Targeted Expression of GLI1 in the Mammary Gland Disrupts Pregnancy-induced Maturation and Causes Lactation Failure*

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The Hedgehog signaling pathway regulates the development and function of numerous tissues and when mis-regulated causes tumorigenesis. To assess the role of a deregulated Hedgehog signaling pathway in the mammary gland we targeted the expression of the Hedgehog effector protein, GLI1, to mammary epithelial cells using a bigenic inducible system. A constitutively active Hedgehog signaling pathway resulted with 100% penetrance in an undifferentiated mammary lobuloalveolar network during pregnancy. GLI1-expressing transgenic females were unable to lactate and milk protein gene expression was essentially absent. The inability to lactate was permanent and independent of continued GLI1 transgene expression. An increased expression of the GLI1 response gene Snail coupled to reduced expression of E-cadherin and STAT5 in the transgenic mammary gland provides a likely molecular explanation, underlying the observed phenotypic changes. In addition, remodeling of the mammary gland after parturition was impaired and expression of GLI1 was associated with accumulation of cellular debris in the mammary ducts during involution, indicating a defect in the clearance of dead cells. Areas with highly proliferative epithelial cells were observed in mammary glands with induced expression of GLI1. Within such areas an increased frequency of cells expressing nuclear Cyclin D1 was observed. Taken together the data support the notion that correct regulation of Hedgehog signaling within the epithelial cell compartment is critical for pregnancy-induced mammary gland development and remodeling.
entire mammary fat pad. During pregnancy, hormonal stimulation alters the morphology of the mammary gland from a ductal to a more lobuloalveolar morphology via branching morphogenesis. At this point, lobuloalveolar progenitor cells in the ducts proliferate and form alveolar buds, which further differentiate into alveoli. After parturition, upon suckling, milk is released until suckling ceases and the gland undergoes involution. During this process most alveolar cells are committed to apoptosis as part of the gland remodeling process. However, a small portion of fully committed alveolar cells escape the involution process and function as alveolar progenitors during subsequent pregnancies (18).

The Hh homologues Shh, Ihh, and Dhh are expressed in the mouse mammary gland (19, 20). Most likely, Ihh and Shh have redundant functions in mammary gland development since the embryonic mammary gland develops normally in Shh and Ihh knock-out mice (21, 22). However, it is interesting to note that Ihh expression is regulated by progesterone, one of the key steroid hormones controlling ductal and alveolar development in the mammary gland (23–25). The core components (Shh, Ihh, Dhh, Ptch1, Smo, Gli1, Gli2, Gli3) of the Hh signaling pathway have been reported to be expressed during essentially all stages of mammary gland development (13, 15, 19). Expression of Hh pathway components varies during the different phases of mammary gland development and is progressively elevated during pregnancy and a peak of expression is detected during lactation (13, 14, 19). Furthermore, the Hedgehog signaling components Ptch1, GLI1, and GLI2 are expressed in normal human mammary progenitor cells (26).

When it comes to studies of the role of Hh signaling during mammary gland development a complication resides in the fact that homozygous null mutations of several key network genes, including Shh, Ihh, Ptch1, Smo, Gli1, Gli2, and Gli3 are embryonic or perinatally lethal. However, hyperplastic changes appear in mammary glands from virgin heterozygous Ptch1+/- mice, in mice expressing constitutively active Smo in the mammary epithelium, in embryonic mammary transplants from Gli2 null mice, and in humanized fat pads of NOD-SCID mice transplanted with Gli2 overexpressing mammosphere initiating cells (14–16, 26). Furthermore, Gli33ex/3ex mutants lack two pairs of mammary buds, which supports a role for Hh signaling during embryonic mammary gland development. In addition, loss of Gli3 expression induces Gli1 mis-expression in the mammary mesenchyme supporting the conclusion that during embryonic mammary gland formation the primary role for Gli3 is to repress Hh inducible target genes (13).

In this report, we show that targeted expression of GLI1 in the mammary epithelial cell compartment results in a reduced mammary epithelial network and alveologenesis during pregnancy. Transgenic female mice are unable to lactate and on a molecular level, the lack of terminal development and differentiation is reflected by impaired milk (WAP, α-lactalbumin, and β-casein) gene expression. We propose that the effects observed are due to GLI1 induction of Snail coupled to reduced expression of E-cadherin and STAT5, which results in a reduced alveolar differentiation. Strikingly, in subsequent pregnancies, without induced GLI1 expression, the glands do not fully develop and the dams fail to lactate, showing profound and long lasting effects of a temporally restricted GLI1 expression. Moreover, the involution process is impaired in GLI1-induced mammary glands, which may explain the sustained defects in the multiparous mice with induced GLI1 expression only during the first pregnancy. In addition, an increase of Cyclin D1 expression and epithelial cell proliferation rate was observed, which frequently was associated with the formation of expanded clusters of epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice and Genotyping—**A tetracycline-regulated GLI1 transgenic construct was made by subcloning a PCR amplified tetracycline responsive promoter consisting of five tetracycline (tet) responsive elements (TRE) and the minimal cytomegalovirus promoter region of pTRELuc (Clontech Inc.) in front of the human full-length GLI1 (human and mouse Gli1 show 85% similarity at the amino acid level). This construct was subcloned into the Sall/BamI-digested Bluescript KS(−) vector (pBSpa) harboring a 0.6-kb fragment of a rabbit β-globin intron and a 1.1-kb fragment containing two 3'-poly(A) signal sequences from the p5′BKSI plasmid, obtained from Dr. Jose Jorcano (CIEMAT, Madrid, Spain). The 3.6-kb fragment of GLI1 cDNA was cleaved with NheI/SnaBI and introduced into the corresponding site of the TRE/pBSA plasmid. The construct was verified by sequencing and tested for its tetracycline inducibility in a reporter-based assay in a 293TetTOn cell line purchased from Clontech. The 5.3-kb transgene was isolated by digestion with NotI/Sall and injected into pronuclei of fertilized [C57BL/6J × CBA] F2 oocytes. The putative founder transgenic mice were genotyped by PCR amplification of sequences specific for the TREGLI1 using tail genomic DNA and primers TREGLI1: R1 primer (5′-CGGTGTCCTTCTATGGAGTCAA-3′), F1 primer (5′-ACCCGGGTGAGTACGGCTGTA-3′). Founders were crossed with negative littermates to establish the F1 generation. The level of expression of GLI1 was studied in five transgenic lines by crossing TREGLI1 F1 mice with KsrtTA F1 mice, which carry a mutated form of the reverse tet activator gene downstream of the skin-specific keratin 5 (K5) promoter (27). As a target line for studies in breast, one of the three TREGLI1 F1 lines that exhibited similar GLI1 expression levels in the skin and profound phenotype was chosen. This TREGLI1 F1 line was crossed with mice carrying the MMTVrtTA promoter upstream of the tet activator gene (28) and the offspring were used for the experiments. Doxycycline (2 mg/ml) and 5% sucrose was added to the drinking water and doxycycline treatment was continued until the animal was sacrificed if not stated otherwise. The bistranergic mice were hemizygous for each transgene and compared with non-treated bigenic animals or WT-treated siblings. Genotyping of mice was performed by PCR and primers for TREGLI1 were described above and for MMTVrtTA: pA primer (5′-ATCCGCACCTTGTGACTCCTG-3′) and pB primer (5′-GGCTAT-CAACAAAACACTGGAC-3′). All transgenic mice were generated within an SPF barrier facility according to local and national regulations, and experimental conditions were approved by the Stockholm South Animal Ethics Committee.
Reverse Transcription-PCR—RNA was isolated using RNAzolB (CRL Inc.) and random hexamer-primed complementary DNA (cDNA) was generated using the reverse transcription system (Promega). Primer pairs specific for Pthch1 (5’-GAATCCAGGCTATCCACACC-3’), Pthch1.B (5’-CCAAGCT-CTGAGCTCAATG-3’), Gli1 (Gli1.a.F 5’-AGACCAGCAG-CGTGACCTGAA-3’), Gli1.b.B (5’-TGGCAGGGTGCACTG-GTC-3’), rtTA (rtTA-404.F 5’-TGACCTCTCATAAGAGA-CACC-3’), Gli1-474r (5’-GGGGCCTTTTTTGTGATTTCA-3’), and Actin (actin.F 5’-GACAGGATGCAAGAGGAT-3’, actin.B 5’-TTGGCTGATCACCACCTGT-GT-3’) were used for analysis of mRNA levels. All experiments were independently repeated at least three times and no amplification was obtained without reverse transcriptase (data not shown). All cDNA products were resolved by electrophoresis using 1–4% agarose gels.

Histological Analysis, Preparation of Mammary Whole Mounts, and TUNEL Assays—Gland fragments from different developmental stages (5 weeks, 10 weeks, 6.5 days of pregnancy (dpc), 18.5 dpc, lactation day 1 (L1, also termed parturition day), lactation day 2 (L2, 1 day after parturition), and involution day 14 (I14, 14 days after parturition)) were embedded in paraffin, sectioned, and hematoxylin/eosin stained. Mammary glands for whole mounts were spread on glass slides and fixed 4 h in Carnoy fixative. The tissue slides were sequentially rehydrated 15 min each in a graded ethanol (70, 50, 30, 10%, and H2O) and stained overnight (0.2% (w/v) carmine (Sigma), 0.5% (w/v) aluminum potassium sulfate (Sigma)). The slides were dehydrated 15 min each in a graded ethanol (70, 95, 100%) and Xylene and mounted with Pertex (Histolab).

To detect apoptotic nuclei, paraformaldehyde-fixed paraffin sections were analyzed by terminal deoxynucleotidyl transferase digoxigenin nick-end labeling using the Apoptag kit (Chemicon) following the manufacturer’s instructions. For quantitative analysis 1000 nuclei from three separate areas in three samples were counted.

Immunohistochemical Analysis—Mammary glands were fixed overnight in 4% paraformaldehyde or Feketes fixative (29) and paraffin embedded. Sections were deparaffinized in xylene, passed through a graded alcohol series and then either micro-sectioned, and hematoxylin/eosin stained. Mammary glands for whole mounts were spread on glass slides and fixed 4 h in Carnoy fixative. The tissue slides were sequentially rehydrated 15 min each in a graded ethanol (70, 50, 30, 10%, and H2O) and stained overnight (0.2% (w/v) carmine (Sigma), 0.5% (w/v) aluminum potassium sulfate (Sigma)). The slides were dehydrated 15 min each in a graded ethanol (70, 95, 100%) and Xylene and mounted with Pertex (Histolab).

Northern Blot Hybridization—RNA from mammary gland tissues was obtained and the Northern blot was performed according to the NorthernMaxTM (Ambion) manual. Probes against WAP, α-lactalbumin, β-casein, and Gapdh were a kind gift from Dr. Stephan Teglund (30). All probes were labeled with 32P using the High Prime labeling kit (Roche).

Immunoprecipitation and Western Blot Analysis—Mammary fat pads were dissected, quickly frozen in liquid nitrogen, and homogenized in 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol with Complete protease inhibitor mixture (Roche Applied Science). Western blot analysis was performed on tissue protein lysates using the primary antibodies anti-GLI1 (1:4,000, Abcam), anti-E-cadherin (1:2,500, BD Biosciences), α-catenin (1:5000, BD Transduction Laboratories), and F4/80 (1:500, Serotec) was performed using mouse monoclonal antibodies and the Histomouse kit (Zymed Laboratories Inc.), according to the manufacturer’s instructions. As negative controls, all experiments were performed without the primary antibody in the presence of equal concentrations of normal rabbit, goat, or mouse IgG.

RESULTS

Generation of Mice with Targeted Expression of the Hh Effector GLI1 in the Mammary Gland—To study the influence of a deregulated Hh signaling pathway during postnatal development in the mouse mammary gland, we employed a bigenic transgenic system to express the hedgehog effector GLI1 in the mammary epithelium (Fig. 1A). The reverse tet-responsive transactivator (rtTA) was expressed under the control of a mammary tumor virus long terminal repeat (MMTV LTR) and administration of doxycycline resulted in activation of the TRE-linked transgene, GLI1. The bitransgenic and WT animals were induced either continuously from mating or starting 3 weeks after birth. The time of GLI1 expression did not influence the phenotypes observed in the GLI1-induced transgenic mammary glands nor did it alter the GLI1 expression levels in the mammary gland during pregnancy as measured by RT-PCR (data not shown). The bigenic doxycycline-treated mice were normal from birth and indistinguishable from their WT litter-
GLI1-expressing Mammary Glands

Expression of Hedgehog Pathway Components in WT and GLI1-expressing Mice—To investigate the expression pattern of Gli1 and Ptch1 in the mammary gland, RT-PCR and immunohistochemistry were performed at various developmental stages. The Gli1 primer pair used for RT-PCR was designed to recognize both human and mouse Gli1 mRNA. The RT-PCR analysis demonstrated that the expression of Gli1 and Ptch1 in the mammary gland from doxycycline-treated bigenic mice was enhanced during pregnancy at all time points analyzed (6.5 dpc, 18.5 dpc, and L1), when compared with WT mice (Fig. 1C). In addition, immunohistochemistry revealed enhanced cytosolic and nuclear expression of the Gli1 protein in mammary tissue from GLI1 expressing mice during pregnancy (6.5 and 18.5 dpc) and parturition (L1) when compared with WT mice at the same developmental stage (Fig. 1D, a–f). The Gli1 expression was essentially homogenous in the mammary epithelial cell compartment. However, a small fraction of predominantly luminal cells were Gli1 negative. The Ptch1 expression in the WT and induced bigenic mammary gland followed the Gli1 expression as expected due to the autoregulatory properties of the Hh signaling pathway (Fig. 1D, g–l) (32). The Gli1 and Ptch1 expression levels were below detection level in induced bigenic virgin (5 and 10 weeks) mice (data not shown).

GLI1-expressing Mice Fail to Lactate—GLI1 transgenic females exposed to doxycycline gave birth to live pups of normal size and number with the expected Mendelian distribution of genotypes. Maternal behavior appeared normal with respect to nursing and the pups showed no craniofacial alterations or apparent neurological defects. All pups, irrespective of genotype, born to GLI1 expressing females died from failure to nurse and consequently no milk spots could be detected (Fig. 1E). The phenotype showed a 100% penetration rate among the GLI1 expressing mothers (n > 30) and could not be compensated for by subsequent pregnancies (n = 10). Furthermore, addition of doxycycline during the second or later pregnancies to previously un-induced females replicated these findings. Non-induced bigenic mice were able to nurse their pups and showed normal development of the mammary glands (data not shown).

Expression of GLI1 Impairs the Lobuloalveolar Development of the Mammary Gland—Whole mount and immunohistological analysis were performed at different developmental stages and compared with WT mice. At 5 and 10 weeks of age, analysis of mammary glands from doxycycline-induced bigenic mice showed no alterations when compared with WT animals at the same age and the mammary fat pads were to an equal degree compensated for by subsequent pregnancies (n = 10). Furthermore, all mammary glands showed no craniofacial alterations or apparent neurological defects. All pups, irrespective of genotype, born to GLI1 expressing females died from failure to nurse and consequently no milk spots could be detected (Fig. 1E). The phenotype showed a 100% penetration rate among the GLI1 expressing mothers (n > 30) and could not be compensated for by subsequent pregnancies (n = 10). Furthermore, addition of doxycycline during the second or later pregnancies to previously un-induced females replicated these findings. Non-induced bigenic mice were able to nurse their pups and showed normal development of the mammary glands (data not shown).

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Role of GLI1 in Mammary Gland Maturation

Expression of GLI1 in mammary epithelial cells delays development during pregnancy and alters the apoptotic rate at parturition. A, whole mounts of carmine-stained mammary glands from GLI1 expressing bigenic and WT mice at 6.5 dpc (a and b), 18.5 dpc (c and d), and L1 (e and f). Histological analysis of hematoxylin and eosin-stained paraffin-embedded GLI1 expressing bigenic (g, i, and k) and WT (h, j, and l) mammary glands at 6.5 dpc, 18.5 dpc, and L1. Reduced branching of the alveolar tree during pregnancy was detected in the mammary gland of GLI1 expressing mice. Scale bar, 250 μm. B, accelerated apoptosis in GLI1 expressing mammary glands at parturition as revealed by the TUNEL assay. Shown are TUNEL positive cells (a and d) and propidium iodide-positive nuclei (b and e) in GLI1 expressing and WT mammary glands. TUNEL positive cells in WT mammary gland are marked with an arrow (d and f). C, quantification of apoptotic cells detected by the TUNEL assay in GLI1 expressing and WT mice at L1.

FIGURE 2. Expression of GLI1 in mammary epithelial cells delays development during pregnancy and alters the apoptotic rate at parturition. A, whole mounts of carmine-stained mammary glands from GLI1 expressing bigenic and WT mice at 6.5 dpc (a and b), 18.5 dpc (c and d), and L1 (e and f). Histological analysis of hematoxylin and eosin-stained paraffin-embedded GLI1 expressing bigenic (g, i, and k) and WT (h, j, and l) mammary glands at 6.5 dpc, 18.5 dpc, and L1. Reduced branching of the alveolar tree during pregnancy was detected in the mammary gland of GLI1 expressing mice. Scale bar, 250 μm. B, accelerated apoptosis in GLI1 expressing mammary glands at parturition as revealed by the TUNEL assay. Shown are TUNEL positive cells (a and d) and propidium iodide-positive nuclei (b and e) in GLI1 expressing and WT mammary glands. TUNEL positive cells in WT mammary gland are marked with an arrow (d and f). C, quantification of apoptotic cells detected by the TUNEL assay in GLI1 expressing and WT mice at L1.

Functional Differentiation of Mammary Epithelium Is Sensitive to Expression of GLI1—The cellular specification and the functional differentiation of WT and GLI1 expressing mammary epithelium were evaluated using immunohistochemical detection of stage-specific protein expression. The water transmammary glands indicating that the polarity of the Slc34a2 expressing luminal epithelial cells was largely maintained (Fig. 3g).

Targeted Expression of GLI1 in the Mammary Gland Regulates STAT5 Activation and the Expression of the STAT5-regulated Milk Genes—Development of the mammary gland during pregnancy is dependent upon the levels of progesterone, estrogen, and prolactin. Prolactin affects cell specification, proliferation, and differentiation through the Jak2-STAT5 pathway and disruption of components in this pathway results in impaired alveolar development and a reduced milk protein production (33). To establish whether GLI1 can affect this process and particular the expression of milk genes, including α-lactalbumin, β-casein, and whey acidic protein (WAP), the presence of milk protein mRNA was determined by Northern blot analysis. Expression of GLI1 in the bigenic mice resulted in a signif-
indicating a defect in epithelial differentiation. The apical surface of epithelial cells in GLI1 expressing mice at parturition showed that the Slc34a2 protein was present in a heterogenic pattern on mammary glands of WT (Fig. 4, A and B) mice at 6.5 dpc and of Slc12a2 in induced bigenic (c) and WT (d) mice at L1. GLI1 expressing mice show a persistence of AQP5 protein expression on the apical membrane of mammary secretory epithelial cells during early pregnancy (6.5 dpc) as well as a maintained ductal epithelial Slc12a2 protein expression at parturition. Immunohistological staining of Slc34a2 in mammary glands of WT (f and h) and GLI1 expressing bigenic (e and g) mice at L1 showed that the Slc34a2 protein was present in a heterogenic pattern on the apical surface of epithelial cells in GLI1 expressing mice at parturition indicating a defect in epithelial differentiation. Scale bar, 20 μm (a–f) and 100 μm (g and h).

A significant decrease of mRNA levels corresponding to all the examined milk proteins (Fig. 4A). The expression levels of β-casein (Fig. 4A, first panel), WAP (Fig. 4A, second panel), and α-lactalbumin (Fig. 4A, third panel) were just above the detection limit in GLI1 expressing mammary glands. These results suggest that targeted expression of GLI1 affects the prolactin-Jak2-STAT5 pathway. To confirm this assumption, immunoblotting using a STAT5 antibody and an antibody specific for STAT5 phosphorylated at Tyr694 were performed using protein extracts from dissected mammary fat pads. Western blot analysis of anti-STAT5 antibody revealed that targeted expression of GLI1 in the mammary gland induced nuclear expression of STAT5 during pregnancy (Fig. 4, A and B). Confirming these results, immunohistology and immunofluorescence revealed a reduced expression of E-cadherin on the basolateral surface of alveolar and ductal epithelial cells in GLI1 expressing mammary glands during pregnancy (Fig. 4, C and D). No change in E-cadherin expression was detected in virgin (5 and 10 weeks) mammary tissue (data not shown).

The E-cadherin expression can be transcriptionally repressed by Snail, a zinc finger transcription factor, which expression has been shown to be enhanced in response to GLI1 (35–37). Immunohistology and immunofluorescence performed with an anti-Snail antibody revealed that targeted expression of GLI1 in the mammary gland induced nuclear expression of Snail during pregnancy (Fig. 4, C and D) but not in virgin (5 or 10 weeks) mammary glands (data not shown).

The Involution Process Is Impaired in GLI1-expressing Mice—In contrast to the reduced mammary ductal tree observed during pregnancy in GLI1 expressing transgenic mammary glands, whole mounts prepared 14 days after parturition (I14) showed a mammary tree with expanded branching and remaining terminal lobuloalveolar outgrowths (Fig. 5A) when compared with WT (Fig. 5B). To be able to make a valid comparison of mammary glands at I14, pups were removed from GLI1 expressing and WT mothers directly after parturition. Histological analysis confirmed the presence of the remaining epithelial clusters at I14 in the mammary glands of GLI1 expressing mice (Fig. 5, c and d). Furthermore, the histological analysis revealed that mammary ducts in GLI1 expressing mice at involution day I14 were occasionally enlarged and filled with cellular debris (Fig. 5C). At the initial stage of involution, apoptotic cells are thought to be engulfed mainly by neighboring epithelial cells; in the later stage, they seem to be cleared by macrophages that migrate into the gland (38, 39). We stained mammary glands at parturition and on day 14 of involution with the anti-F4/80 antibody, which detect an antigen expressed on a wide range of active macrophages. No F4/80+ cells were detected at parturition in either WT or GLI1 expressing mammary glands (data not shown). In contrast, GLI1 expressing mammary glands analyzed at I14 show F4/80 staining (Fig. 5e), whereas no F4/80 staining was observed in WT mammary glands taken at I14 (Fig. 5f). Further analysis of mRNA levels corresponding to all the examined milk proteins (Fig. 4A). The expression levels of β-casein (Fig. 4A, first panel), WAP (Fig. 4A, second panel), and α-lactalbumin (Fig. 4A, third panel) were just above the detection limit in GLI1 expressing mammary glands. These results suggest that targeted expression of GLI1 affects the prolactin-Jak2-STAT5 pathway. To confirm this assumption, immunoblotting using a STAT5 antibody and an antibody specific for STAT5 phosphorylated at Tyr694 were performed using protein extracts from dissected mammary fat pads. Western blot analysis of anti-STAT5 antibody revealed that targeted expression of GLI1 in the mammary gland induced nuclear expression of STAT5 during pregnancy (Fig. 4, A and B). Confirming these results, immunohistology and immunofluorescence revealed a reduced expression of E-cadherin on the basolateral surface of alveolar and ductal epithelial cells in GLI1 expressing mammary glands during pregnancy (Fig. 4, C and D). No change in E-cadherin expression was detected in virgin (5 and 10 weeks) mammary tissue (data not shown).

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The E-cadherin expression can be transcriptionally repressed by Snail, a zinc finger transcription factor, which expression has been shown to be enhanced in response to GLI1 (35–37). Immunohistology and immunofluorescence performed with an anti-Snail antibody revealed that targeted expression of GLI1 in the mammary gland induced nuclear expression of Snail during pregnancy (Fig. 4, C and D) but not in virgin (5 or 10 weeks) mammary glands (data not shown).

The Involution Process Is Impaired in GLI1-expressing Mice—In contrast to the reduced mammary ductal tree observed during pregnancy in GLI1 expressing transgenic mammary glands, whole mounts prepared 14 days after parturition (I14) showed a mammary tree with expanded branching and remaining terminal lobuloalveolar outgrowths (Fig. 5A) when compared with WT (Fig. 5B). To be able to make a valid comparison of mammary glands at I14, pups were removed from GLI1 expressing and WT mothers directly after parturition. Histological analysis confirmed the presence of the remaining epithelial clusters at I14 in the mammary glands of GLI1 expressing mice (Fig. 5, c and d). Furthermore, the histological analysis revealed that mammary ducts in GLI1 expressing mice at involution day I14 were occasionally enlarged and filled with cellular debris (Fig. 5C). At the initial stage of involution, apoptotic cells are thought to be engulfed mainly by neighboring epithelial cells; in the later stage, they seem to be cleared by macrophages that migrate into the gland (38, 39). We stained mammary glands at parturition and on day 14 of involution with the anti-F4/80 antibody, which detect an antigen expressed on a wide range of active macrophages. No F4/80+ cells were detected at parturition in either WT or GLI1 expressing mammary glands (data not shown). In contrast, GLI1 expressing mammary glands analyzed at I14 show F4/80 staining (Fig. 5e), whereas no F4/80 staining was observed in WT mammary glands taken at I14 (Fig. 5f). Further
FIGURE 4. The mammary glands of GLI1-expressing mice display an altered milk gene, STAT5, E-cadherin, and Snail expression during pregnancy. A, Northern blot analysis of milk gene expression in two WT (lanes 1 and 2) and two GLI1 expressing (lanes 3 and 4) mice at L1. Total mammary gland RNA was isolated at L1 and subjected to Northern blot analysis using probes specific for the milk genes β-casein, Wap, and α-lactalbumin. The milk gene expression was drastically reduced in the induced bitransgenic mice at parturition. GAPDH expression and the S28 ribosomal band served as controls for RNA loading. B, whole cell extracts of WT and induced bigenic mammary glands at 18.5 dpc and L1 were analyzed by immunoblotting for expression of GLI1, inactive and phosphorylated STAT5, E-cadherin, and Actin. GLI1 protein levels are raised at all time points analyzed in the induced bigenic mammary glands compared with WT, whereas the inactive and the phosphorylated STAT5 and E-cadherin protein expression is reduced. Actin expression was used as a loading control. C, immunohistochemical detection of E-cadherin in induced bigenic (a, c, and e) and WT mammary glands (b, d, and f) and of Snail in induced bigenic (g, i, and k) and WT (h, j, and l) mammary glands at 6.5 dpc, 18.5 dpc, and L1. The E-cadherin expression is present on the basolateral surface of all alveolar and ductal cells in the WT mammary gland during pregnancy, whereas the GLI1 expressing mammary glands show a dramatic reduction of E-cadherin expression at all time points analyzed during pregnancy. In contrast, Snail expression is low in the WT mammary gland, whereas the induced bigenic mammary glands show a nuclear localization of Snail during pregnancy. Individual cells appearing in panel e reflects an increase in stromal fibroblasts. D, immunofluorescence analysis of E-cadherin and Snail in WT (a–d) and GLI1 expressing bigenic (e–h) mammary glands at 6.5 dpc confirm the reduced E-cadherin and increased nuclear Snail expression. E, schematic illustration of the proposed signaling pathway underlying the GLI1 induced mammary gland maturation defects. Scale bar, 50 μm.
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FIGURE 5. Targeted expression of GLI1 impairs mammary involution. Whole mount and carmine-stained mammary glands from GLI1 expressing mice at involution day 14 reveal retained epithelial clusters and enlarged ducts filled with membranous materials (a–d). Sections of mammary glands from GLI1 expressing (e) and WT (f) mice on day 14 of involution were stained with the F4/80 antibody recognizing macrophages. Mammary glands from GLI1 expressing mice reveal F4/80 staining at I14 as well as areas with inflammation (e). Scale bar, 100 μm.

FIGURE 6. Presence of permanent alterations in GLI1-expressing mammary glands. A, GLI1 and rtTA expression was determined by RT-PCR analysis of total RNA extracted from two induced bigenic (GLI1(L1)), two non-induced bigenic (GLI1(−dox)) and one multiparous bigenic mouse treated with doxycycline until and during the first parturition (GLI1(+/dox, −dox)) (L2). Analysis of Actin expression was used as a control. B, whole mounts and immunohistological stainings prepared from multiparous bigenic mammary glands only treated with doxycycline until and during the first parturition (GLI1(+/dox, −dox)) (a and c) show enlarged ducts and a reduced lobuloalveolar network 1 day after parturition (L2) when compared with WT mammary glands at parturition (L1) (b and d). In addition, mammary glands from multiparous bigenic (GLI1(+/dox, −dox)) mice show heterogeneity in Slc34a2 expression when compared with WT (e and f). Immunohistochemical analysis reveals that GLI1(+/dox, −dox) and WT mammary glands when stained with antibodies against GLI1 (g and h), Snail (i and j), and E-cadherin (k and l) show a similar expression pattern. Scale bar, 100 μm.

thermore, the abnormal involution in the GLI1-induced mammary glands induced an inflammatory response (Fig. 5e).

GLI1 Expression during Pregnancy Induces Irreversible Changes in the Mammary Gland Epithelium—Bigenic females, which have gone through one pregnancy under the influence of doxycycline and another pregnancy without GLI1 induction, were still not able to lactate. Semi-quantitative RT-PCR analysis of mRNA levels showed that these multiparous animals (GLI1(+dox, −dox)) did not express detectable levels of GLI1 in the mammary gland 1 day after parturition (L2), whereas bigenic mothers receiving doxycycline (GLI1(+dox)) expressed high levels of GLI1 in the mammary gland at parturition (L1) (Fig. 6A). Without doxycycline exposure the bigenic mammary glands GLI1(−dox) did not show detectable GLI1 expression at parturition (Fig. 6A). All animals expressed rtTA and Actin at similar levels (Fig. 6A). Immunohistochemical analysis confirmed the low levels of GLI1 expression 1 day after parturition (L2) in the GLI1(+dox, −dox) mammary glands (Fig. 6B, g). Whole mount and immunohistological analysis revealed that multiparous GLI1(+dox, −dox) mice only induced with doxycycline until after the first parturition showed a reduction in alveolar epithelial cells 1 day after parturition (L2) when compared with WT mice at parturition (L1) (Fig. 6B, a–d). The histological defects detected in the GLI1(+dox, −dox) mammary glands were similar to the changes observed in GLI1 expressing mice with permanent doxycycline exposure (Fig. 2, e and k). Moreover, enlarged ducts filled with cellular debris were found in the GLI1(+dox, −dox) mammary glands 1 day after parturition (Fig. 6B, c). The enlarged ducts found in the GLI1(+dox, −dox) mammary glands appeared similar to the dilated ducts found at 114 in GLI1 expressing mammary glands (Fig. 5c). The multiparous GLI1(+dox, −dox) mammary glands showed a similar heterogeneous expression pattern of the differentiation marker Slc34a2 1 day after parturition (Fig. 6B, e) as that observed in induced bigenic mice at parturition (Fig. 3e). In contrast, WT mammary glands expressed Slc34a2 protein on the apical membrane of all alveolar epithelial cells at parturition (Fig. 6B, f). The multiparous GLI1(+dox, −dox) mammary glands did not
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![Figure 7](Image)

**FIGURE 7.** GLI1-expressing mice show an increase in mammary epithelial cell proliferation. A, immunohistochemical detection of in situ BrdUrd incorporation in GLI1 expressing (a, c, and e) and WT mice (b, d, and f) and of Cyclin D1 protein in induced bigenic (g, i, and k) and WT (h, j, and l) mammary glands at 6.5 dpc, 18.5 dpc, and L1. A marked increase of BrdUrd and Cyclin D1-labeled nuclei were observed in induced bigenic epithelial cells during pregnancy. B, immunohistological analysis of β-catenin in induced bigenic (a) and WT (b) mammary glands at 6.5 dpc reveal no increase in β-catenin expression. Scale bar, 50 μm (A) and 20 μm (B).

show an increased expression of Snail (Fig. 6B, i) or reduced expression of E-cadherin (Fig. 6B, k) 1 day after parturition as observed in induced bigenic mice at parturition (Fig. 4C, k and e).

**Targeted Expression of GLI1 Lead to an Increased Proliferation Rate in the Mammary Epithelial Cells and Expansion of Epithelial Cell Clusters—**To determine whether the phenotypic defects in the GLI1 expressing bigenic mice were associated with changes in the level of proliferation in the mammary gland, we measured the number of BrdUrd-positive epithelial cells undergoing active DNA synthesis. A dramatic increase in proliferation and the number of S-phase cells was observed during pregnancy (6.5 and 18.5 dpc) and at parturition (L1) in the induced bigenic mammary epithelial cells when compared with WT (Fig. 7A, a–f). Previously, it has been shown that Hh signaling promotes cellular growth through activation of transcription of Cyclin D1, a principal regulator of the cell cycle (40). Immunohistological analysis revealed an increased expression level and nuclear localization of Cyclin D1 at the same time points when BrdUrd incorporation was increased (Fig. 7A, g–l). 

**Cyclin D1** is further a Wnt-signaling target gene and because GLI1 expressing mammary epithelial cells showed a diminished expression of E-cadherin we explored the pattern of β-catenin expression. Immunohistochemical analysis revealed no nuclear or increased cytosolic expression of β-catenin during pregnancy in the induced bigenic mammary epithelial cells when compared with WT (Fig. 7B and data not shown). Morphological analysis of mammary glands taken from pregnant GLI1 expressing mice revealed epithelial defects as early as 6.5 dpc (n = 5). These changes appeared sporadically throughout the GLI1 expressing mammary gland and consisted of a thickening of the mammary epithelia and occasional occlusion of the epithelial luminal space (Fig. 8A). Furthermore, an unusually dense layer of fibroblastic stroma was sporadically detected in the mammary ducts of parous GLI1 expressing mice (Fig. 8A). Immunofluorescence with an anti-Laminin antibody detected the basement membrane around all the epithelial changes, the staining was continuous, suggesting correct deposition of the extracellular matrix in the GLI1 expressing mammary glands at 6.5 dpc (Fig. 8B). In accordance with the myoepithelial smooth muscle actin expression, the most basal epithelial cells revealed continuous positive staining in WT and induced bigenic mammary glands (Fig. 8B). Morphological examination of GLI1 expressing and WT virgin mammary glands (5 and 10 weeks) revealed no alterations in the mammary alveolar epithelial cells, consistent with low/absent GLI1 expression (data not shown).

**DISCUSSION**

In this study we describe novel and fully penetrant features as the result of targeted expression of GLI1 in the mammary gland during pregnancy. First, all pups born to GLI1 expressing bigenic mice die after parturition. This dramatic effect cannot be explained by any craniofacial (mouth or tongue) or neurological defects in the newborn pups, or by the behavior of the mothers, which was normal. This led us to the conclusion that the GLI1 expressing mothers suffered from a lactation defect supported also by the lack of milk gene expression. Second, mammary glands of GLI1 expressing mice did not regress fully to the pre-pregnant state, instead expanded epithelial clusters and enlarged ducts could be detected when involution was expected to be complete. Moreover, animals that have gone through one pregnancy with induced GLI1 expression are not able to lactate again, even though doxycycline treatment is withdrawn and the GLI1 mRNA is no longer detectable. This shows that the permanent changes are not dependent on maintained GLI1 expres-
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Corroborating such a link, we found that induced GLI1 expression in the mammary gland during pregnancy renders a reduced level of STAT5 expression as well as reduced phosphorylation of the STAT5 protein at regulatory amino acid Tyr694, which may then explain the inability of the induced bigenic mice to lactate. In contrast to STAT5a-null mice and Prl-R heterozygous mutants, which after multiple pregnancies were able to attain functional mammary gland development, GLI1 expressing mice, just like Jak2-deficient females were unable to form functional alveolar cells even after several pregnancies (41–43). The permanent inability to form functional alveolar epithelial cells after multiple gestation cycles indicates that neither other components of the prolactin signaling cascade nor other growth factors and their signal transducers were able to restrain or circumvent the GLI1-induced repression and activate STAT5 in vivo.

We propose that the Hh pathway is indirectly coupled to STAT5 during functional differentiation of mammary epithelium, via Snail and E-cadherin. In support of this notion, we show that targeted expression of GLI1 during pregnancy dramatically increases the expression level of Snail and reduces the expression of the adhesion molecule E-cadherin. Interestingly, conditional knock-out of E-cadherin in the mammary gland during pregnancy results in the accumulation of histologically similar defects as observed in STAT5a knock-out and GLI1 expressing mice, i.e. consisting of a reduced lobuloalveolar network and inability to support their offspring at parturition (34). In addition, conditional knock-out of E-cadherin results in reduced expression of the prolactin-dependent transcription factor STAT5 at parturition (34). E-cadherin is a direct transcriptional target of Snail, and whereas regulation of E-cadherin by Snail has not previously been implicated in pregnancy-induced mammary gland development a role during breast tumorigenesis and metastasis has been indicated (44). The expression pattern of Snail during mammary gland development is not fully clear. However, Snail is enriched in the epithelial compartment of the terminal end buds during the virgin stage of mammary gland development (19). We here demonstrate that targeted expression of GLI1 dramatically induces the nuclear Snail expression in mammary epithelial cells during pregnancy. This is consistent with previous reports where Snail was found to be a target gene and transcriptionally
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regulated by Gli1 (37). In addition, transgenic GLI1 expression rapidly induces Snail expression in murine skin and lesions, which exhibit molecular similarities with basal cell carcinoma (45). The results described above depict a signaling cascade comprising GLI1, Snail, E-cadherin, and STAT5 (Fig. 4E) that when altered culminates in the inability to form fully functional mammary alveolar epithelial cells resulting from impaired functional differentiation and maturation of mammary epithelial cells during pregnancy. Interestingly, induction of mammary cell differentiation is enhanced by inhibition of the Hh signaling pathway in a mammosphere assay, which supports the reduction in mammary differentiation that we observe in the mammary glands from GLI1 expressing mice (26).

We also present evidence that expression of GLI1 during one pregnancy is enough to elicit sustained damage to the mammary gland. One plausible explanation for the permanent changes is the impaired involution detected in the GLI1 expressing mammary gland. Correct remodeling of the mammary gland is necessary for subsequent pregnancies and previous reports have shown that impaired remodeling can cause impaired lactation during following pregnancies (46). During involution of the mammary gland apoptotic cells are engulfed by neighboring epithelial cells and by macrophages that migrate into the mammary gland. We find that mice expressing GLI1 at I14 and multiparous mice expressing GLI1 only during the first pregnancy at L2 contain enlarged ducts filled with cellular debris (Figs. 5c and 6B, c), indicating a defective clearance. Furthermore, we show a remaining macrophage population at I14 in mammary glands of GLI1 expressing mice, indicating an incomplete involution process.

Consistent with the remaining and expanded epithelial cell population observed at I14, we detect an increase in the epithelial proliferation rate, resulting in clusters of epithelial cells already by 6.5 days of pregnancy in the GLI1 expressing mammary gland. The changes consist of a thickening of the mammary epithelium, and in many cases the luminal space is totally occluded by epithelial cells. The occlusion of the luminal space could perhaps be explained by the reduction of the adhesion molecule E-cadherin as blocking of E-cadherin disrupts the extracellular matrix in the mammary epithelial changes detected in the GLI1 expressing mice analyzed at 6.5 dpc. Furthermore, the cell polarity is maintained in a fraction but not all of the GLI1 expressing luminal epithelial cells. These observations show that also the functional maturation of luminal cells is compromised in GLI1 expressing mice. The increase in epithelial cell proliferation in the GLI1 expressing mammary gland is associated with an increase in Cyclin D1 expression. This is in agreement with previous reports, showing that Hh signaling can affect the expression levels of Cyclin D1 and induce proliferative changes (2, 40). Moreover, it was recently shown that the HH pathway components PTCH1, GLI1, and GLI2 are expressed in human mammary progenitor cells and that Hh signaling enhances mammosphere formation (26). It is an interesting possibility that the epithelial changes detected in the early pregnant GLI1 expressing mammary gland are a consequence of induced proliferation of the mammary stem cells. Bigenic virgin mice treated with doxycycline do not exhibit any histological changes, consistent with non-detectable levels of GLI1 transgene expression and low expression of the MMTVrTA transactivator in such mice (28). Thus, we cannot at present rule out that changes may occur also in the virgin mammary gland if the GLI1 expression was substantially increased at an earlier stage. This latter possibility is in fact strongly supported by a very recent study showing that expression of a dominant active Smo in the mammary gland causes increased proliferation and ductal dysplasia in non-pregnant female mice (16). Moreover, mammary gland epithelial cells may display a differential responsiveness to increased GLI1 expression and in this respect cells where Gli3 normally repress Hh-pathway signaling represent a potential target population (13).

In summary, this study reveals that correct regulation of the Hedgehog signaling pathway is of critical importance in pregnancy-induced mammary gland maturation and plays a central role in mammary histogenesis. Based on our data, we propose that induced expression of GLI1 culminates in the reduction of phosphorylated STAT5 in mammary gland epithelial cells dependent on a signaling cascade involving the intermediate components Snail and E-cadherin. Furthermore, we show that expression of GLI1 during one pregnancy is enough to elicit sustained changes during forthcoming pregnancies. The detailed molecular mechanisms that regulate the different Hh responses in breast epithelium are not yet fully elucidated but will be a future area of research.

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