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# Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland

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## SUMMARY

**The steroid hormones 17β-estradiol and progesterone play a central role in the pathogenesis of breast cancer and regulate key phases of mammary gland development. This suggests that developmental regulatory molecules whose activity is influenced by ovarian hormones may also contribute to mammary carcinogenesis. In a screen designed to identify protein kinases expressed in the mammary gland, we previously identified a novel SNF1-related serine/threonine kinase, Hunk (hormonally upregulated Neu-associated kinase). During postnatal mammary development, *Hunk* mRNA expression is restricted to a subset of mammary epithelial cells and is temporally regulated with highest levels of expression occurring during early pregnancy. In addition, treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in a subset of mammary epithelial cells, suggesting that the expression of this kinase may be regulated by ovarian hormones. Consistent with the tightly regulated pattern of *Hunk* expression during pregnancy, mammary glands from transgenic mice engineered to misexpress *Hunk* in the mammary epithelium manifest temporally distinct defects in epithelial proliferation and differentiation during pregnancy, and fail to undergo normal lobuloalveolar development. Together, these observations suggest that Hunk may contribute to changes in the mammary gland that occur during pregnancy in response to ovarian hormones.**

Key words: Mammary gland, Protein kinase, Transgenic, Cell differentiation, Hormone, Mouse, Hunk

## INTRODUCTION

A wealth of epidemiological evidence indicates that ovarian hormones play a crucial role in the etiology of breast cancer (Kelsey et al., 1993). Specifically, the observations that early menarche, late menopause and postmenopausal hormone replacement therapy are each associated with increased breast cancer risk, whereas early oophorectomy is associated with decreased breast cancer risk, have led to the hypothesis that breast cancer risk is proportional to cumulative estradiol and progesterone exposure (Henderson et al., 1988; Pike et al., 1993). As such, elucidating the mechanisms by which hormones contribute to mammary carcinogenesis is a central goal of breast cancer research.

In addition to their roles in the pathogenesis of breast cancer, estradiol and progesterone are the principal steroid hormones responsible for regulating the development of the mammary gland during puberty, pregnancy and lactation (Topper and Freeman, 1980). For example, estradiol action is required for epithelial proliferation and ductal morphogenesis during puberty, whereas progesterone action is required for ductal arborization and alveolar differentiation during pregnancy (Bocchinfuso and Korach, 1997; Humphreys et al., 1997; Topper and Freeman, 1980). The effects of estradiol and progesterone in a given tissue are ultimately determined by the activation and repression of their respective target genes. Consequently, understanding the effects of estradiol and progesterone in the breast will require the identification of downstream targets of these hormones, and the analysis of these targets will undoubtedly contribute to our understanding of both mammary development and carcinogenesis.

Protein kinases function as molecular switches in signal transduction pathways that regulate cellular processes such as proliferation and differentiation. Accordingly, aberrant expression or mutations in several members of the protein kinase family have been shown to be involved in the pathogenesis of breast cancer both in humans and in rodent model systems (Cardiff and Muller, 1993; Cooper, 1990; Di Fiore et al., 1987; Muller et al., 1988). Moreover, overexpression of protein kinases such as ERBB2/neu in

human breast cancers has been shown to provide prognostic information relevant to clinical outcome and response to therapy (Klijn et al., 1993; Slamon et al., 1987, 1989). Given the important roles played by protein kinases in development and carcinogenesis, we previously carried out a PCR-based screen to identify kinase family members expressed either during mammary development or in epithelial cell lines derived from different transgenic mouse models of breast cancer (Chodosh et al., 1999, 2000). A total of 41 protein kinases were identified in this screen including 33 tyrosine kinases and eight serine/threonine kinases, three of which were novel (Chodosh et al., 2000; Gardner et al., 2000a,b; Stairs et al., 1998).

One of these novel kinases, Hunk, is an 80 kDa putative serine/threonine kinase that bears homology to the SNF1 family of protein kinases (Gardner et al., 2000b). Several members of this family, including SNF1 in *Saccharomyces cerevisiae* and AMP-activated protein kinase in mammals, regulate metabolic changes that occur in response to nutritional and environmental stresses (Hardie et al., 1994). Other SNF1-related kinases, including PAR1, MARK1, MARK2 and MARK3/KP78/C-TAK1, have been implicated in the regulation of developmental processes (Bohm et al., 1997; Guo and Kemphues, 1995; Peng et al., 1997; Ruiz et al., 1994). For example, in both *Caenorhabditis elegans* and *Drosophila, par-1* is required for the establishment of anterior-posterior axis formation during embryogenesis (Guo and Kemphues, 1995; Shulman et al., 2000). Analogously, a mammalian homolog of PAR1, MARK2/EMK, is asymmetrically localized in polarized epithelial cells, and expression of a dominant negative form of MARK2 disrupts cell polarity (Bohm et al., 1997). Consistent with a developmental role for this molecule, disruption of murine *Emk*/*MARK* results in dwarfism, pituitary defects and hypofertility (Bessone et al., 1999). Additional SNF1-related kinases such as *Msk* and SNRK have been implicated in vertebrate development on the basis of their temporal and spatial patterns of expression (Becker et al., 1996; Ruiz et al., 1994). These data suggest a role for SNF1 family members in development in higher eukaryotes.

We have previously described the cloning, activity and chromosomal localization of *Hunk* and have shown that *Hunk* mRNA expression is temporally and spatially regulated during embryonic development (Gardner et al., 2000b). In the developing embryo, *Hunk* is expressed at high levels within a subset of organs during mid-gestation and is downregulated in some, but not all, tissues prior to parturition. As in the embryo, *Hunk* expression in the adult mouse is tissue specific, with highest levels observed in ovary, lung and brain. Interestingly, within multiple tissues *Hunk* mRNA expression is restricted to specific compartments and within these compartments is further restricted to a subset of cells. For example, *Hunk* expression in the duodenum is limited to a subset of epithelial cells in duodenal crypts, whereas little or no expression is observed in more differentiated cells of the duodenal epithelium. The tissue-specific, temporally regulated and spatially restricted pattern of *Hunk* expression in the mouse suggest a developmental role for this kinase in multiple tissues.

In this report, we investigate the role of *Hunk* in the mammary gland. During postnatal mammary development, *Hunk* mRNA expression is spatially restricted to a subset of epithelial cells and is tightly regulated, with highest levels of expression occurring early in pregnancy. Moreover, treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic induction of *Hunk* expression in the mammary epithelium, suggesting that *Hunk* upregulation during early pregnancy may be due to increases in circulating levels of ovarian hormones. Finally, misexpression of *Hunk* in the mammary epithelium of MMTV-*Hunk* transgenic mice results in decreased proliferation and impaired differentiation of alveolar epithelial cells during distinct periods of pregnancy and lactation. Taken together, our data suggest that Hunk may contribute to mammary development by regulating pregnancy-induced changes in the alveolar epithelium that occur in response to estrogen and progesterone.

## MATERIALS AND METHODS

### Animal and tissue preparation

FVB mice were housed under barrier conditions with a 12-hour light/dark cycle. Mammary glands from pregnant females were harvested at specified timepoints after timed matings. Female mice were housed with male mice every third night and day 0.5 was defined as noon of the day on which a vaginal plug was observed. Gestational stage was confirmed by analysis of embryos. Transgenic mothers were housed with wild-type mothers immediately after parturition to ensure pup survival and equivalent suckling stimuli. Both transgenic and wild-type females were observed to nurse pups. For experiments involving chronic hormone treatment, adult female FVB mice were subject to bilateral oophorectomy and allowed to recover for two weeks prior to hormonal injections that were administered as previously described (Marquis et al., 1995). For short-term hormone administration experiments, four-month-old virgin female FVB mice were injected subcutaneously with either phosphate buffered saline (PBS) or a combination of 5 mg progesterone in 5% gum arabic and 20 μg of 17β-estradiol in PBS. Four animals from each treatment group were sacrificed 24±1 hours after injection. Tissues used for RNA analysis were snap frozen on dry ice. Tissues used for in situ hybridization analysis were embedded in OCT compound.

For whole mount analysis, number four mammary glands were spread on glass slides and fixed for 24 hours in 10% neutral buffered formalin. Glands were subsequently immersed in 70% ethanol for 15 minutes followed by 15 minutes in deionized water prior to staining in 0.05% Carmine/0.12% aluminum potassium sulfate for 24-48 hours. Glands were dehydrated sequentially in 70%, 90% and 100% ethanol for 10 minutes each, and then cleared in toluene or methyl salicylate overnight. For histological analysis, mammary glands were fixed as above and transferred to 70% ethanol prior to paraffin embedding. Sections 5 μm thick were cut and stained with Hematoxylin and Eosin. For BrdU analysis, animals were injected with 50 μg BrdU per g total bodyweight two hours before sacrifice followed by fixation and paraffin embedding as above.

### Generation of MMTV-*Hunk* transgenic mice

A full-length cDNA clone, G3, encoding *Hunk*, was digested with *Sma*I and *Spe*I to liberate a 3.2 kb fragment containing the complete coding sequence for *Hunk* (GenBank Accession number AF167987). This fragment was cloned downstream of the mouse mammary tumor virus long terminal repeat (MMTV LTR) into the multiple cloning site of pBS-MMTV-pA (E. Gunther, unpublished), which consists of the MMTV LTR upstream of the H-ras leader sequence (Huang et al., 1981) and SV40 splicing and polyadenylation signals. Linearized plasmid DNA was injected into fertilized oocytes harvested from superovulated FVB mice. Tail-derived DNA was prepared as described (Hogan et al., 1994). Mice were genotyped by Southern hybridization analysis and by two independent PCR reactions designed to amplify a region within the SV40 portion of the transgene,

and a region spanning the junction between *Hunk* and SV40 sequences. A portion of the *Gapdh* (glycelaldehyde-3-phosphate dehydrogenase) locus was amplified as a positive control for PCR reactions. Oligonucleotide primer sequences were Gapdh.F, CTCACTCAAGATTGTCAGCAATGC; Gapdh.B, AGGGTTTCT-TACTCCTTGGAGGC; SV40.F, CCTTAAACGCCTGGTGCTA-CGC; SV40.B, GCAGTAGCCTCATCATCACTAGATGG; Hunk.F, CTTTCTTTTTCCCCTGACC; PolyA.B, ACGGTGAGTAGCGTC-ACG. Southern hybridization analysis of tail-derived genomic DNA digested with *Spe*I was performed according to standard methods using a probe specific to the SV40 portion of the transgene. Four founder mice were identified harboring the MMTV-*Hunk* transgene in tail-derived DNA that passed the transgene to offspring in a Mendelian fashion. These were screened for transgene expression by Northern hybridization and RNase protection analysis. One founder line, MHK3, was identified that expressed the MMTV-*Hunk* transgene at high levels. Of note, a subset of transgene-positive MHK3 animals was found not to express the MMTV-*Hunk* transgene. All MHK3 non-expressing animals were analyzed by Southern hybridization analysis to confirm transgene presence and the expected MHK3-specific integration site.

### RNA preparation and analysis

RNA preparation, northern hybridization and labeling of cDNA probes was performed as previously described (Marquis et al., 1995). The $^{32}$P-labeled cDNA probe for *Hunk* encompassed nucleotides 275 to 793 (GenBank Accession number AF167987). Probes for milk protein gene expression were: β-casein, nt 181-719 (GenBank Accession number X04490); κ-casein, nt 125-661 (GenBank Accession number M10114); lactoferrin, nt 993-2065 (GenBank Accession number D88510); WAP, nt 131-483 (GenBank Accession number X01158) and ε-casein, nt 83-637 (GenBank Accession number V00740).

Ribonuclease protection analysis was performed as described using body-labeled antisense riboprobes specific to nucleotides 276-500 of *Hunk* and 1142-1241 of β-actin (GenBank Accession number X03672; Marquis et al., 1995). In order to distinguish transgenic from endogenous *Hunk* expression in MHK3 animals, RNase protection analysis was performed using an antisense riboprobe spanning the 3′ end of the *Hunk* cDNA and the 5′ end of the SV40 polyadenylation signal sequence. A β-actin antisense riboprobe was added to each reaction as an internal control. Signal intensities were quantitated by phosphorimager analysis (Molecular Dynamics).

In situ hybridization was performed as described (Marquis et al., 1995; Rajan et al., 1997) using a PCR template containing nucleotides 276 to 793 of *Hunk.* Exposure times were 6 weeks in all cases.

### Protein analysis

Generation of anti-Hunk antisera, immunoblotting and immunoprecipitation were performed as described (Gardner et al., 2000b). Protein was extracted from mammary glands by dounce homogenization in EBC buffer as described (Gardner et al., 2000b). For immunoprecipitation, 500 μg of protein (3 mg/ml) was precleared with 1/10 vol of 1:1 protein A-sepharose in PBS overnight at 4°C. Precleared lysates were incubated overnight at 4°C in EBC (50 mM Tris-HCl, pH 7.9; 120 mM NaCl; 0.5% NP40) plus 5% Tween 20 (Biorad) with or without affinity-purified antisera raised against the C terminus of Hunk (0.4 μg/ml). Immune complexes were precipitated by incubating with 40 μl of 1:1 protein A-sepharose in PBS for 1 hour at 4°C. Complexes were washed sequentially with EBC plus 5% Tween 20, EBC (2×), and PBS (2×). One-fifth of the precipitated complexes were used in an in vitro kinase reaction as previously described with 5 μM ATP and 0.5 μg/μl histone H1 (Gardner et al., 2000b). The remaining precipitate was electrophoresed on a 10% SDS-PAGE gel, transferred onto a PVDF membrane, and immunoblotted with an antibody against the C terminus of Hunk as described (Gardner et al., 2000b).

### Immunohistochemistry

Mammary glands from nulliparous wild-type and MHK3 transgenic females were fixed in 4% paraformaldehyde overnight and transferred to 70% ethanol prior to paraffin embedding. 5 μm sections were dewaxed in xylene and sequentially rehydrated in 100%, 95% and 70% ethanol, followed by PBS. Sections were incubated in Antigen Unmasking Solution (Vector) for 30 minutes at 100°C and then transferred to PBS at room temperature (RT). Sections were incubated for 2 hours at RT with antibody raised against the C terminus of Hunk, washed in PBS (×3), then incubated with 1:500 biotinylated goat anti-rabbit antibody (Vector) in 1%BSA/PBS for 30 minutes at RT. After washing in PBS (×3), slides were incubated in a 1:250 dilution of Avidin (Vector) for 15 minutes at RT and washed in PBS (3×). NBT and BCIP substrate addition was performed in alkaline phosphate buffer for 3 minutes according to manufacturer instructions (BMB). Sections were counterstained for 10 minutes in 0.5% (w/v) Methyl Green in 1.0 M NaOAc, pH 4.0.

### BrdU

Paraffin-embedded 5 μm sections were dewaxed as above, pretreated in 2N HCl for 20 minutes at RT, washed in 0.1 M borate buffer, pH 8.5 (×2) and rinsed in PBS. BrdU immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories), rat anti-BrdU IgG (Vector), and a secondary biotinylated rabbit anti-rat IgG antibody according to manufacturer instructions. Sections were counterstained with Methyl Green as described above. The areas of BrdU-positive and -negative nuclei were quantitated by color segmentation analysis of digitally captured images using Image-Pro Plus software (Media Cybernetics). The percentage of BrdU-positive epithelial cells was determined after normalizing nuclear area to the average nuclear size of either BrdU positive or negative cells.

## RESULTS

*Hunk,* initially termed *Bstk1*, was identified as a 207 bp RT-PCR product isolated from an epithelial cell line derived from a mammary adenocarcinoma arising in an MMTV-*neu* transgenic mouse (Chodosh et al., 1999, 2000; Gardner et al., 2000b). *Bstk1* was subsequently renamed *Hunk* to reflect the upregulation of this kinase in the mammary gland both during pregnancy and in response to ovarian hormones, as well as the preferential expression of this kinase in transgenic murine mammary epithelial cell lines overexpressing the *neu/Erbb2* oncogene (this report and data not shown).

### *Hunk* expression is developmentally regulated in the mammary gland

RNase protection analysis was used to determine the temporal pattern of *Hunk* expression during the postnatal development of the murine mammary gland (Fig. 1A). Mammary glands were harvested from male FVB mice, virgin mice at developmental time points prior to puberty (2 weeks), during puberty (5 weeks) and after puberty (10 weeks and 15 weeks), as well as from mice during early, mid and late pregnancy (day 7, 14 and 20), lactation (day 9), and postlactational regression (days 2, 7 and 28). This analysis revealed that steady-state levels of *Hunk* mRNA were low and remained relatively constant throughout virgin development. During early pregnancy (day 7), when alveolar buds begin to proliferate rapidly and differentiate, *Hunk* mRNA levels underwent a dramatic increase and then returned to baseline by mid-pregnancy (Fig. 1A,B). The apparent decline in β-actin expression seen by RNase protection analysis during late

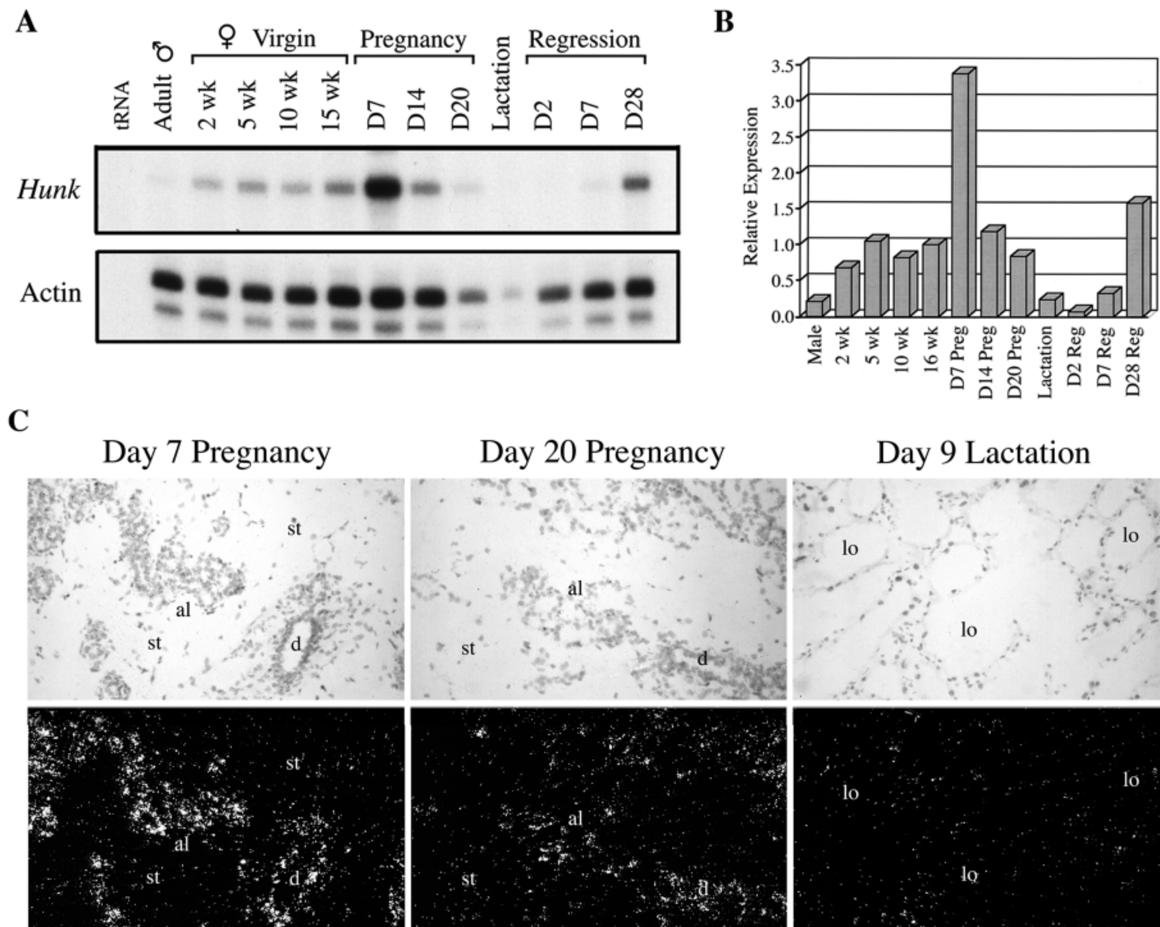


**Fig. 1.** Temporal regulation of *Hunk* expression during mammary gland development. (A) RNase protection analysis of *Hunk* mRNA expression during postnatal developmental of the murine mammary gland. 40 μg of total RNA isolated from mammary glands at the indicated timepoints was hybridized to a $^{32}$P-labeled antisense RNA probe specific for *Hunk*. A $^{32}$P-labeled antisense RNA probe specific for β-actin was included in the same hybridization reaction as an internal loading control. (B) Phosphorimager analysis of RNase protection analysis in A. *Hunk* expression was quantitated and normalized to β-actin expression to correct for dilutional effects due to large-scale increases in expression of milk protein genes during late pregnancy and lactation. Expression levels are shown relative to adult virgin (15 wk). (C) In situ hybridization analysis of *Hunk* expression during pregnancy and lactation. Bright-field (top panel) and dark-field (bottom panel) photomicrographs of mammary gland sections from day 7 pregnant, day 20 pregnant or day 9 lactating animals hybridized with an $^{35}$S-labeled *Hunk*-specific antisense probe. No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were identical for all dark-field photomicrographs to illustrate changes in *Hunk* expression during pregnancy. al, alveoli; d, duct; lo, lobule; st, adipose stroma.

pregnancy, lactation and early postlactational regression results from a dilutional effect that is due to large-scale expression of genes for milk proteins during these developmental stages (Buhler et al., 1993; Gavin and McMahon, 1992; Marquis et al., 1995). Normalization of *Hunk* expression to β-actin to control for this dilutional effect confirmed that *Hunk* expression returned to baseline levels by mid-pregnancy and decreased further during lactation and early postlactational regression (Fig. 1B). An essentially identical expression profile was observed during pregnancy when *Hunk* mRNA levels were normalized to cytokeratin 18, an epithelial-specific marker, indicating that developmental changes in *Hunk* expression are not the result of changes in epithelial cell content in the gland during pregnancy (data not shown). This conclusion is supported by the finding that *Hunk* mRNA expression levels decreased from day 7 to day 14 of pregnancy, despite ongoing increases in epithelial cell content that occur during this stage of development. Furthermore, changes in *Hunk* expression did not appear to be the result of increased cellular proliferation, since the pattern of *Hunk* expression observed during pregnancy did not correlate with levels of epithelial proliferation that, unlike *Hunk* expression, remained elevated during mid-pregnancy (see Fig. 5B).

In order to determine whether the observed pregnancy-induced changes in *Hunk* mRNA expression levels represent global changes in expression throughout the mammary gland, or changes in expressing subpopulations of cells, in situ hybridization was performed (Fig. 1C and data not shown). Consistent with the results from RNase protection analysis, in situ hybridization confirmed that *Hunk* expression in the mammary gland was highest at day 7 of pregnancy and decreased progressively throughout the remainder of pregnancy and lactation. This analysis also revealed that *Hunk* was expressed exclusively in the epithelium throughout mammary gland development and that *Hunk* upregulation during pregnancy appeared to result from both the upregulation

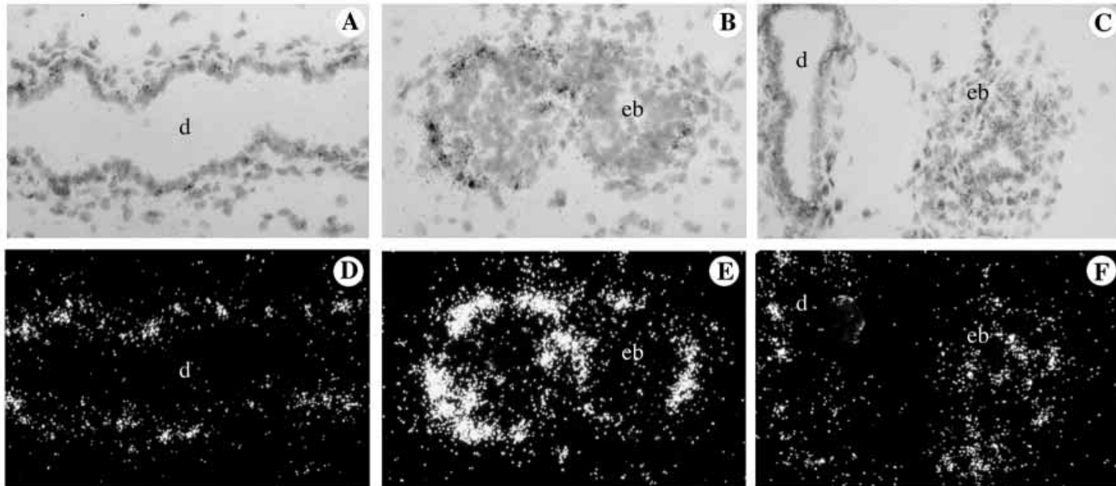


**Fig. 2.** Heterogeneous expression of *Hunk* in the mammary epithelium (A-F). In situ hybridization analysis of *Hunk* expression in the virgin mammary gland using an $^{35}$S-labeled *Hunk*-specific antisense probe. Bright-field (A-C) and dark-field (D-F) photomicrographs of in situ hybridization analysis performed on mammary gland sections from 5-week-old nulliparous females. In all cases note the heterogeneous expression pattern of *Hunk* in both epithelial ducts (A,C,D,F) and terminal end buds (B,C,E,F). No signal over background was detected in serial sections hybridized with a sense *Hunk* probe. Exposure times were optimized for each dark-field panel. d, duct; eb, terminal end bud.

of *Hunk* in a subset of cells and an increase in the proportion of *Hunk*-expressing epithelial cells (Fig. 1C and data not shown).

The observation that cells highly expressing *Hunk* are found adjacent to non-expressing cells indicates that, as in other organs of the adult mouse, *Hunk* expression in the mammary gland is spatially restricted (Figs 1C and 2). This heterogeneous expression pattern is particularly striking in terminal end buds and epithelial ducts of the adolescent gland (Fig. 2). These data suggest that the murine mammary epithelium is composed of *Hunk*-expressing and *Hunk* non-expressing cell types.

### *Hunk* expression is regulated by ovarian hormones

The observation that *Hunk* mRNA levels in the mammary gland increase during pregnancy suggests that the expression of this gene may be modulated by estrogen and progesterone. In order to test this possibility, oophorectomized FVB mice were treated for fourteen days with 17β-estradiol alone, progesterone alone, or a combination of both hormones. Intact (sham) and oophorectomized, non-hormone treated (OVX) animals were used for comparison.

*Hunk* mRNA levels were quantitated by RNase protection analysis of RNA prepared from mammary glands or uteri pooled from at least 10 animals in each experimental group (Fig. 3). Steady-state *Hunk* mRNA levels were approximately fourfold lower in the mammary glands of oophorectomized mice compared with intact mice, suggesting that maintenance of basal levels of *Hunk* expression in the mammary glands of nulliparous mice requires ovarian hormones (Fig. 3A). Treatment of oophorectomized animals with 17β-estradiol alone increased *Hunk* mRNA expression but to levels below those observed in intact animals, whereas treatment with progesterone alone increased *Hunk* mRNA expression to levels comparable with those observed in intact animals. In contrast, treatment of oophorectomized animals with both 17β-estradiol and progesterone resulted in a 14-fold increase in the level of *Hunk* mRNA relative to control oophorectomized animals and a 3-fold increase relative to intact animals, similar to increases in *Hunk* expression observed during early pregnancy. These observations suggest that the increase in *Hunk* mRNA expression observed in the mammary gland during early pregnancy may result, either directly or indirectly, from increases in circulating levels of estrogens and progesterone.

Treatment of mice with ovarian hormones also affected *Hunk* expression in the uterus (Fig. 3B). Steady-state *Hunk* mRNA levels were nearly two-fold higher in oophorectomized animals compared with intact mice suggesting that circulating levels of 17β-estradiol may repress *Hunk* expression in the uteri of nulliparous mice. Consistent with this suggestion, treatment of oophorectomized animals with 17β-estradiol either alone or in combination with progesterone decreased *Hunk* expression to levels below those observed in either intact or oophorectomized animals. In contrast to findings in the mammary gland, progesterone treatment had little if any effect on *Hunk* expression in the uterus. These results suggest that the increase in *Hunk* mRNA expression observed in the uterus following oophorectomy is due, either directly or indirectly, to loss of tonic inhibition of *Hunk* expression by estradiol. The observation that the combination of estradiol and progesterone has opposing effects on *Hunk* expression in the mammary gland and uterus is consistent with the opposing physiological effects of these hormones on proliferation and differentiation in these tissues.

The effects of estradiol and progesterone on *Hunk* expression in the mammary gland and uterus were confirmed by in situ hybridization analysis performed on tissues from the experimental animals described above (Fig. 3D and data not shown). Consistent with RNase protection results, oophorectomy resulted in a marked decrease in *Hunk* mRNA expression in the mammary epithelium and the combination of 17β-estradiol and progesterone resulted in a synergistic increase in *Hunk* expression. Reminiscent of *Hunk* expression during early pregnancy, the upregulation of *Hunk* mRNA levels

in oophorectomized animals treated with a combination of 17β-estradiol and progesterone occurred in a subset of epithelial cells in both ducts and developing alveolar buds.

Since the above experiments involved the chronic administration of hormones, sufficient time elapsed during hormone treatment for significant developmental changes to occur in both the mammary glands and uteri of oophorectomized animals. As such, these experiments do not distinguish whether changes in *Hunk* expression reflect direct regulation by ovarian hormones, or are a consequence of the changes in epithelial proliferation and differentiation that occur in response to the chronic administration of ovarian hormones. To address this issue, intact mice were treated with a combination of 17β-estradiol and progesterone for 24 hours before sacrifice (Fig. 3C). Mice treated in such a manner do not develop the marked morphological changes characteristic of long-term hormone administration. Analysis of *Hunk* mRNA expression levels in these mice revealed a pattern similar to that observed in mice treated chronically with hormones. Within 24 hours of the administration of 17β-estradiol and progesterone, steady-state levels of *Hunk* mRNA increased in the mammary gland and decreased in the uterus. These findings suggest that the regulation of *Hunk* expression by estradiol and progesterone is not solely a consequence of changes in mammary and uterine tissue architecture that occur in response to chronic hormone treatment, but rather may result from direct regulation by these hormones.

### Generation of MMTV-*Hunk* transgenic animals

The tightly regulated expression of *Hunk* observed in the mammary gland during pregnancy and in response to ovarian hormones suggests the possibility that *Hunk* may play a role in mediating pregnancy-induced changes in the mammary gland. To test this hypothesis, transgenic mice overexpressing *Hunk* in a mammary-specific fashion were generated using the MMTV LTR. Activity of the MMTV LTR is upregulated in mammary epithelial cells during pregnancy and lactation in response to rising levels of prolactin, progesterone and glucocorticoids. Since endogenous *Hunk* expression is heterogeneous and is transiently upregulated during early pregnancy, MMTV-driven expression of *Hunk* in transgenic mice would be predicted to alter the temporal and spatial profile of *Hunk* expression in the mammary gland.

A cDNA encoding the full-length Hunk protein was cloned downstream of the MMTV LTR and injected into superovulated FVB mice. One of four founder lines, MHK3, was found to express the *Hunk* transgene at high levels in the mammary gland and was therefore studied further (Fig. 4A). The tissue specificity of transgene expression in the MHK3 line was determined by RNase protection analysis using a transgene-specific probe (Fig. 4B). This analysis confirmed that nulliparous MHK3 transgenic females express high levels of the MMTV-*Hunk* transgene in the mammary gland and lower but detectable levels of transgene expression in the spleen, salivary gland, lung and thymus, as has been observed for other MMTV transgenic mouse models.

The hormonally responsive nature of the MMTV LTR often results in low levels of expression in the mammary glands of nulliparous transgenic animals and high levels of transgene expression during pregnancy that peak during lactation. In contrast, MHK3 animals express high levels of

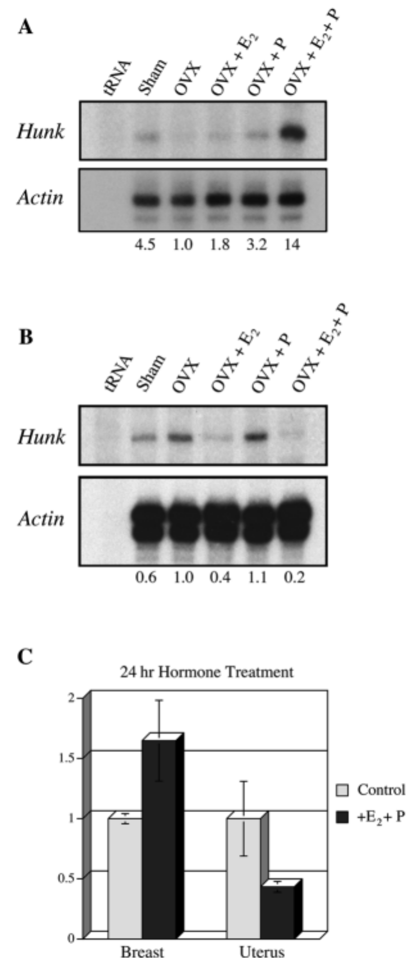


**Fig. 3.** Ovarian hormones alter *Hunk* expression in vivo. *Hunk* mRNA expression in mammary glands and uteri of mice treated with ovarian hormones. (A,B) Tissues were harvested from either intact females (sham) or oophorectomized females that received daily subcutaneous injections of either vehicle alone (OVX), 17β-estradiol (OVX+$E_2$), progesterone (OVX+P), or both 17β-estradiol and progesterone (OVX+$E_2$+P) for fourteen days. Each sample represents a pool of at least 10 mice. 20 μg of total RNA isolated from the mammary glands (A) or uteri (B) of treated animals was hybridized overnight with $^{32}$P-labeled antisense RNA probes specific for *Hunk* and β-actin. Signal intensities were quantitated by phosphorimager analysis and *Hunk* expression was normalized to β-actin expression levels. *Hunk* expression relative to expression in oophorectomized (OVX) controls is shown below each lane. (C) Quantitation of *Hunk* expression in mammary glands and uteri from intact FVB female mice killed 24 hours after injection with PBS (control; light shaded boxes) or a combination of 5 mg progesterone in 5% gum arabic and 20 μg of 17β-estradiol in PBS (+$E_2$+P; dark shaded boxes). RNase protection analysis was performed on either 20 μg (breast) or 40 μg (uterus) of total RNA using $^{32}$P-labeled antisense RNA probes specific for *Hunk* and β-actin. *Hunk* expression was quantitated by phosphorimager analysis and normalized to β-actin. Values are shown relative to control animals. Each bar represents the average of four animals±s.e.m. for each group. (D) In situ hybridization analysis of *Hunk* expression in mammary gland sections from oophorectomized mice treated with hormones as described in A. Dark-field exposure times were identical in all cases. al, alveoli; d, duct; st, adipose stroma.

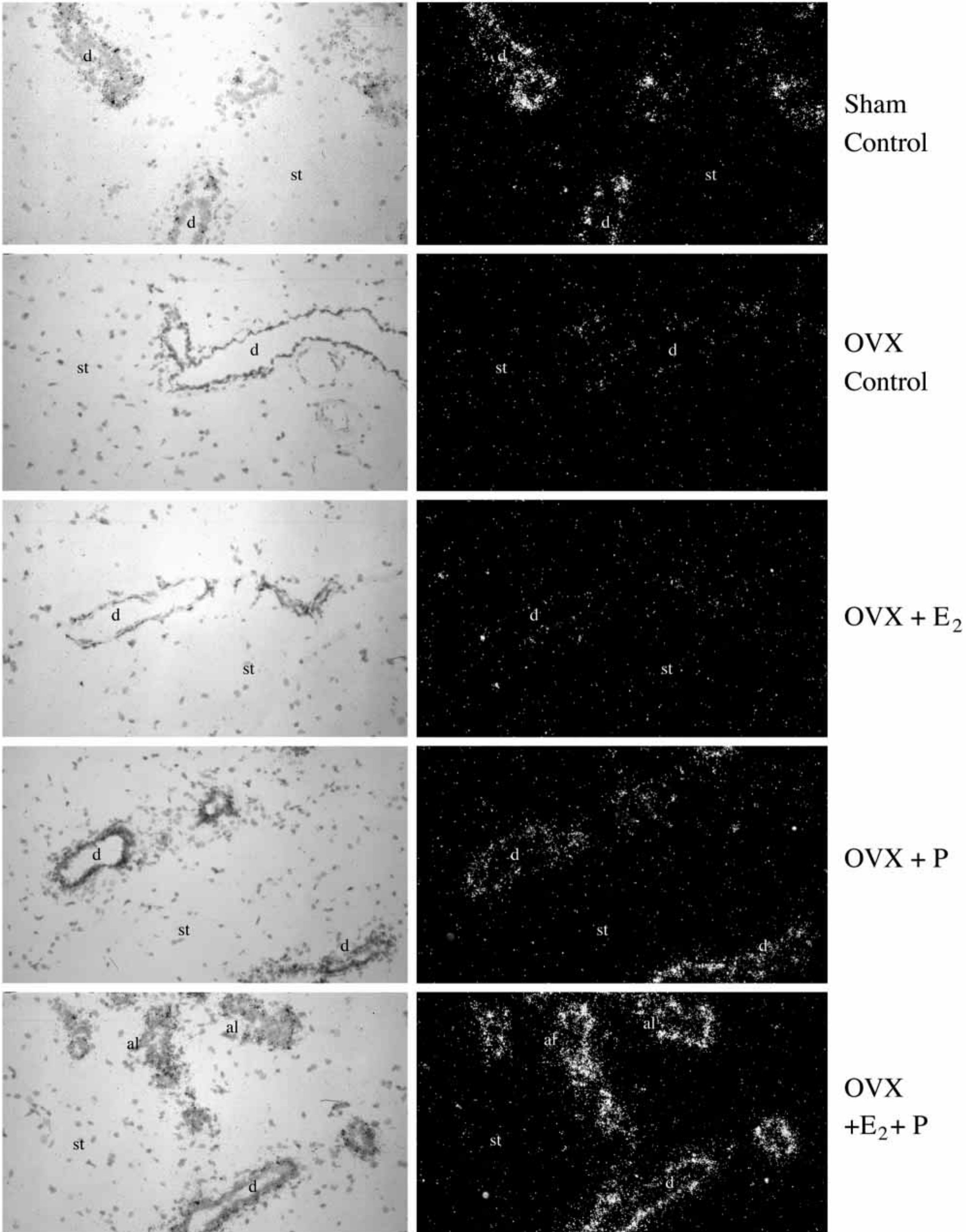


**Fig. 3D**

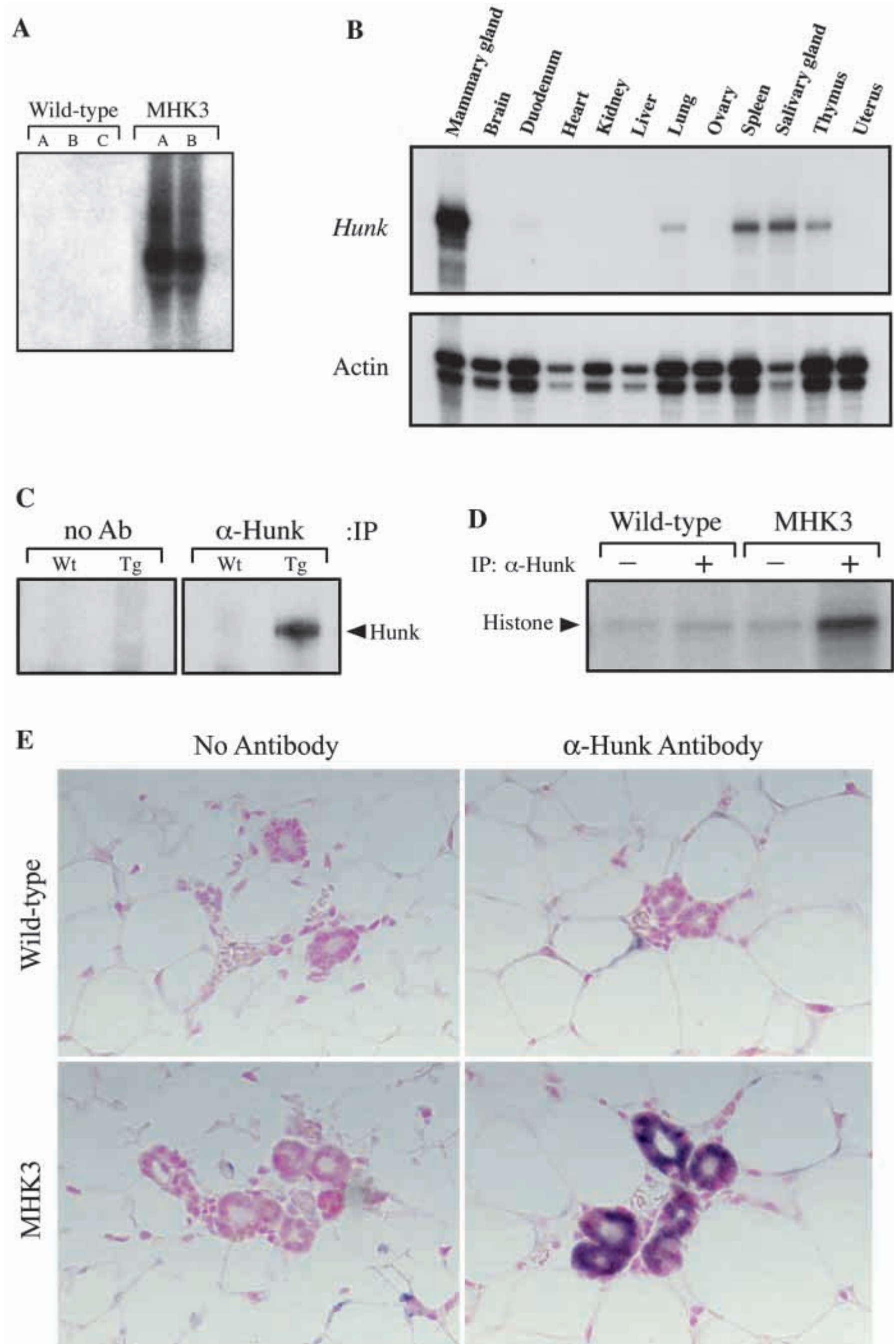


**Fig. 4.** MMTV-*Hunk* transgene expression in MHK3 transgenic mice. The MMTV LTR was used to generate transgenic mice misexpressing *Hunk* in a mammary-specific manner. (A) Northern hybridization analysis of MMTV-*Hunk* transgene expression in mammary glands from 7- to 9-week-old nulliparous wild-type or MHK3 transgenic mice using a $^{32}$P-labeled probe specific for *Hunk.* The detected mRNA transcript corresponds to the expected size of the MMTV-*Hunk* transgene. (B) RNase protection analysis of MMTV-*Hunk* transgene expression in organs from a 7-week-old nulliparous MHK3 transgenic female mouse. A $^{32}$P-labeled antisense RNA probe spanning the junction of the 3′ end of the *Hunk* cDNA and the 5′ end of the SV40 polyadenylation signal was used to specifically detect transgene expression in 20 μg of total RNA. A $^{32}$P-labeled antisense RNA probe for β-actin was used in the same reaction to control for RNA loading and sample processing. (C) Immunoprecipitation of Hunk protein from lactating MHK3 transgenic animals. Affinity-purified antisera raised against the C terminus of Hunk (α-Hunk) was incubated with 500 μg of protein extract prepared from mammary glands harvested from either MHK3 transgenic (Tg) or wild-type (Wt) mice during lactation. A control reaction was performed without antisera (no Ab). Immunoprecipitated protein was analyzed by immunoblotting using C terminal anti-Hunk antisera. The expected migration of Hunk is indicated. (D) In vitro kinase assay of anti-Hunk immunoprecipitates. Histone H1 was used as an in vitro kinase substrate for protein immunoprecipitated with (+) or without (–) anti-Hunk antisera from extracts containing equal amounts of protein as in (C). The relative migration of histone H1 is indicated. (E) Immunohistochemical analysis of Hunk protein expression in MHK3 transgenic mice. Anti-Hunk antisera from (C) and (D) above was used to detect Hunk protein in sections from paraffin-embedded mammary glands harvested from 14-week-old nulliparous wild-type or MHK3 transgenic females. A control assay was performed by omitting primary antisera from the protocol. Detection reaction times were identical in all cases.

the MMTV-*Hunk* transgene in the nulliparous state. In addition, MMTV-*Hunk* transgene expression levels in mammary glands from pregnant or lactating MHK3 animals were found to vary less than threefold relative to nulliparous MHK3 animals, a range of expression that is far less than that typically found in MMTV-based transgenic mouse models (data not shown). Together, these data indicate that MMTV-*Hunk* transgene expression is high relative to endogenous *Hunk* expression during all stages of postnatal mammary development.

To determine if *Hunk* mRNA levels in transgenic mice resulted in changes in Hunk protein levels, antisera specific to Hunk were used to analyze Hunk expression levels in extracts prepared from lactating mammary glands of MHK3 transgenic and wild-type mice (Fig. 4C; Gardner et al., 2000b). Western analysis of immunoprecipitated Hunk using Hunk-specific antisera revealed increased amounts of Hunk protein in extracts prepared from transgenic when compared with wild-type mammary glands (Fig. 4C). The inability to detect Hunk protein in extracts from wild-type lactating glands was consistent with the barely detectable levels of endogenous *Hunk* mRNA expression during this developmental stage (Fig.

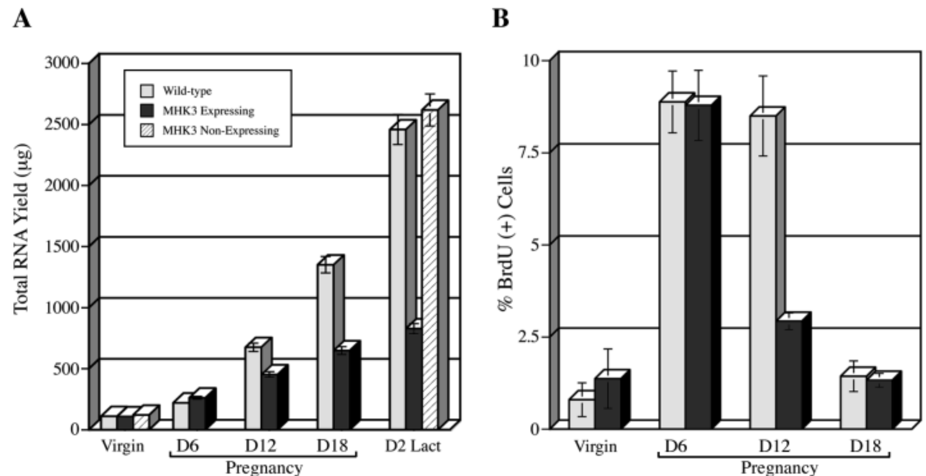


**Fig. 5.** Effect of Hunk overexpression on RNA content and mammary epithelial proliferation. (A) Amount of total RNA isolated from either wild-type (light-shaded boxes), expressing MHK3 transgenic (dark-shaded boxes), or non-expressing MHK3 transgenic (hatched boxes) female mice during mammary development. Total RNA was isolated from number 3 and number 5 mammary glands harvested from female mice at the indicated developmental timepoints. The average total RNA yield for each group is represented as the mean±s.e.m. At least three mice were analyzed from each group. A significant difference in RNA content was observed between wild-type and transgenic mammary glands at day 18.5 of pregnancy and day 2 of lactation (*t*-test, $P$=0.047 and 0.0007, respectively). (B) Relative percentage of BrdU-positive epithelial cells in the mammary glands of wild-type and MHK3 transgenic mice during development. Two hours before being killed, mice received injections of 50 µg BrdU per g total body weight. Following fixation and paraffin embedding, BrdU incorporation was detected using an anti-BrdU antibody followed by ABC detection method (Vector). The fraction of BrdU-positive and negative epithelial cells was determined by quantitative analysis using Phase 3 Imaging Software. At least four different fields per animal and three animals per timepoint were analyzed for BrdU incorporation. A significant difference in the fraction of BrdU-positive cells was observed between wild-type and transgenic mammary glands only at day 12.5 of pregnancy (*t*-test, $P$=0.004).

1). Conversely, MMTV-*Hunk* transgene expression was very high during lactation (data not shown).

In order to demonstrate that Hunk-associated kinase activity is also elevated in MHK3 transgenic animals, in vitro kinase assays were performed. Hunk was immunoprecipitated from protein extracts prepared from the lactating mammary glands of wild-type or transgenic mice as above (Fig. 4D). Control immunoprecipitation reactions were carried out in the absence of anti-Hunk antisera. The resulting immunoprecipitates were incubated with $\gamma$-$^{32}$P-ATP and histone H1. As predicted, based on the relative quantities of Hunk in these extracts, Hunk-associated kinase activity was substantially greater in immunoprecipitates prepared from transgenic when compared with wild-type mammary glands. These experiments confirm that MHK3 transgenic animals manifest increased levels of both Hunk protein and Hunk-associated kinase activity.

To investigate the spatial pattern of Hunk protein expression in MHK3 transgenic animals, immunohistochemistry was performed on mammary glands harvested from nulliparous transgenic and wild-type female mice (Fig. 4E). Consistent with high levels of MMTV-Hunk mRNA expression in MHK3 mice, this analysis revealed high levels of Hunk protein expression in transgenic compared with wild-type mammary glands. As described for other MMTV transgenic models, exogenously expressed Hunk was restricted to the epithelium of MHK3 mice. In addition, Hunk expression in the mammary epithelium of MHK3 animals was found to be relatively homogeneous, unlike the heterogeneous patterns of transgene expression observed in other MMTV transgenic models or the heterogeneous expression of endogenous *Hunk* mRNA. These data indicate that compared with wild-type animals, MHK3 transgenic animals overexpress Hunk in a mammary epithelial-specific and relatively homogenous manner.

Notably, some MHK3 transgenic animals did not express the MMTV-*Hunk* transgene. The presence of the MHK3-specific transgene integration site was confirmed by Southern hybridization analysis for all non-expressing MHK3 transgenic mice. A similar type of transgene silencing has been observed in other MMTV transgenic models (Betzl et al., 1996; Sternlicht et al., 1999).

## Hunk overexpression results in impaired lactation

Consonant with the hypothesis that Hunk plays a role in mammary gland development during pregnancy, we initially noted that the number of pups successfully reared by MHK3 transgenic females was significantly reduced compared with wild-type animals, with many pups dying within 1-2 days of birth independent of pup genotype. In contrast, offspring of transgenic males mated to wild-type females displayed survival rates comparable with those observed for offspring of wild-type crosses. These observations suggested that the inability to successfully rear pups was due to a defect in the ability of MHK3 transgenic females to lactate.

Consistent with the presence of a lactation defect in MHK3 mice, we initially noted that mammary glands from pregnant or lactating transgenic animals contained lower amounts of RNA compared with their wild-type counterparts. The amount of total RNA isolated from wild-type murine mammary glands is highly dependent upon developmental stage and can increase almost two orders of magnitude from the nulliparous state to the peak of lactation. The dramatic increase in RNA content during pregnancy and lactation is due to a combination of increased epithelial cell number and increased milk protein gene expression by individual alveolar epithelial cells. To confirm our initial observations regarding reduced RNA content in MHK3 mammary glands, we determined the yield of total RNA isolated from mammary glands harvested from either wild-type or MHK3 transgenic females during mammary development (Fig. 5A). As expected, in wild-type animals this analysis revealed an approximately 20-fold increase in RNA yield from lactating when compared with nulliparous mammary glands. In contrast, the increase in RNA yield over this developmental interval was significantly lower in MHK3 transgenic glands, with the difference between wild-

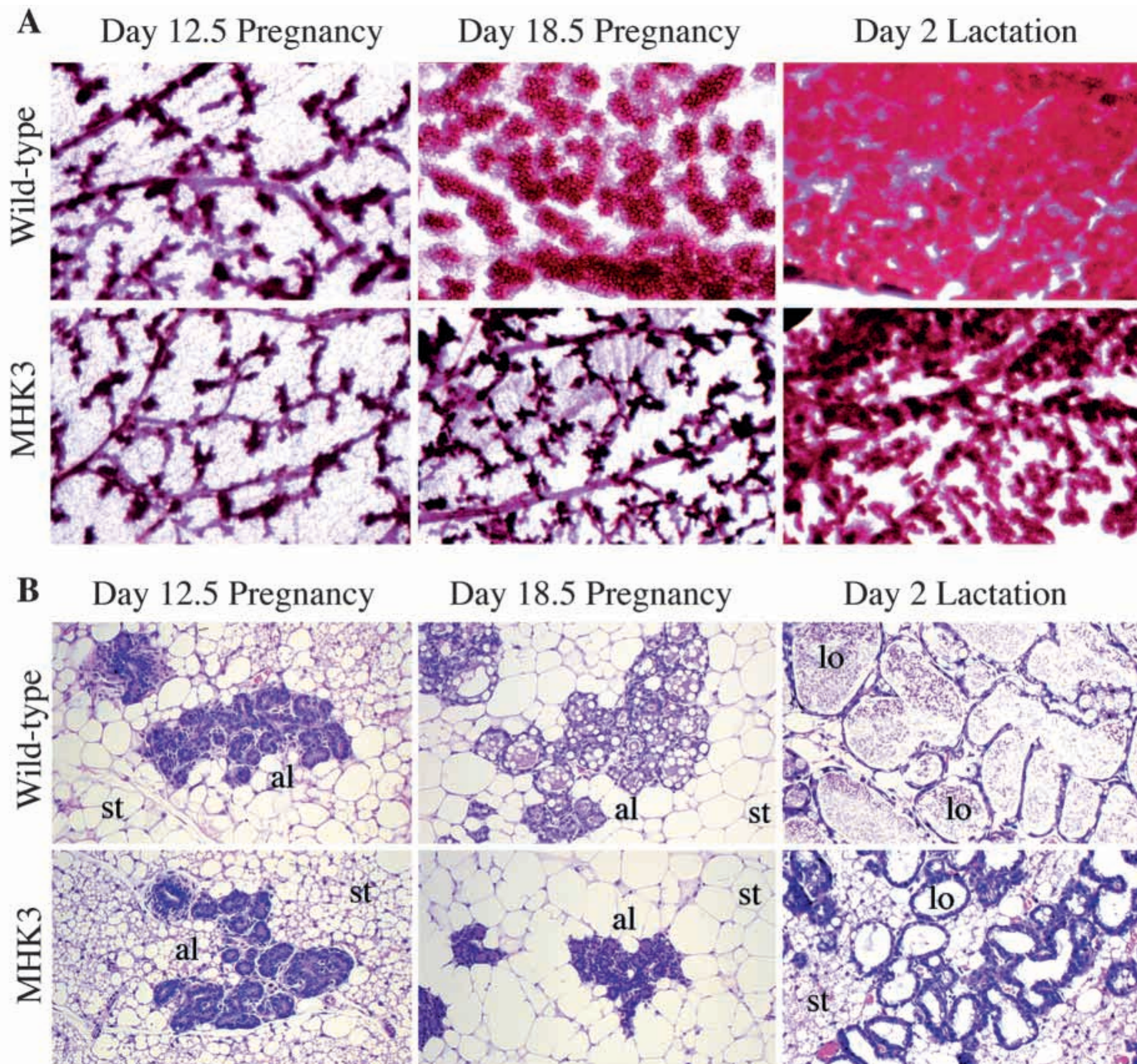


**Fig. 6.** Morphological defects in MHK3 transgenic mice during late pregnancy and lactation. Number four mammary glands from MHK3 transgenic and wild-type females were harvested at day 12.5 and day 18.5 of pregnancy, and day 2 of lactation. At least three transgene-expressing mice and three wild-type mice were analyzed for each timepoint. A representative photomicrograph is shown for each group. (A) Whole-mount analysis of transgenic and wild-type mammary glands at the indicated timepoints. Harvested glands were fixed and stained with Carmine dye in order to visualize epithelial ducts and alveoli. (B) Representative Hematoxylin and Eosin-stained sections of paraffin-embedded transgenic and wild-type mammary glands. al, alveoli; lo, lobule; st, adipose stroma.

type and transgenic glands becoming more pronounced towards late-pregnancy and lactation (wild-type versus transgenic, *t*-test $P$=0.047 (day 18.5 of pregnancy) and $P$=0.0007 (day 2 of lactation). In fact, at day 2 of lactation only one third of the total amount of RNA was isolated from transgenic when compared with wild-type glands. Non-expressing MHK3 transgenic females exhibited RNA yields indistinguishable from wild-type animals, indicating that the reduction in RNA observed in MHK3 animals was dependent upon expression of the *Hunk* transgene (Fig. 5A). These data suggest the possibility of impaired mammary development in MHK3 animals during pregnancy and lactation.

## Hunk overexpression decreases epithelial proliferation during mid-pregnancy

Lobuloalveolar development during pregnancy involves both proliferation and differentiation of alveolar epithelial cells. Alveolar cell proliferation occurs primarily during the first two trimesters of pregnancy, while alveolar differentiation occurs in a graded and progressive manner throughout pregnancy. To determine whether the decrease in RNA yield obtained from MHK3 transgenic glands during pregnancy is related to a decrease in cellular proliferation in these mice, we compared BrdU incorporation rates in epithelial cells from wild-type and transgenic mammary glands (Fig. 5B). Wild-type and MHK3 transgenic female mice at different developmental stages were pulse labeled with BrdU before sacrifice and the percentage of BrdU-positive epithelial cells was determined by quantitative analysis of anti-BrdU-stained sections. As predicted based upon the similar morphology of wild-type and transgenic mammary glands in nulliparous animals (data not shown), no significant difference in the percentage of BrdU-positive cells

was observed between wild-type and transgene-expressing mammary glands harvested from nulliparous animals. Moreover, a dramatic increase in epithelial proliferation was observed at day 6.5 of pregnancy both in wild-type and transgenic animals relative to nulliparous females. In contrast, at day 12.5 of pregnancy, epithelial proliferation rates remained high in wild-type glands but dropped markedly in glands from MHK3 animals (wild-type versus transgenic at day 12.5, *t*-test, $P$=0.004). By comparison, no differences in epithelial proliferation rates were observed between wild-type and transgenic glands at day 18.5 of pregnancy. Furthermore, no differences in apoptosis rates were observed between wild-type and MHK3 transgenic glands during virgin development, pregnancy or lactation, as evidenced by similar levels of TUNEL-positive cells (data not shown). Since MMTV-*Hunk* transgene expression levels in MHK3 animals are roughly comparable in the mammary gland throughout pregnancy and do not coincide with the observed defect in proliferation, we conclude that *Hunk* overexpression inhibits mammary epithelial proliferation specifically during mid-pregnancy.

## Hunk overexpression impairs lobuloalveolar development

Our finding of increased pup death among offspring of MHK3 females, together with the decreased RNA content of mammary glands from lactating MHK3 animals, suggested that MHK3 female glands may have a defect in lobuloalveolar development. To address this hypothesis directly, MHK3 transgenic females were sacrificed at different stages of pregnancy and lactation for morphological analysis. Analysis of both whole mounts and Hematoxylin and Eosin stained sections at day 6.5 and day 12.5 of pregnancy revealed no obvious morphological differences between the mammary glands of wild-type and MHK3 transgenic animals, despite the fact that epithelial cell proliferation is markedly impaired in MHK3 female mice at day 12.5 of pregnancy (Figs 5B and 6, and data not shown). In contrast, marked morphological differences were observed between wild-type and transgenic animals at day 18.5 of pregnancy. Analysis of whole mounts and Hematoxylin and Eosin stained sections at this stage of development consistently showed decreased lobuloalveolar development in MHK3 transgenic animals (Fig. 6). In addition to their larger size, alveoli in wild-type mice at day 18.5 of pregnancy contained copious amounts of lipid, whereas those of MHK3 mice did not.

In addition to the abnormalities observed at day 18.5 of pregnancy, decreased lobuloalveolar development was also observed in MHK3 females at day 2 of lactation. Normally during lactation the mammary gland is filled with casein-secreting lobules such that by whole-mount analysis the gland is entirely opaque, and by histological analysis no white adipose tissue is seen (Fig. 6). In contrast, lobuloalveolar units in lactating Hunk-overexpressing transgenic animals were smaller and appeared less developed by whole-mount analysis compared with wild-type and non-expressing MHK3 females (Fig. 6A and data not shown). Consequently, only half of the mammary fat pad of lactating MHK3 mice was occupied by secretory alveoli (Fig. 6B). While this may be due in part to decreased epithelial cell proliferation observed during mid-pregnancy, morphometric analysis of Hematoxylin and Eosin stained sections from MHK3 mice at day 18.5 of pregnancy and day 2 of lactation revealed that compared with their wild-type counterparts, the mammary glands of MHK3 animals consist of a normal number of alveoli that are uniformly smaller and less differentiated morphologically, rather than a smaller number of morphologically normal alveoli. (Fig. 6B and data not shown). Moreover, alveoli in lactating transgenic animals were less distended with milk when compared with wild-type glands. In contrast, similar analyses performed on the mammary glands of non-expressing MHK3 transgenic animals during lactation revealed no morphological defects (data not shown). These observations suggest that dysregulated expression of *Hunk* impairs terminal differentiation of the mammary gland during late pregnancy and lactation in a manner potentially distinct from the observed defect in epithelial proliferation.

## Hunk overexpression inhibits mammary epithelial differentiation

The dramatic changes in epithelial differentiation that occur in the mammary gland during lobuloalveolar development are reflected on a molecular level by the tightly regulated and temporally ordered expression of genes for milk proteins (Robinson et al., 1995). While steady-state mRNA levels for each of these genes typically increase throughout pregnancy, each gene undergoes a maximal increase in expression at a characteristic time during pregnancy. These differential expression profiles permit individual genes to be classified as early (β-casein), intermediate (κ-casein, lactoferrin), late-intermediate (WAP) or late (ε-casein) markers of mammary epithelial differentiation (Robinson et al., 1995; C. D'Cruz, unpublished). As such, the expression of these genes can be used as a molecular correlate for the extent of mammary epithelial differentiation. Accordingly, analysis of temporal expression patterns of milk protein genes permits the degree of lobuloalveolar differentiation to be reproducibly and objectively determined at the molecular level.

To confirm that the defect in lobuloalveolar development observed in MHK3 transgenic mice included a defect in differentiation, and was not simply a consequence of reduced epithelial cell numbers, we examined the expression of a panel of molecular differentiation markers in wild-type and MHK3 animals during lobuloalveolar development. We reasoned that if the defect in lobuloalveolar development was solely due to reduced epithelial cell mass, then the absolute level of expression of milk protein genes in MHK3 animals should be similar to that observed in wild-type animals when normalized for epithelial content. Similarly, if alveolar cells present in MHK3 glands differentiate normally during pregnancy, then the levels of expression of early, mid and late differentiation markers relative to each other should be similar to that observed in wild-type animals. As such, the observation that the absolute levels of expression of multiple differentiation markers are reduced despite normalizing for epithelial content, or that the expression of these differentiation markers relative to each other is altered compared to wild-type animals, would indicate that mammary epithelial differentiation is impaired in MHK3 animals and is independent of the observed proliferation defect.

To determine whether MHK3 animals manifest a defect in differentiation in addition to the defect in proliferation demonstrated above, we determined mRNA expression levels

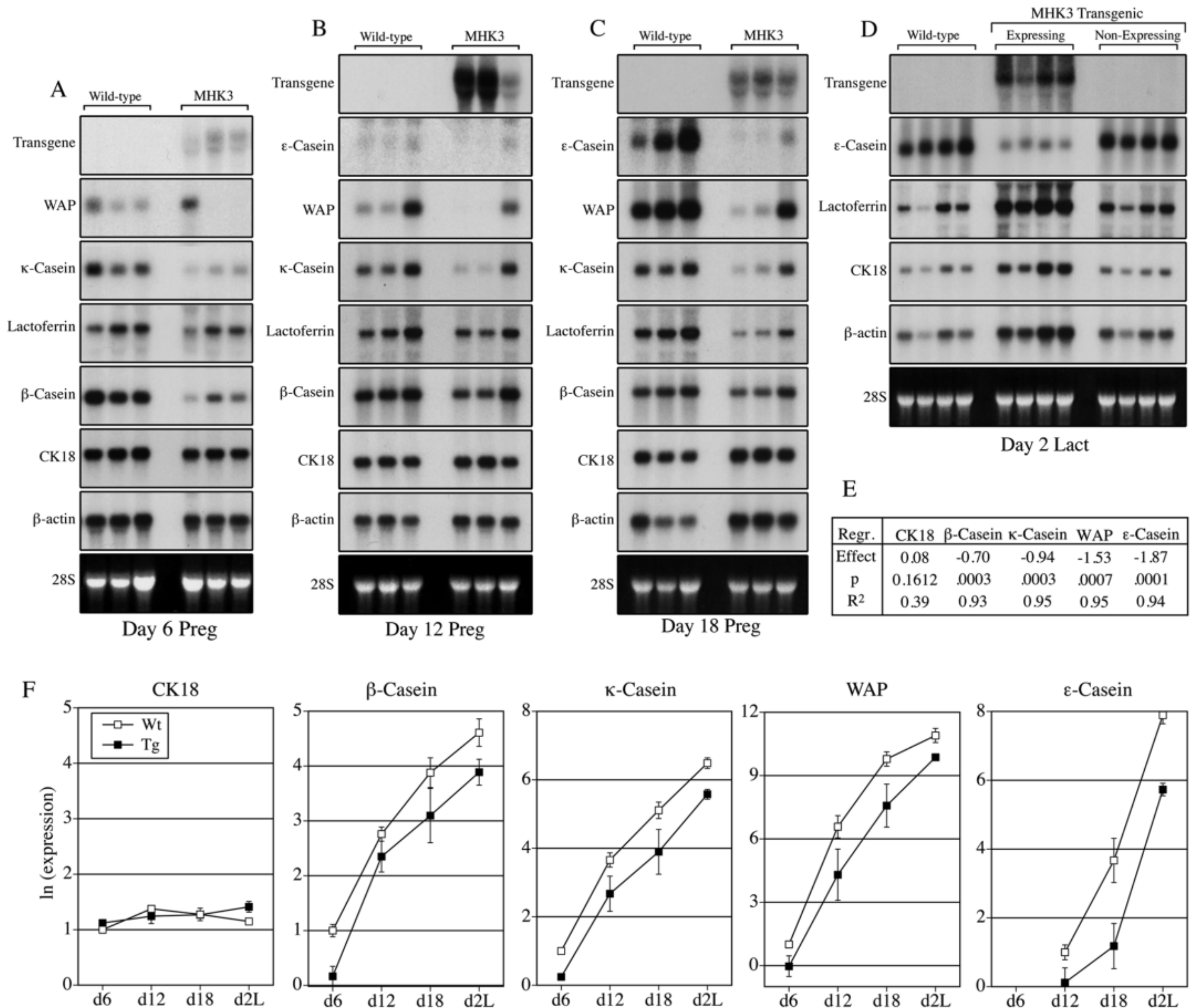


**Fig. 7.** Differentiation defects in MHK3 transgenic mice during pregnancy and lactation. (A-D) Northern analysis of gene expression for epithelial differentiation markers (β-casein, κ-casein, lactoferrin, WAP and ε-casein) in the mammary glands of wild-type or MHK3 transgene-expressing animals at day 6.5 of pregnancy (A), day 12.5 of pregnancy (B), day 18.5 of pregnancy (C) or at day 2 of lactation (D). Differentiation marker expression in the mammary glands of non-expressing MHK3 transgenic animals is also shown in D. β-actin expression is shown as a control for dilutional effects and the 28S ribosomal RNA band is shown as a loading control. (E) Multivariate regression analysis of (A-D) demonstrating the effects of transgene expression and developmental stage on the natural logarithm of cytokeratin 18 and expression levels of milk protein genes. All expression levels were normalized to β-actin. The average effect of transgene expression (Effect) on the expression of each milk protein gene is represented as the natural logarithm of the average fold-difference between transgenic and wild-type values. The respective *P* value (significance of transgene effect) is shown for each milk protein gene. Note that transgene expression has no effect on cytokeratin 18 expression and results in an average decrease in the expression levels of differentiation markers ranging from 2.0-fold (β-casein) to 6.5-fold (ε-casein). The $R^2$ value represents the degree to which the difference in the observed data from the null hypothesis is due to transgene expression. The *P* value for the significance of the regression model was < 0.01 for all differentiation markers shown.
(F) Phosphorimager quantitation of northern analyses in A-D. Expression levels of milk protein genes were normalized to β-actin expression and are shown on a logarithmic scale in arbitrary units relative to expression levels first detected in wild-type animals. Values are shown as the mean±s.e.m. for each point. The number of mice analyzed in each group is: 4 Wt, 5 Tg (d6.5); 3 Wt, 3 Tg (d12.5 and d18.5); and 4 Wt, 4 Tg, 4 non-expressing Tg (d2 Lact).

for a panel of early, intermediate and late markers of mammary epithelial differentiation in mammary glands from transgenic and wild-type animals during pregnancy and lactation (Fig. 7). Although few if any morphological differences were noted in transgenic mice before day 18.5 of pregnancy, when normalized to β-actin expression, steady-state levels of expression for all five milk protein genes were reduced in mammary glands from MHK3 transgenic mice compared with

wild-type mice beginning as early as day 6.5 of pregnancy and persisting throughout pregnancy and into lactation (Fig. 7). In contrast, expression levels of the epithelial cell marker, cytokeratin 18, did not differ significantly between wild-type and transgenic glands at any stage of pregnancy or lactation when normalized to β-actin expression (Fig. 7).

Accurate interpretation of gene expression levels in the mammary glands of mice bearing defects in lobuloalveolar development requires normalization to reference genes, such as β-actin, in order to control for differences in dilutional effects cause by expression of milk protein genes. Although β-actin levels do not change significantly on a per cell basis during pregnancy and lactation, the enormous contribution of the expression of milk protein genes to the total RNA pool results in an apparent decrease in the expression of reference genes when comparing equal amounts of total RNA (Figs 1 and 7). The magnitude of this dilutional effect correlates with the differentiation state of the mammary gland. Thus, the lower levels of expression of milk protein genes observed in the less differentiated MHK3 glands results in a less severe dilutional effect and apparent increases in β-actin and cytokeratin 18 expression in the mammary glands of MHK3 animals compared with wild-type animals at day 18 of pregnancy and day 2 of lactation. Therefore, in aggregate our findings indicate that the reduced expression of differentiation markers in MHK3 animals during pregnancy and lactation is not simply due to a reduction in epithelial cell content and suggests that mammary glands from *Hunk*-overexpressing transgenic mice are less differentiated than wild-type glands at each stage of lobuloalveolar development.

As further controls for these experiments, expression of milk protein genes was analyzed in non-expressing MHK3 transgenic females at day 2 of lactation (Figs 7 and 8, and data not shown). No differences in the expression either of cytokeratin 18 or of alveolar differentiation markers were observed between non-expressing MHK3 glands and glands from wild-type mice, consistent with the lack of morphological or functional defects in non-expressing MHK3 glands. Together, these findings strongly suggest that the abnormalities in mammary epithelial differentiation observed in MHK3 mice are due to MMTV-*Hunk* transgene expression rather than to site-specific integration effects such as the insertional disruption of an endogenous gene.

To analyze further the impact of MMTV-*Hunk* transgene expression on lobuloalveolar development, a multivariate regression analysis was performed on the above normalized gene expression data to quantitate the effects of transgene expression on mammary epithelial differentiation during a developmental interval from day 6.5 of pregnancy to day 2 of lactation (Fig. 7E,F). This analysis revealed that the expression of four epithelial differentiation markers (β-casein, κ-casein, WAP and ε-casein) was significantly lower in the mammary glands of transgenic animals compared with wild-type animals across all developmental time points. No differences were observed in cytokeratin 18 expression between wild-type and transgenic glands, confirming that normalization to β-actin expression was sufficient to control for differences in epithelial cell content. These results indicate that the mammary glands of MHK3 animals are significantly less differentiated than wild-type glands throughout pregnancy and into lactation.

Interestingly, the average reductions in mRNA expression levels observed for the late differentiation marker, ε-casein (Tg effect=–1.87; 6.5-fold), and the late-intermediate differentiation marker, WAP (Tg effect=–1.53; 4.6-fold), were considerably more pronounced than the reductions in expression observed for the early differentiation marker, β-casein (Tg effect=–0.70; 2.0-fold), and the intermediate differentiation marker, κ-casein (Tg effect=–0.94; 2.6-fold) (Fig. 7). The observation that transgene expression had a greater effect on the expression of late differentiation markers compared with early differentiation markers suggests that late events in mammary epithelial differentiation are disproportionately affected during lobuloalveolar development in MHK3 mice. This finding is consistent with the morphological defects observed in these mice during late pregnancy.

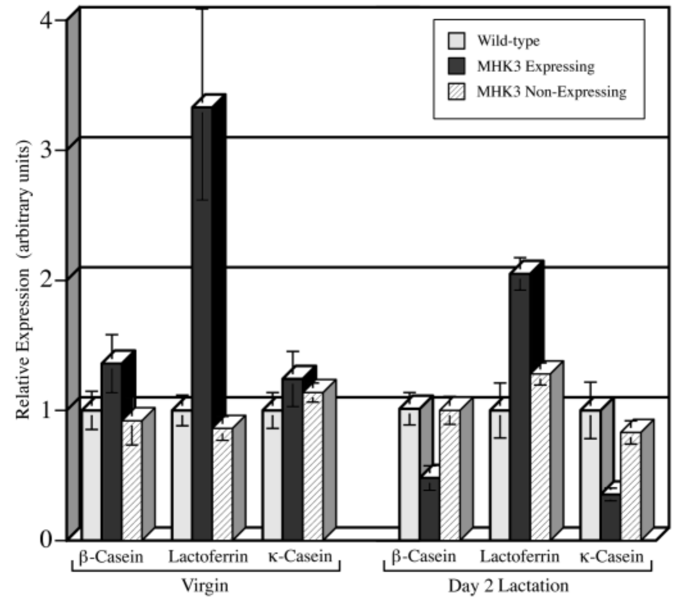


**Fig. 8.** Upregulation of lactoferrin expression at specific developmental stages in MHK3 mammary glands. Analysis of differentiation marker expression in mammary glands from either wild-type (light-shaded boxes), MHK3 transgene-expressing (dark-shaded boxes) or non-expressing MHK3 transgenic (hatched boxes) female mice during puberty or day 2 of lactation, as described in Fig. 7. Sample sizes were 16, 10 and 8, respectively for adolescent mice and 4 animals per group for lactation points. Northern hybridization analysis and quantitation was performed on 3 μg (virgin) or 5 μg (day 2 lactation) of total RNA isolated from mammary glands using $^{32}$P-labeled cDNA probes specific for milk protein genes as indicated. Expression of these genes was normalized to that of β-actin. Wild-type expression values were set to 1.0 and are represented as the mean±s.e.m. for each group.

## Hunk upregulates lactoferrin expression in MHK3 mice

Surprisingly, while the expression of all 5 epithelial differentiation markers examined was reduced in the mammary glands of MHK3 transgenic animals throughout pregnancy, expression of the gene for lactoferrin was actually higher in transgenic animals compared with wild-type animals at day 2 of lactation (Figs 7D and 8). This finding prompted us to analyze the impact of Hunk overexpression on lactoferrin

expression in nulliparous MHK3 mice. Consistent with results obtained in lactating MHK3 animals, steady-state levels of lactoferrin mRNA were significantly higher in the mammary glands of nulliparous MHK3 expressing transgenic animals compared with either non-expressing MHK3 transgenic animals or age-matched nulliparous wild-type animals (Fig. 8). In contrast to lactoferrin, mRNA expression levels of the epithelial differentiation markers, β-casein, κ-casein, α-lactalbumin (Lalba – Mouse Genome Informatics), WDNM1 (Expi – Mouse Genome Informatics) and WAP, in adolescent nulliparous females were not significantly affected by *Hunk* overexpression (Fig. 8 and data not shown). Consistent with this finding, the rate of ductal elongation and extent of epithelial side-branching in mammary glands from 5- to 6-week-old nulliparous transgenic mice was comparable with that observed in wild-type mice as analyzed by whole-mount and histological analysis (data not shown). These observations suggest that Hunk does not cause precocious differentiation of the mammary gland during puberty, but may specifically activate pathways resulting in *lactoferrin* upregulation. Similarly, the observation that lactoferrin expression is upregulated in the mammary glands of lactating MHK3 animals, despite the global inhibitory effect of Hunk overexpression on mammary epithelial differentiation during late pregnancy and lactation, suggests that the effects of Hunk on lactoferrin expression are distinct from those on mammary epithelial differentiation.

## DISCUSSION

The data presented in this report demonstrate that expression of the novel SNF1-related serine/threonine kinase, *Hunk*, in the mammary gland is: tightly regulated during mammary development with a transient peak during early pregnancy; rapidly and synergistically induced in response to estradiol and progesterone; and spatially restricted within a subset of mammary epithelial cells throughout postnatal development. These data suggest a role for Hunk in mammary development, particularly with respect to pregnancy-induced changes in the mammary gland. Consistent with this hypothesis, misexpression of Hunk in the mammary gland disrupts normal lobuloalveolar development during pregnancy and lactation. Specifically, dysregulated Hunk expression results in decreased epithelial cell proliferation exclusively during mid-pregnancy as well as impaired alveolar cell differentiation throughout pregnancy and lactation. Together, these data suggest that Hunk may contribute to pregnancy-induced changes in the mammary gland, and that Hunk may play a role in the response of the mammary epithelium to ovarian hormones.

Although *Hunk* mRNA expression levels are markedly upregulated during early pregnancy, a developmental stage that is characterized by rapid alveolar cell proliferation, multiple lines of evidence suggest that *Hunk* expression is not simply a correlate of proliferation. For instance, the temporal profile of *Hunk* expression in the mammary gland during development is distinct from that of bona fide markers of proliferation such as cyclin A, cyclin D1, PCNA and PLK (Chodosh et al., 2000; Master et al., unpublished). Specifically, the upregulation of *Hunk* expression in the mammary gland is confined to early pregnancy, whereas the above proliferation markers are not only upregulated during early pregnancy, but also during mid-pregnancy as well as puberty. Moreover, *Hunk* is not preferentially expressed in proliferative as compared to non-proliferative compartments in the mammary gland (i.e. terminal end buds versus ducts during puberty, alveoli versus ducts during early pregnancy). Finally, analysis of actively growing versus confluent or serum-starved mammary epithelial cells reveals no difference in *Hunk* mRNA levels (H. P. G., unpublished). These observations suggest that *Hunk* expression does not simply reflect the proliferative state of the mammary epithelium, but rather may reflect other developmental pathways or events in the mammary gland.

*Hunk* upregulation in the mammary gland during early pregnancy is transient. This observation raises the intriguing possibility that the tightly regulated pattern of *Hunk* expression during pregnancy is required for normal lobuloalveolar development. We have tested this hypothesis by misexpressing *Hunk* in the mammary glands of transgenic mice. Forced overexpression of an MMTV-*Hunk* transgene in the mammary epithelium throughout postnatal development results in a defect in lobuloalveolar development with molecular abnormalities first discernible during early pregnancy, cellular abnormalities discernible during mid-pregnancy and morphological abnormalities discernible late in pregnancy. Specifically, Hunk overexpression results in a defect in epithelial proliferation that is restricted to mid-pregnancy and a defect in differentiation that is manifest throughout the developmental interval spanning day 6.5 of pregnancy to day 2 of lactation. In contrast, forced overexpression of *Hunk* in nulliparous animals has no obvious effect on patterns of proliferation or differentiation, or on the morphology of the mammary epithelial tree. Together, our findings suggest the possibility that the defects observed in lobuloalveolar development in MHK3 mice are due to the failure to downregulate Hunk expression during mid-pregnancy, rather than to Hunk overexpression per se.

Our finding that Hunk overexpression inhibits alveolar proliferation during mid-pregnancy was surprising given that *Hunk* is normally upregulated in the mammary gland during early pregnancy – the stage of pregnancy associated with maximum alveolar proliferation. This suggests either that the normal role of Hunk may be to negatively regulate mammary epithelial proliferation during pregnancy, or that the inhibitory effect of Hunk on proliferation at day 12.5 of pregnancy is a consequence of overexpression during a developmental stage at which Hunk is normally downregulated. Alternatively, the developmental profile of endogenous Hunk activity may be different from that of steady-state levels of *Hunk* mRNA. Interestingly, the human SNF1-related kinase, C-TAK1/KP78/MARK3, has been shown to phosphorylate and inactivate Cdc25c, thereby preventing activation of Cdc2 and presumably inhibiting entry of cells into mitosis (Peng et al., 1997, 1998). Consistent with this hypothesis, expression of KP78 protein has been reported to be downregulated in adenocarcinomas of the pancreas (Parsa, 1988). Thus, there is precedent for the negative regulation of cellular proliferation by mammalian SNF1 family members. Whether Hunk interacts with Cdc25c, negatively regulates cellular proliferation when expressed at physiological levels or is altered in human malignancies is unknown.

We have demonstrated defects in both mammary epithelial

proliferation and differentiation in MHK3 animals during pregnancy. For example, the lower total RNA yield obtained from transgenic glands as compared with wild-type glands during late pregnancy and lactation probably reflects, in part, the reduced epithelial cell content of MHK3 transgenic glands, since the increase in total RNA present in the mammary gland during lobuloalveolar development is a result both of increases in epithelial cell number and increases in expression of milk protein genes on a per-cell basis (Fig. 5B). As such, we initially considered the possibility that the decreased expression of markers for mammary epithelial differentiation observed in MHK3 animals during pregnancy and lactation is a consequence of the decreased alveolar proliferation evident in MHK3 mice at day 12.5 of pregnancy, and the resulting decrease in epithelial cell mass. However, several lines of evidence indicate that the abnormalities in mammary epithelial differentiation that we have described in MHK3 animals cannot be explained by a decrease in epithelial cell mass. First, the fact that defects in alveolar differentiation in MHK3 animals actually precede the reduction in epithelial proliferation that occurs at day 12.5 strongly argues that defects in differentiation cannot solely be a consequence of defects in proliferation. In addition, RNA extracted from a mammary gland composed of a smaller number of appropriately differentiated epithelial cells would be predicted to give rise to a normal distribution of milk protein gene expression (i.e. early versus late), and to normal levels of expression of milk protein genes when normalized to epithelial cell content. In contrast, our observations indicate that both the level and the composition of milk protein RNA produced by the mammary glands of MHK3 animals during pregnancy and lactation is abnormal even after controlling for differences in epithelial content between wild-type and transgenic glands. Consistent with this conclusion, the morphology of the alveolar epithelial cells present in the mammary glands of MHK3 animals at day 18.5 of pregnancy is less differentiated compared with those present in their wild-type counterparts. Thus, we conclude that the reduced expression of differentiation markers in MHK3 transgenic glands reflects the less differentiated state of the mammary epithelial cells present, rather than a reduced number of appropriately differentiated mammary epithelial cells. As such, our data indicate that the defects in differentiation that occur in MHK3 animals as a consequence of Hunk overexpression are separable from and, at least in part, independent of the defects in proliferation that occur in these animals.

With regard to the analysis presented in this manuscript, it is important to note that during pregnancy and lactation, a similar magnitude of reduction in the expression of differentiation markers was observed in the mammary glands of MHK3 animals compared with wild-type animals regardless of whether levels of expression of milk protein genes were normalized to β-actin or to the epithelial cell marker, cytokeratin 18 (Fig. 7 and data not shown). That is, when normalized to β-actin expression, cytokeratin 18 expression levels do not differ between MHK3 transgenic animals and wild-type animals at any stage of lobuloalveolar development. Presumably, this reflects the fact that mammary epithelial cells contribute the vast majority of RNA to the total RNA pool during pregnancy and lactation, an observation that explains why cytokeratin 18 levels show little change during pregnancy when normalized to β-actin expression. Thus, normalizing mRNA expression levels to β-actin mRNA levels itself effectively controls for the decreases in epithelial cell content that occur in MHK3 animals.

Surprisingly, lactoferrin expression in the mammary glands of both nulliparous and lactating mice was elevated in Hunk-overexpressing MHK3 animals compared either with wild-type animals or with non-expressing MHK3 transgenic animals. This observation suggests that while Hunk overexpression may inhibit mammary epithelial differentiation globally during pregnancy and lactation, the effects of Hunk overexpression on lactoferrin expression may be more specific. In support of this hypothesis, we have compared gene expression patterns in wild-type and MHK3 nulliparous transgenic glands using oligonucleotide-based cDNA microarrays. These microarray studies revealed that of the approx. 5500 genes analyzed, the gene for lactoferrin is one of only 16 genes whose expression changes by more than 2.5-fold in transgenic when compared with wild-type glands (H. P. G., unpublished). As noted above, the mammary glands of nulliparous MHK3 animals are morphologically indistinguishable from those of wild-type littermates. Thus, our findings indicate that the effects of Hunk overexpression on lactoferrin gene regulation are relatively specific, and are unlikely to be secondary to marked abnormalities in mammary gland morphology or to global changes in gene expression. Since lactoferrin expression in several tissues has been shown to be regulated by 17β-estradiol, EGF, protein kinase C and cAMP-mediated pathways (Teng, 1995), the specific upregulation of lactoferrin expression in MHK3 transgenic mice may provide a clue to signal transduction pathways in which Hunk may be involved.

*Hunk* is expressed in a heterogeneous, epithelial-specific manner throughout postnatal mammary development. This heterogeneous expression pattern is particularly striking in the terminal end bud during puberty and throughout the mammary epithelium during pregnancy, and suggests that *Hunk* may be a marker for a previously undescribed subtype of mammary epithelial cell or may be a marker for a particular cellular state. An intriguing hypothesis regarding the heterogeneous pattern of *Hunk* expression in both developing alveolar buds and epithelial ducts during pregnancy is that this heterogeneity may reflect the differing ability of mammary epithelial cells to respond to ovarian hormones. Testing this hypothesis will require the ability to colocalize the expression of endogenous Hunk protein with steroid hormone receptors and other markers of hormone responsiveness.

Finally, we have demonstrated that treatment of mice with 17β-estradiol and progesterone results in the rapid and synergistic upregulation of *Hunk* expression in the mammary gland. These findings suggest that the upregulation of *Hunk* expression in response to hormones is not a consequence of the marked changes in epithelial differentiation or epithelial cell number that occur either during early pregnancy or in response to the chronic administration of 17β-estradiol and progesterone. Interestingly, unlike the effect of steroid hormones on *Hunk* expression in the mammary gland, treatment of mice with 17β-estradiol either alone or in combination with progesterone results in downregulation of *Hunk* expression in the uterus. The opposing effects of combined estradiol and progesterone treatment on *Hunk* expression in the mammary gland and uterus is reminiscent

of the dichotomous effects of these hormones on epithelial proliferation in these tissues. As such, our finding that the effect of steroid hormones on *Hunk* expression in the mammary gland and uterus parallels the dichotomous response of these tissues to estradiol and progesterone provides additional support for the hypothesis that Hunk may be a downstream effector of estrogen and progesterone, and suggests a potential explanation for the dichotomous response of these tissues to steroid hormones.

In aggregate, our findings raise the possibility that the upregulation of *Hunk* expression in the mammary gland by estradiol and progesterone may contribute to changes in the mammary epithelium that occur during pregnancy by mediating the effects of steroid hormones. Ultimately, elucidating the mechanisms by which hormones regulate *Hunk* expression, and by which Hunk may regulate mammary epithelial proliferation and differentiation, may yield insights into the complex role which hormones play in mammary development and carcinogenesis.

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