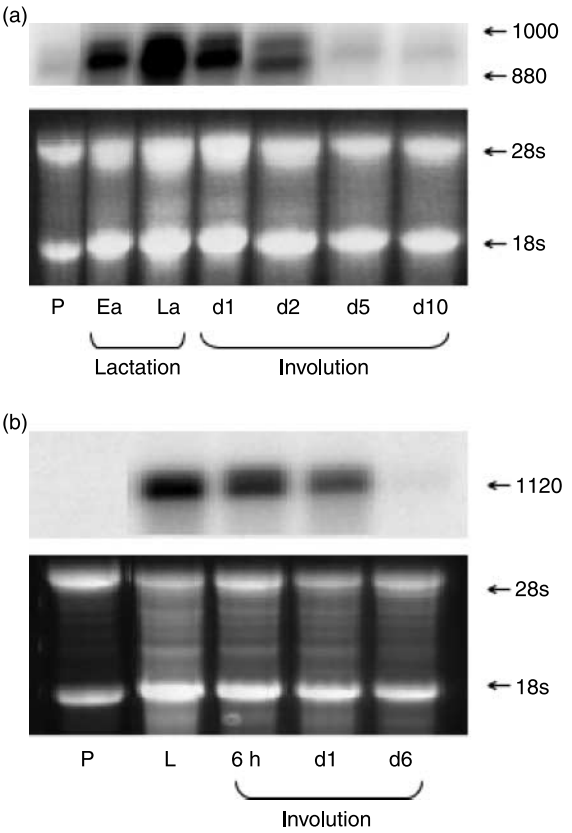

Figure 1 Analysis of gene expression during the lactation cycle (a) β-lactoglobulin gene expression in the tammar and (b) β-casein gene expression in the mouse. Total RNA (10 μg, lower panels) was assayed by northern analysis (upper panels) using [α-^{32}P]dCTP-labelled probes for β-lactoglobulin (a; tammar) or β-casein (b; mouse). (a) Mammary gland tissue from pregnant tammars (P), lactation (early (Ea) and late (La)) and forced involution by removal of pouch young at day 160 of lactation, at day 1 (d1), day 2 (d2), day 5 (d5) and day 10 (d10). (b) Mammary gland tissue from pregnant mice (P), lactation (L) and forced involution by removal of young at day 10 of lactation at 6 h (6 h), day 1 (d1) and day 6 (d6). Arrows indicate transcript size in nucleotides and RNA ribosomal bands.

insulin, cortisol and prolactin (IFP_3). The BLG and β-casein transcripts were not detected in mammary explants from the tammar and mouse respectively after 10 days of culture in the absence of exogenous growth factors or hormones (NH_{10}). Subsequent addition of IFP to the media for 3 days after culture in NH for 10 days resulted in the detection of BLG and β-casein transcripts ($NH_{10} \rightarrow IFP_3$). To investigate whether an initial incubation in IFP affects explant survival, mammary explants were first cultured with IFP for 3 days to induce milk protein expression and mimic lactation. Removal of exogenous IFP from the media resulted in the down-regulation of BLG and β-casein gene expression in tammar and mouse explants respectively ($IFP_3 \rightarrow NH_{10}$). The re-introduction of IFP to the media for 3 days after culture for 10 days in the absence of hormones resulted in the re-detection of BLG and β-casein ($IFP_3 \rightarrow NH_{10} \rightarrow IFP_3$).

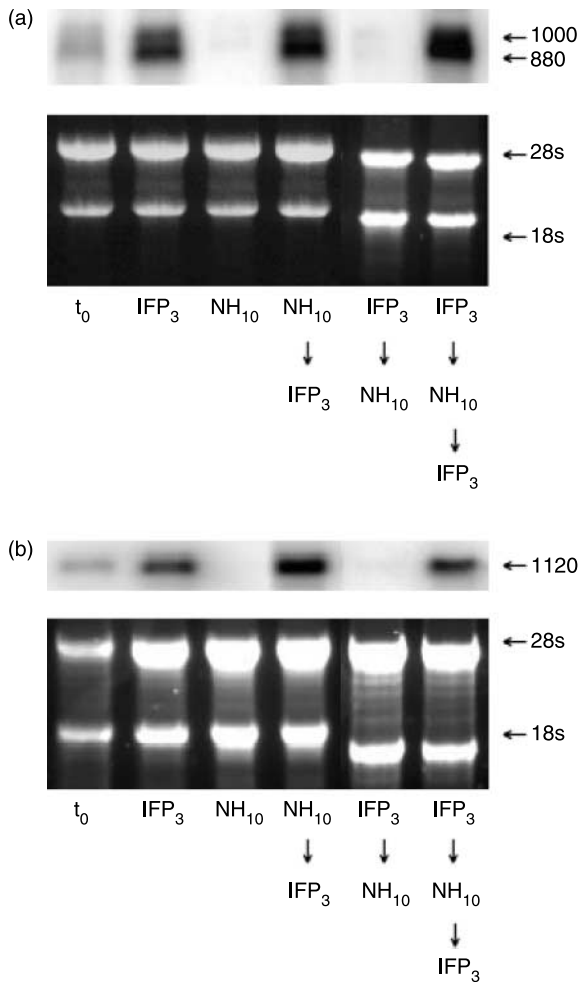

Figure 2 Analysis of gene expression in mammary explants. (a) β-lactoglobulin gene expression in tammar mammary explants from day 24 pregnant animals and (b) β-casein gene expression in mouse mammary explants from day 13 pregnant animals. Total RNA (10 μg, lower panels) was assayed by northern analysis (upper panels) using [α-^{32}P]dCTP-labelled probes for β-lactoglobulin (a; tammar) or β-casein (b; mouse). (a and b) t_0, mammary explants from day 24 pregnant tammars and day 13 pregnant mice; IFP_3, mammary explants from pregnant tammars and pregnant mice were cultured in M199 containing insulin (I; 1 μg/ml), cortisol (F; 50 ng/ml) and prolactin (P; 1 μg/ml) for 3 days; NH_{10}, mammary explants from pregnant tammars and pregnant mice were cultured in M199 containing no hormone or growth factors for 10 days; $NH_{10}\rightarrow IFP_3$, mammary explants from pregnant tammars and pregnant mice were cultured in M199 containing no hormone or growth factors for 10 days followed by introduction of insulin, cortisol and prolactin for 3 days; $IFP_3\rightarrow NH_{10}$, mammary explants from pregnant tammars and pregnant mice were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media; $IFP_3\rightarrow NH_{10}\rightarrow IFP_3$, mammary explants from pregnant tammars and pregnant mice were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media, after which insulin, cortisol and prolactin were reintroduced for 3 days. Arrows indicate transcript size in nucleotides and RNA ribosomal bands.

To determine whether the mammary tissue responds differently when milk protein genes are induced *in vivo* prior to culture, mammary explants from tammars at day 70 of lactation (Fig. 3a) and mice at day 4 of lactation (Fig. 3b) were cultured in NH for 10 days. Northern analysis showed the detection of elevated levels of BLG and β-casein transcripts from lactating mammary gland tissue for the tammar and mouse respectively, which was maintained when cultured as explants in the presence of IFP for 3 days (IFP_3). Removal of exogenous IFP from the media for 10 days resulted in down-regulation of BLG and β-casein expression (NH_{10}). However, subsequent addition of IFP to the media for 3 days resulted in a significant up-regulation in BLG and β-casein expression for the tammar and mouse respectively ($NH_{10}\rightarrow IFP_3$). All sets of experiments were performed in triplicate using different groups of animals ($n=3$), and other milk protein genes from the wallaby (α-lactalbumin, β-casein and early lactation protein) and mouse (α-lactalbumin and α-casein) showed similar expression patterns to BLG and β-casein respectively (data not shown).

Morphological and apoptosis evaluation of mammary tissue and explants from the tammar wallaby

Cellular architecture of tammar mammary tissue and explants was examined by haematoxylin and eosin stained sections. Histological examination of the mammary tissue from a pregnant tammar shows the organisation of immature alveoli in preparation for the onset of lactation at parturition and is mostly composed of stroma (Fig. 4a and f). Slides prepared from the mammary tissue of a pregnant tammar: after culture for 3 days in IFP media (IFP_3), after culture for 3 days in IFP media followed by the removal of hormones for a subsequent 10 days ($IFP_3\rightarrow NH_{10}$) and after culture for 3 days in IFP media followed by the removal of hormones for 10 days and the subsequent re-introduction of IFP for a further 3 days ($IFP_3\rightarrow NH_{10}\rightarrow IFP_3$) indicate that the cellular architecture of the tissue was well conserved throughout the culture period regardless of hormone conditions. The gland is still predominantly composed of stroma, however, the alveoli have expanded and epithelial cells are flattened, which is consistent with a lactating phenotype (Fig. 4b–d and g–i). In contrast, slides prepared from day 5 of an involuting tammar mammary gland show the complete disorganisation and collapse of the alveoli and supporting stroma (Fig. 4e and j).

Induction of DNA fragmentation as a biochemical feature of apoptosis was examined by the ApopTag assay for tammar mammary tissue and explants (Fig. 5A). All control slides show non-specific background stromal staining not associated with apoptosis. The mammary tissue from a pregnant tammar showed an absence of apoptotic cells (Fig. 5A a–c), while explanted mammary tissue showed the presence of many apoptotic cells (Fig. 5A d–l). However, there is also a non-apoptotic population of mammary epithelial cells that are maintained within the alveolar architecture in all explanted mammary tissue. In comparison, the involuting mammary

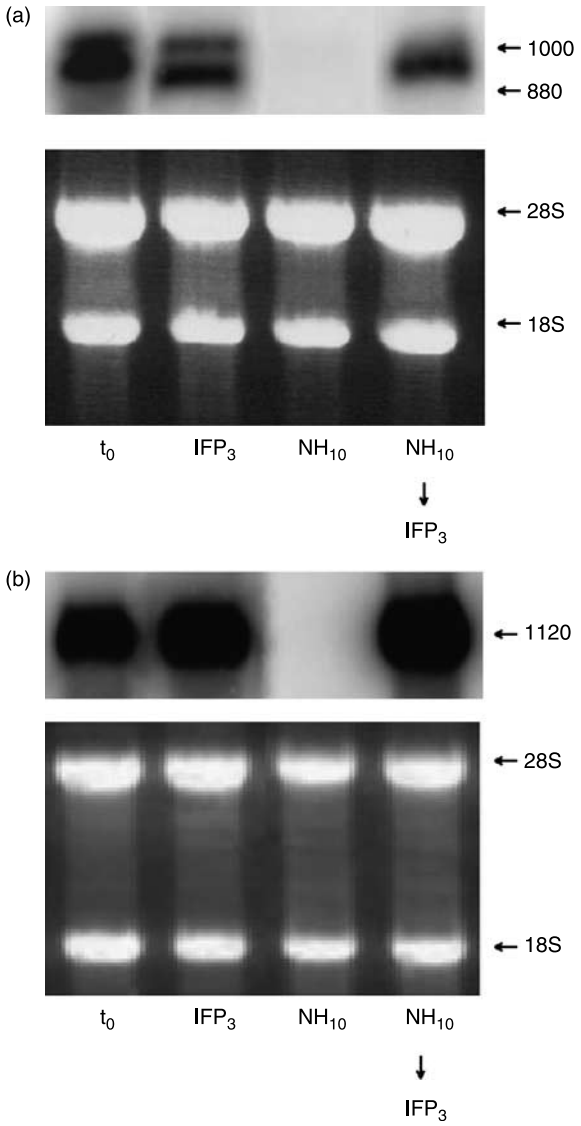

Figure 3 Analysis of gene expression in mammary explants. (a) β-lactoglobulin gene expression in tammar mammary explants from day 70 lactating animals and (b) β-casein gene expression in mouse mammary explants from day 4 lactating animals. Total RNA (10 μg, lower panels) was assayed by northern analysis (upper panels) using [α-^{32}P]dCTP-labelled probes for β-lactoglobulin (a; tammar) or β-casein (b; mouse). (a and b) t_0, mammary explants from day 70 lactating tammars and day 4 lactating mice; IFP_3, mammary explants from lactating tammars and lactating mice were cultured in M199 containing insulin (I; 1 μg/ml), cortisol (F; 50 ng/ml) and prolactin (P; 1 μg/ml) for 3 days; NH_{10}, mammary explants from lactating tammars and lactating mice were cultured in M199 containing no hormone or growth factors for 10 days; $NH_{10} \rightarrow IFP_3$, mammary explants from lactating tammars and lactating mice were cultured in M199 containing no hormone or growth factors for 10 days followed by introduction of insulin, cortisol and prolactin for 3 days. Arrows indicate transcript size in nucleotides and RNA ribosomal bands.

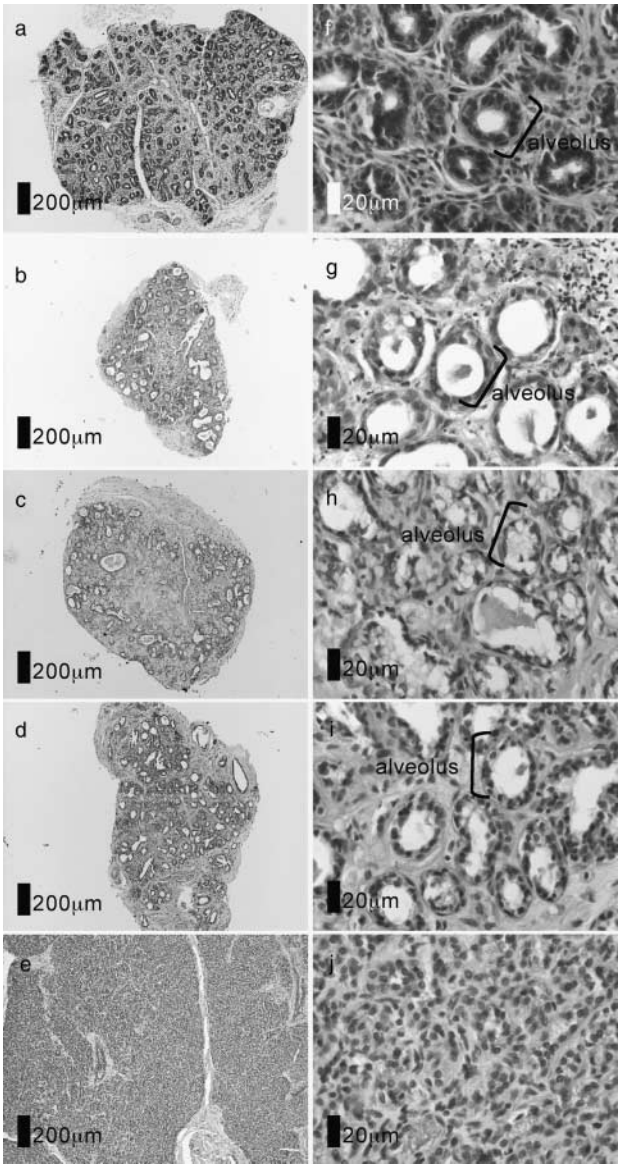

Figure 4 Morphology of tammar wallaby mammary tissue and explants. Haematoxylin and eosin stained sections at 5× (a–e) and 40× (f–j) magnification. (a and f) Mammary explants from day 24 pregnant tammars (t_0), (b and g) mammary explants from pregnant tammars were cultured in M199 containing insulin (I; 1 μg/ml), cortisol (F; 50 ng/ml) and prolactin (P; 1 μg/ml) for 3 days (IFP_3), (c and h) mammary explants from pregnant tammars were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media, (d and i) mammary explants from pregnant tammars were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media, after which insulin, cortisol and prolactin were reintroduced for 3 days and (e and j) involuting tammar mammary tissue at 5 days. Pregnant and treated tissue shows adequate maintenance of the epithelial and stromal compartments in comparison to involution. Examples of alveoli are indicated.

tissue shows a higher proportion of apoptotic cells within the gland and the collapse of alveoli (Fig. 5A m–o). To quantitatively illustrate the apoptotic index of tammar mammary explants, and the mammary tissue from pregnant and involuting tammars, the average number of immunoreactive nuclei per total number of epithelial cells was calculated and expressed as a percentage ($n=8$, Fig 5b). Mammary tissue from a pregnant tammar showed 1·5% apoptotic cells while a statistically significant increase in the percentage of apoptotic epithelial cells was observed after explant culture showing an apoptotic index of: 47·3% after IFP_3 treatment; 50·3% after $IFP_3 \rightarrow NH_{10}$ treatment and 54·8% after $IFP_3 \rightarrow NH_{10} \rightarrow IFP_3$ treatment. In contrast, the percentage of apoptotic epithelial cells in involuting mammary tissue from a tammar (71·9%) was significantly higher than explant culture tissue. A one-way ANOVA of the number of apoptotic cells in the different groups showed a significant effect ($F_{(5,\ 5)}=114{\cdot}983$, $P<0{\cdot}001$). However, there was no significant difference in the apoptotic index of mammary explants cultured in different treatments (Table 2).

Analysis of gene expression in tammar wallaby mammary explants and involuting mammary gland

In order to characterise gene expression profiles of mammary tissue from these experiments, microarray slides with 10 000 mammary ESTs representing genes expressed throughout the tammar lactation cycle (Lefèvre, manuscript in preparation) were used to compare transcript profiles. Genes were considered differentially expressed if there was a twofold or more increase or decrease of intensity between slides, with an intensity of at least one S.D. greater than the background. The tissue from day 24 pregnant animals and explants cultured for 4 days in no hormone were compared. There were 55 genes up-regulated (annotated=25, unannotated=30) and 122 genes down-regulated (annotated=43, unannotated=79) after culture without hormones for 4 days (68 differentially expressed annotated genes in total: see Supplementary Table 1 data in the online of version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol196/issue3/). These genes were compared with the differentially expressed genes from microarrays of mammary tissue collected at different stages of involution in the tammar wallaby. There were 43 (annotated=24, unannotated=19) genes that showed up-regulation and 61 genes (annotated=21, unannotated=40) that showed down-regulation common to both explants and involution (Fig. 6b). Datasets were integrated and analyzed for function to investigate mammary epithelial cell survival in mammary explants using the Ingenuity Pathways Analysis software (Ingenuity Systems, http://www.ingenuity.com, Fig. 6a). The functional analysis identified the biological functions that were most significant to the dataset. Genes from the datasets that were associated with biological functions in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fisher's exact test was used to calculate a P value determining the probability that each biological function assigned to that dataset was due to chance alone. We identified a total of 17 genes encoding proteins linked to apoptosis that showed altered expression in cultured mammary tissue. When differentially expressed genes found in explants cultured without hormones for 4 days were compared with differentially expressed genes found in involution, there were nine common genes in the functional dataset associated with apoptosis (Fig. 7a). Anti-apoptotic genes that were uniquely up-regulated in mammary explants included three novel anti-apoptotic genes that have not been characterised in the mammary gland, heme oxygenase-1 (HMOX1), testicular-enhanced gene transcript (TEGT) and Id protein 3 (ID3). Only one apoptotic gene, e-cadherin (CDH1), was uniquely down-regulated in mammary explants. Opposite differential gene expression was observed for the anti-apoptotic membrane metallo-endopeptidase (MME) and the apoptotic dipeptidyl peptidase 4 (DPP4). Microarray expression was validated by RT-PCR of two genes (HMOX1 and TEGT), and presented relative to expression of the GAPDH gene using ImageJ for PCR band quantification ($n=3$, Fig. 7b). Furthermore, analysis using days 1, 2, 5 and 10 of involution showed explants cultured in no hormone for 4 days have a gene profile most similar to involution at 5 days (Fig. 8). Values were calculated by determining the number of differentially expressed genes common in both comparisons as a percentage of the total number of differentially expressed genes found at days 1, 2, 5 and 10 of involution.

Discussion

Mammary explants from pregnant tammar wallabies and mice do not require exposure to exogenous macromolecules during culture to maintain a population of mammary epithelial cells in a hormone-responsive state. Previous reports have argued that rodent mammary explants require addition of either serum, insulin or a growth factor to retain the tissue viability in culture (Topper & Freeman 1980, Kulski *et al.* 1983, Topper *et al.* 1984). It was reported that the withdrawal of hormones and growth factors triggers a programme of epithelial cell death in mammary explants within 48 h, indicated by an increase in the intensity of non-random DNA degradation and apoptosis-associated genes (Atwood *et al.* 1995, Jaggi *et al.* 1996, Accorsi *et al.* 2002). Similarly, immunohistochemical analysis of the tammar wallaby mammary gland shows an apoptotic population of mammary epithelial cells in explants. However, we show for the first time, a population of surviving cells that remain hormone responsive are capable of milk protein gene expression and maintain alveolar architecture in the absence of exogenous hormones and growth factors for at least 10 days.

Mammary-specific stem cells do not undergo apoptosis during post-lactational remodelling and persist throughout a female's life (Wagner & Smith 2005). Furthermore, they are

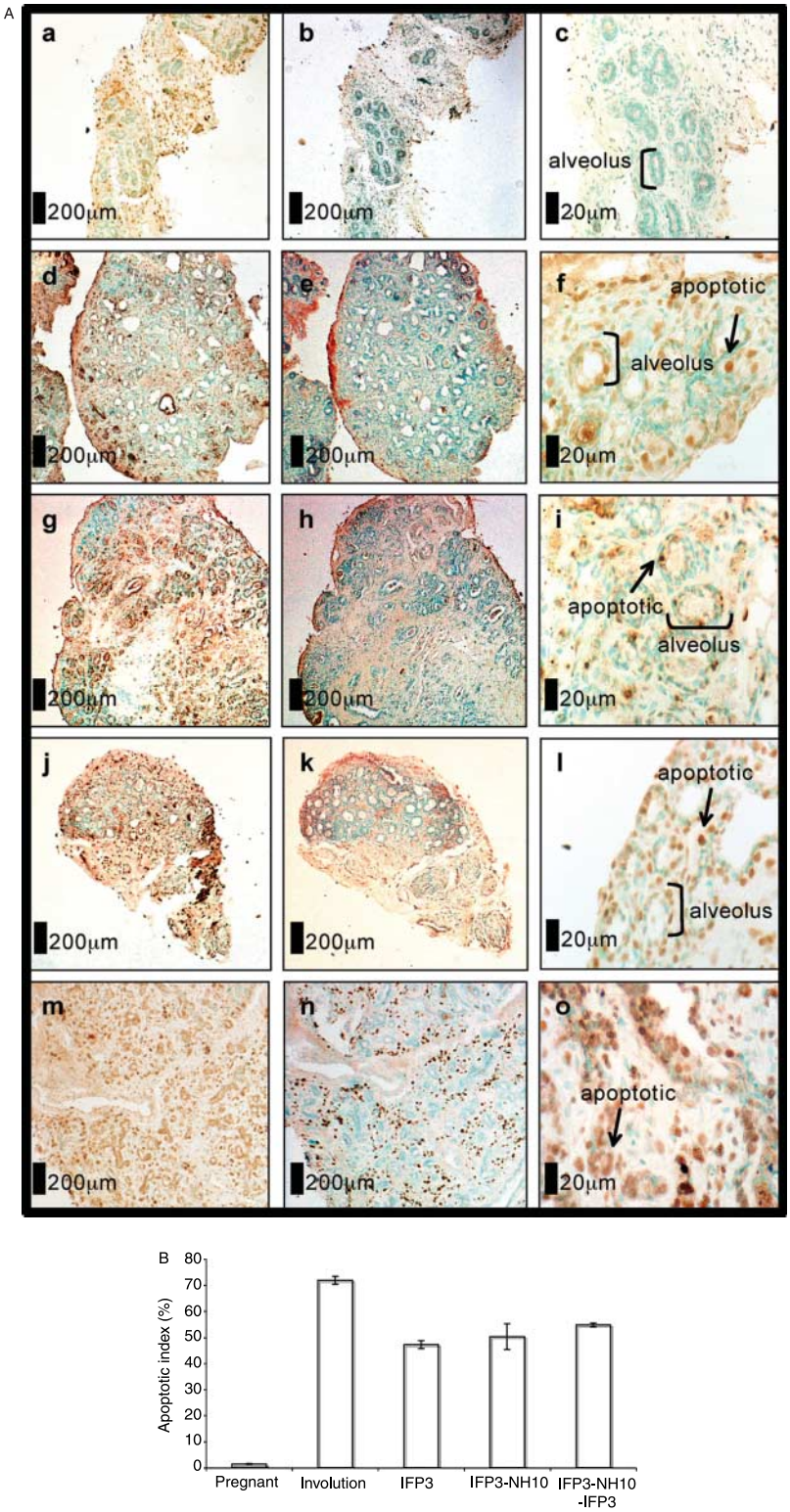

Table 2 Matrix of pairwise comparison probabilites for the apoptotic index

	IFP_3	$IFP_3 \rightarrow NH_{10}$	$IFP_3 \rightarrow NH_{10} \rightarrow IFP_3$	Involution	Pregnant
Treatment					
IFP_3	1·000				
$IFP_3 \rightarrow NH_{10}$	0·906	1·000			
$IFP_3 \rightarrow NH_{10} \rightarrow IFP_3$	0·236	0·699	1·000		
Involution	0·000*	0·000*	0·001*	1·000	
Pregnant	0·000*	0·000*	0·000*	0·000*	1·000

Tukey's multiple comparisons for the apoptotic index of mammary explants and mammary tissue from pregnant and involuting tammars. Asterisks denote significant difference.

scattered throughout the epithelium and are defined by their ability to self-renew and to produce differentiated and functional cells (Clarke *et al.* 2003). Similarly, the population of apoptotic and live cells in mammary explants is randomly distributed throughout the epithelium of the alveoli. It is unlikely, however, that the surviving population of mammary epithelial cells in mammary explants is mammary stem cells since the percentage of stem cells in the mouse mammary gland is estimated to be only 2–3% of all epithelial cells (Welm *et al.* 2002). In addition, the evidence from *in vitro* studies suggests that stem cells are not capable of producing milk proteins (Smith & Medina 1988, Chepko & Smith 1997). This contrasts with the results of this study, which shows that a significant percentage of mammary epithelial cells survive culture in the absence of hormones, and that milk protein gene expression is significantly up-regulated when lactogenic hormones are introduced to the culture media.

It is well established in the rodent that involution takes place in at least two stages. The first, reversible stage is triggered by mammary-derived signals and lasts ~48–72 h (Li *et al.* 1997). This is followed by a non-reversible phase associated with a decline in the circulating galactopoietic factors and a reduction in milk protein gene expression (Wilde *et al.* 1999). These stages are evident in the mouse and wallaby, which show a progressive decrease in the expression of milk protein genes 6-days post-weaning (Atwood *et al.* 1995). However, it is unlikely that apoptosis in mammary explants is initiated by local milk factors. Although milk proteins are synthesised and secreted by mouse and tammar mammary explants incubated with lactogenic hormones, milk constituents do not accumulate to elevated concentrations such as those at weaning (Nagamatsu & Oka 1980, Nicholas & Tyndale-Biscoe 1985). Histology also shows that alveoli within explants do not become engorged, which eliminates apoptosis due to stretching of cells or dissociation of tight junctions (Stelwagen *et al.* 1994, 1997, Singh *et al.* 2005). Furthermore, different hormone treatments do not have a significant effect on the apoptotic index. Therefore, it is possible that the population of apoptotic cells within mammary explants is due to the absence of a galactopoietic lactogenic hormone (other than insulin, cortisol or prolactin) that also declines during phase 2 of involution. The presence of apoptotic nuclei in the epithelial layer of the mammary explant alveoli is consistent with Watson (2006), who showed activated caspase-3 was present in a population of cells within the alveoli only during the second, irreversible phase of involution.

Microarray analysis also shows tammar mammary explants cultured without hormones for 4 days have a profile of differentially expressed genes most similar to day 5 of involution, which includes nine common apoptosis-associated genes. However, the tissue taken at day 5 of involution has a significantly higher proportion of apoptotic cells than explanted tissue and shows complete disorganisation and collapse of the mammary alveoli. The difference in morphology and gene regulation suggests that the occurrence of apoptotic mammary epithelial cells in mammary explants may not be a result of either hormone withdrawal or representative of cell death and mammary gland remodelling in the second phase of involution in the tammar mammary gland. This argument is strengthened

Figure 5 Apoptotic cells in tammar wallaby mammary tissue and explant sections. (A) The first vertical panel shows Apoptag-stained apoptotic cells within the mammary tissue at 5× magnification. Sections were counterstained with methyl green to show the presence of non-apoptotic cells. The second vertical panel shows control slides at 5× magnification. The third vertical panel shows Apoptag-stained apoptotic cells within the mammary tissue at 40× magnification. (a–c) Mammary explants from day 24 pregnant tammars (t_0), (d–f) mammary explants from pregnant tammars were cultured in M199 containing insulin, cortisol and prolactin for 3 days, (g–i) explants from pregnant tammars were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media, (j–l) explants from pregnant tammars were cultured in M199 containing insulin, cortisol and prolactin for 3 days, which were removed and explants were cultured for 10 days with no hormone or growth factors in the media, after which insulin, cortisol and prolactin were reintroduced for 3 days and (m–o) involuting tammar mammary tissue at 5 days. Tissue from pregnant wallabies and explants shows adequate maintenance of the epithelial and stromal compartments in comparison to involution. Examples of alveoli and apoptotic cells are indicated. (B) The apoptotic index was calculated by determining the average number of immunoreactive nuclei per total number of epithelial cells and expressed as a percentage. Each value represents ±S.E.M. ($n=5$). Different letters denote significant differences in the apoptotic index.

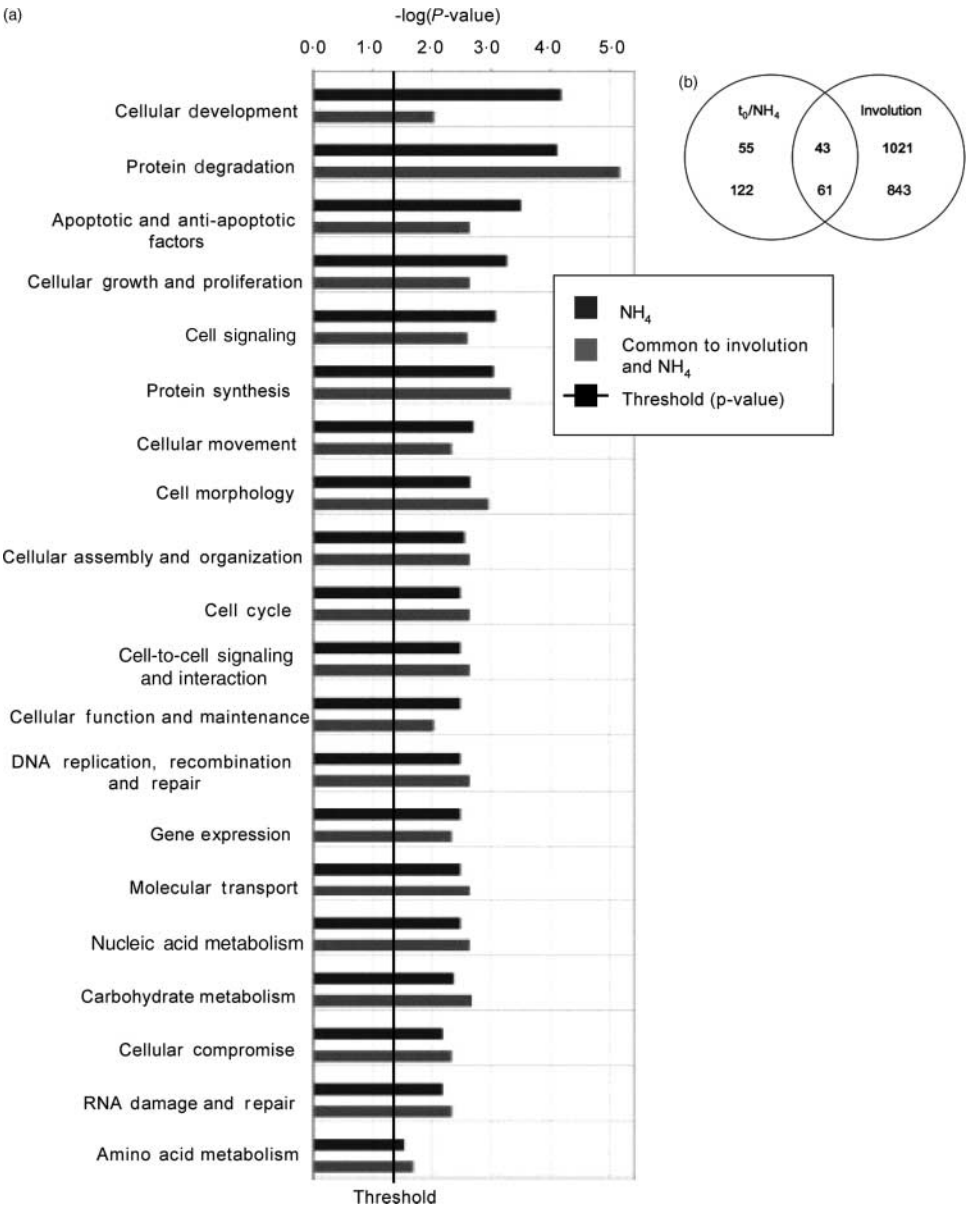

Figure 6 Comparison of tammar wallaby mammary explant gene expression with involution. Tammar wallaby EST microarray chips were hybridised to cDNA probes generated from RNA from pregnant tammar wallaby mammary glands ($n=3$), explanted mammary tissue from pregnant tammar wallabies cultured without hormones for 4 days ($n=3$), and involution ($n=4$). (a) Global functional analysis. The significance value associated with a function in global analysis is a measure for how likely it is that genes from the dataset file under investigation participate in that function. The significance is expressed as a P value that is calculated using the right-tailed Fisher's exact test. Datasets were integrated and analyzed for function using the Ingenuity Pathways Analysis software (Ingenuity Systems, http://www.ingenuity.com). (b) The venn diagram represents the number of genes up- (bold) or down-regulated greater than twofold in the mammary gland of the involuting tammar and mammary explants from pregnant tammars cultured for 4 days in the absence of hormones as determined by EST microarray analysis. Forty-three gene spots showed up-regulation common in both comparisons. Sixty-one gene spots showed down-regulation common in both comparisons.

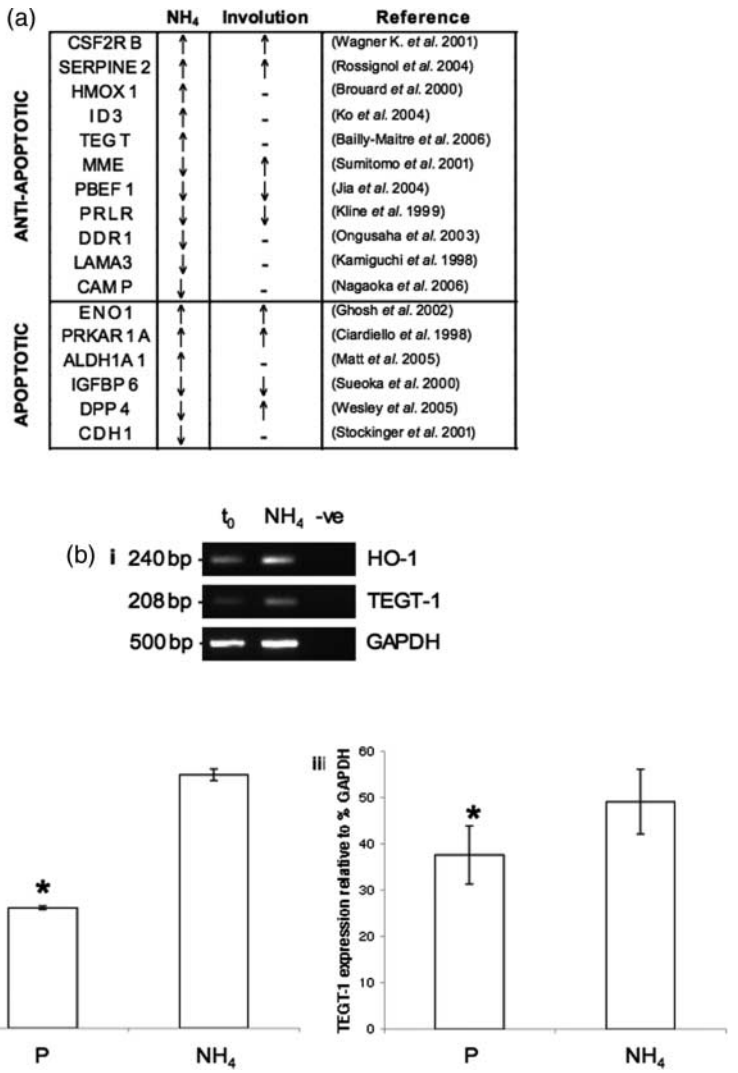

Figure 7 Regulation of tammar wallaby mammary gland gene expression found in mammary explants after culture for 4 days without hormones. Tammar wallaby EST microarray chips were hybridised to cDNA probes generated from RNA from pregnant tammar wallaby mammary glands ($n=3$) and explanted mammary tissue from pregnant tammar wallabies cultured without hormones for 4 days ($n=3$). (a) Apoptotic and anti-apoptotic genes up- and down-regulated in tammar mammary explants cultured for 4 days without hormones, and genes in common with involution. (b) RT-PCR analysis of up-regulated anti-apoptotic/survival gene expression in cultured tammar mammary tissue. Total RNA was prepared from the mammary tissue of a pregnant tammar (t_0), and the mammary explants of a pregnant tammar cultured in no hormone for 4 days (NH_4). RT-PCR was performed using specific primers. i) Anti-apoptotic genes significantly up-regulated only in mammary tissue after culture in no hormone for 4 days. HMOX, heme oxygenase-1; TEGT, testicular enhanced gene transcript. ii) Expression of heme oxygenase-1 relative to %GAPDH using ImageJ for quantitative evaluation. iii) Expression of testicular enhanced gene transcript relative to %GAPDH using ImageJ for quantitative evaluation. Each value represents ± S.E.M. ($n=3$), $P<0{\cdot}05$. PCRs were performed for 25 cycles.

by global functional microarray analysis that shows the gene expression similarities between explants and involution are related to many cellular functions other than apoptosis. Therefore, it is likely that the apoptotic cells detected within mammary explants may be a consequence of tissue culture, as different hormone treatments do not have a significant effect on the apoptotic index.

A number of genes encoding proteins linked to apoptosis that showed altered expression in cultured mammary tissue were identified. It is not surprising that seven of these genes

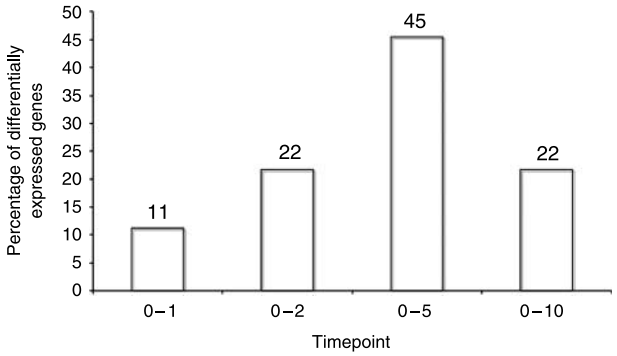

Figure 8 Comparison of tammar wallaby mammary explant gene expression with stages of involution. Tammar wallaby EST microarray chips were hybridised to cDNA probes generated from RNA from pregnant tammar wallaby mammary glands ($n=3$), explanted mammary tissue from pregnant tammar wallabies cultured without hormones for 4 days ($n=3$) and involution at days 1, 2, 5 and 10 ($n=1$). Values were calculated by determining the number of differentially expressed genes in explanted mammary tissue from pregnant tammar wallabies cultured without hormones for 4 days as a percentage of the total number of differentially expressed genes found at days 1, 2, 5 and 10 of involution.

showed parallel regulation in both mammary explants cultured without hormones and in involution, as explants exposed to different hormone treatments had significantly higher apoptotic indices than mammary tissue from a pregnant animal. However, we identified five differentially expressed genes in mammary explants that may contribute to the survival of mammary epithelial cells. For example, the down-regulation of CDH1 and DPP4 may contribute to the suppression of cell death, as both genes induce apoptosis of epithelial cells when transiently expressed (Stockinger *et al.* 2001, Wesley *et al.* 2005). Similarly, the anti-apoptotic ID3 gene that allows thymocytes to survive selection events in the thymus is up-regulated only in culture (Ko *et al.* 2004). We also found that two stress-induced anti-apoptotic genes are up-regulated only when explants are cultured in the absence of hormones. The first gene, HMOX-1 is the rate-limiting enzyme in the catabolism of heme-generating biliverdin, carbon monoxide and iron (Alam *et al.* 2000, Chen *et al.* 2004), and is an inducible enzyme activated by most oxidative stress inducers and cytokines (Chen *et al.* 2004). The almost universal stimulation of HMOX expression by pro-oxidants and the observation that biliverdin and bilirubin are potent anti-oxidants have led to the assumption that enhancement of HMOX activity represents an adaptive, and ultimately protective response to cellular stress (Alam *et al.* 2000). The HMOX enzyme is believed to exert an anti-apoptotic action by decreasing intracellular pro-oxidant levels (Fang *et al.* 2004). For example, in renal immune injury, it is proposed that activation of HMOX protects against NO-mediated toxicity by negatively modulating expression or activity of iNOS (Chen *et al.* 2004). The second gene, TEGT suppresses apoptosis induced by bax, etopsoside, staurosporine and growth factor deprivation (Grzmil *et al.* 2003). Interestingly, TEGT has been evolutionarily conserved as it regulates cell death in both plant and animal cells (Chae *et al.* 2003). In mammalian species, TEGT regulates cell death pathways controlled by bcl-2 and bax, and appears to function in general cell survival (Chae *et al.* 2003). When HMOX or TEGT are up-regulated the rejection rate of organ transplants is significantly reduced (Wagner *et al.* 2003, Bailly-Maitre *et al.* 2006). Therefore, the cytoprotective nature of these genes suggests up-regulation may be a response to cellular stress caused by culture contributing to the survival of a cell population.

Alternatively, as the accumulation of milk initiates cell death cascades in the first phase of apoptosis, several death pathways may be avoided in mammary explants if milk constituents do not accumulate to elevated concentrations such as those at weaning. Transgenic mouse studies have been used to identify genes that can inactivate apoptosis pathways in the epithelium. For example, incomplete involution results when the survival factor insulin-like growth factor-I (IGF-I) with reduced affinity for IGF binding proteins (IGFBPs) is overexpressed in the mammary glands of transgenic mice (Hadsell *et al.* 1996). Similarly, the conditional knockout of mammary-specific Stat3 delays involution because Stat3 and Stat5 are reciprocally activated in the mammary gland; Stat5 promotes survival and milk secretion, while Stat3 stimulates apoptosis (Chapman *et al.* 1999, Humphreys *et al.* 2002). Importantly, microarray analysis of tammar mammary explants shows that 'classic' phase 1 markers defined in Clarkson *et al.* (2004), and Green & Streuli (2004), are not differentially expressed.

This investigation clearly demonstrates that a population of mammary epithelial cells have the capacity to remain viable and hormone responsive for extended periods in chemically defined media without any exogenous macromolecules. These results are in contrast to previous reports that have argued rodent mammary explants require addition of serum, insulin or a growth factor to retain tissue viability in culture. The fact that apoptosis can be reduced in the first phase without the total loss of alveoli or tissue remodelling demonstrates that the two phases can occur independently of each other (Stein *et al.* 2007). We propose that the mammary explant culture model uncouples the first phase of involution, as milk accumulation that normally provides involution stimuli is absent in this culture model allowing a population of cells to survive, therefore providing a mechanism to further investigate apoptosis in the mammary gland. These data are consistent with the argument that the accumulation of milk at weaning regulates the initiation of involution and progression to apoptosis in the mammary gland.

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