INTRODUCTION

In response to hypoxia, tissues attempt to restore homeostasis by regulating cellular metabolism and by inducing angiogenesis (reviewed by Semenza, 2000). Both of these processes are primarily regulated by a heterodimeric transcription factor complex known as the hypoxia-inducible factor (HIF) 1 complex, HIF1α, was deleted in the murine mammary gland. Although vascular density was unchanged in the HIF1α null mammary gland, loss of HIF1α impaired mammary differentiation and lipid secretion, culminating in lactation failure and striking changes in milk composition. Transplantation experiments confirmed that these developmental defects were mammary epithelial cell autonomous. These data make clear that HIF1α plays a critical role in the differentiation and function of the mammary epithelium.

SUMMARY

During pregnancy the mammary epithelium and its supporting vasculature rapidly expand to prepare for lactation, resulting in dramatic changes in the micro-environment. In order to investigate the role of oxygenation and metabolism in these processes, the oxygen-responsive component of the hypoxia-inducible factor (HIF) 1 complex, HIF1α, was deleted in the murine mammary gland. Although vascular density was unchanged in the HIF1α null mammary gland, loss of HIF1α impaired mammary differentiation and lipid secretion, culminating in lactation failure and striking changes in milk composition. Transplantation experiments confirmed that these developmental defects were mammary epithelial cell autonomous. These data make clear that HIF1α plays a critical role in the differentiation and function of the mammary epithelium.

Key words: Hypoxia, HIF1, Mammary gland, Lactation, Differentiation, Metabolism, Mouse

INTRODUCTION

In response to hypoxia, tissues attempt to restore homeostasis by regulating cellular metabolism and by inducing angiogenesis (reviewed by Semenza, 2000). Both of these processes are primarily regulated by a heterodimeric transcription factor complex known as the hypoxia inducible factor 1 or HIFI (Semenza, 2000). The HIFI heterodimer includes HIF1α, a basic helix-loop-helix (bHLH) protein induced and stabilized by hypoxia, and the aryl hydrocarbon receptor nuclear translocator (ARNT) protein (also termed HIF1β), which is expressed constitutively and heterodimerizes with multiple bHLH partners.

Under normoxic conditions, HIF1α protein is rapidly degraded through targeted ubiquitination mediated by direct binding of its oxygen dependent domain to the β subunit of the von Hippel Lindau (VHL) tumor suppressor protein (reviewed by Kondo and Kaelin, 2001). In response to hypoxia, HIF1α protein accumulates, owing to decreased interaction with VHL (Krek, 2000). An increase in HIF1α protein is first detectable at partial pressures of oxygen equivalent to 6% O2 and is maximal between 0.5-1.0% O2 (Stroka et al., 2001). In a hypoxic environment, HIF1 activates the hypoxic response elements (HREs) of target gene regulatory sequences (Huang et al., 1998; Salceda and Caro, 1997), resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress (reviewed by Giordano and Johnson, 2001). Some of the direct targets include erythropoietin, the angiogenic factor vascular endothelial growth factor (VEGF), glucose transporters and multiple glycolytic enzymes. The connection between the hypoxic response and angiogenesis is also clear from the study of individuals with VHL disease, an autosomal, dominantly inherited cancer syndrome. Individuals heterozygous for one inactivating mutation in VHL are predisposed to developing a variety of tumor types, including renal clear cell carcinomas that are massively hypervascular with highly elevated levels of VEGF expression caused by constitutive HIF1 activity in response to inactivation of the second VHL allele (Kondo and Kaelin, 2001).

Recently, HIF1α has been demonstrated to be upregulated in a variety of human solid tumors, in particular breast tumors that exhibit high rates of proliferation (Bos et al., 2001; Zhong et al., 1999). Zhong et al. reported that HIF1α protein was overexpressed in breast tumors, as well as bordering ‘normal’ areas adjacent to tumors, but not in normal breast tissue (Zhong et al., 1999). These observations in breast tumors are consistent with our previous findings that HIF1α functions as a positive
regulator of tumor growth (Ryan et al., 2000; Seagroves and Johnson, 2002). In a subsequent study, the level of HIF1α expression in breast tumors was correlated with other prognostic factors. Specifically, in ductal carcinoma in situ (DCIS) lesions, relatively high levels of HIF1α expression were associated with increased proliferation, as well as increased expression of VEGF and the estrogen receptor (Bos et al., 2001). However, HIF1α expression did not correlate with p53, supporting the observations of our own laboratory that the effects of loss of Hif1a on cell growth, metabolism or tumorigenesis are independent of p53 expression (Ryan et al., 2000). In order to provide a foundation for understanding the role of HIF1 in mammary tumorigenesis, the function of HIF1α was investigated during normal mammary gland development.

Several laboratories have demonstrated that HIF1α is required to regulate the response to lowered oxygen levels in developing murine tissues (Iyer et al., 1998; Ryan et al., 1998; Schipani et al., 2001; Yun et al., 2002). With respect to known functions of HIF1, there were several compelling reasons to study HIF1α function in the context of normal mammary gland development. First, the normal mammary parenchyma undergoes tremendous expansion as it prepares for lactation during the course of pregnancy (Matsumoto et al., 1992), including formation of new blood vessel networks to provide oxygen and nutrients to the lactating mammary gland. For example, in the rat, the vasculature doubles by mid-pregnancy through angiogenesis via sprouting and intussusception (Djonov et al., 2001).

In addition, in preparation for lactation, there is a requirement for glucose to provide energy as well as to synthesize lactose, the primary carbohydrate in milk. Notably, the increased activity of several glycolytic enzymes involved in glucose metabolism has been reported at the transition from pregnancy to lactation (Mazurek et al., 1999). The transition from differentiation during pregnancy to successful milk secretion at lactation is complex, and has been divided into two stages, recently termed secretory differentiation and secretory activation (McManaman and Neville, 2003). Secretory differentiation begins at mid-gestation with the production of significant quantities of milk protein and lipid. Secretory activation is coordinated with the birth of pups, and depends on the completion of secretory differentiation. The increased demands for energy for synthesis of milk components that begin during pregnancy persist during lactation, as the gland is actively making and secreting milk. Because the developing mammary gland is both highly vascularized, and metabolically active, with a requirement for glucose to produce milk, it serves as an ideal tissue to determine the in vivo role of HIF1 and its subunit HIF1α in a developmentally regulated metabolic switch.

The clear increase in demands for energy during lactation, as well as the striking and extensive angiogenesis that occurs during pregnancy, led us to hypothesize that during both secretory differentiation and activation, HIF1α may be required to alleviate transient hypoxia through angiogenesis, increased dependence on glycolysis and regulation of substrates for the production of milk. In order to test this hypothesis, we have specifically removed Hif1a from the mammary epithelium using previously characterized HIF ‘floxed’ mice (Ryan et al., 2000) that express MMTV-Cre (Wagner et al., 2001; Wagner et al., 1997). In these mice, multiple facets of the differentiation process were impaired, culminating in a functional failure of the mammary gland.

### MATERIALS AND METHODS

#### Animals and tissue collection

Animals were housed in an AAALAC-approved facility in filter-topped cages and provided with food and water ad libitum. All animal experiments were conducted using the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice harboring two alleles of exon 2 of Hif1a flanked by loxP sites (Hif1a<sup>fl/fl</sup> or ‘floxed’) (Ryan et al., 2000) were bred to MMTV-Cre (line A) transgenic mice (Wagner et al., 2001). Wild type (Hif1a<sup>fl/fl</sup>, MMTV-Cre-negative) and HIF1α ‘null’ (Hif1a<sup>ΔE+</sup>, MMTV-Cre-positive) littermate females were bred with CD-1 males (day of plug=0). Mammary glands were harvested from mice at day 10 (<i>n</i>=3/genotype), day 15 (<i>n</i>=6/genotype) and day 18 of pregnancy (<i>n</i>=5/genotype), day 1 of lactation (date of birth, <i>n</i>=4/genotype), or day 10 of lactation (<i>n</i>=25/genotype). At sacrifice, one inguinal gland was fixed for 6 hours at room temperature with 10% neutral buffered formalin (NBF) prior to paraffin wax embedding, sectioning and staining with Hematoxylin and Eosin.

#### Quantitation of DNA, RNA and protein

A piece of inguinal mammary gland harvested at day 18 of gestation was finely ground to a powder under liquid nitrogen and homogenized in a modified RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 400 mM NaCl, 1 mM EDTA in RNase-free water). DNA was quantitated after Hoechst 33258 staining using a Horiba Micromax fluorometer (excitation, 350 nm; emission 473 nm). To measure RNA, homogenates were first treated for 90 minutes with DNase I, and the fluorescence intensity quantitated following incubation with Ribogreen Dye (Molecular Probes). Protein was measured using a Bradford assay (BioRad).

#### Milk collection and analysis

At birth pups were removed from their natural mothers, randomized and 10-12 pups placed with each dam. The average pup weight per litter per day was determined until mid-lactation (day 9-11 lactation), when milk and mammary tissues were collected. Milk was collected under gentle vacuum into tared tubes on ice from weaned dams injected with oxytocin (1.5 U per leg, i.m.). For each sample, the water, fat, nitrogen, lactose, sodium and chloride contents were measured according to standard protocols (Jensen, 1995). Briefly, the percentage of water (% w/w) was measured as weight loss after drying, and sodium and chloride were measured by inductively coupled plasma spectrometry using a Spectro-CIROSC<sup>CD</sup> (Spectro Analytical Instruments). To compare wet weight of the lactating glands, both inguinal glands were dissected following milking, flash frozen and weighed immediately.

#### Primary culture, adenoviral infection and transplantation

Primary mammary epithelial cells (MEC) were isolated from Hif1a<sup>ΔE+</sup> pregnant mice according to Pullan and Streuli (Pullan and Streuli, 1997). Equal volumes of cells were allowed to spread onto plastic dishes in plating medium (Ham’s F12 containing 10% FBS, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 5 ng/ml cholera toxin and 50 μg/ml gentamicin, 100 U Penicillin/100 U Streptomycin) for 48 hours before replacing this medium with growth medium (same as plating, but no cholera toxin or 5% FBS). The next day, the cells were infected overnight with either Adenovirus-β-galactosidase (Adeno-βgal) or Adenovirus-Cre (Adeno-Cre, generously provided by Dr Frank Giordano) at a multiplicity of infection of 60-65 particles per cell (Rijnkels and Rosen, 2001). The next day, the cells washed several times with PBS and fresh growth medium was added. Cells were allowed to recover from infection for 24-48 hours. To compare mRNA expression of target genes of cultured MEC or to prepare nuclear extracts for western blotting, the medium was changed to growth medium.
containing 25 mM HEPES pH 7.4 at 0 hours. Cells were then left at normoxia or transferred to a hypoxic incubator (0.5% O2 balanced with N2) for 24 hours. For transplantation into host mice, cells were trypsinized, washed and resuspended to 50,000-100,000 cells/µl in HBSS. Approximately 10-15 µl of cells were injected into the cleared inguinal fat pads of 3-week old immunocompromised Rag1−/− females (Jackson Labs). After a period of MEC outgrowth of at least 10 weeks, the hosts were then mated and the outgrowths (4R, Adeno-β-gal-infected, wild type; 4L, Adeno-Cre infected, Hif1a−/−) harvested and fixed in 10% NBF.

**Nuclear extract preparation and western blotting**

Nuclear extracts (NE) were prepared as described previously (Ryan et al., 1998). HIF1α protein was detected by western blotting using 60 µg input of NE resolved by a 6% SDS-PAGE gel and transferred to PVDF membrane. The membrane was blocked overnight at 4°C in 10% nonfat dry milk, followed by a 3 hour incubation in a 1:1000 dilution of anti-mouse HIF1α antibody (Novus, NB100-123). The blot was then incubated in a 1:10,000 dilution of anti-mouse whole IgG-HRP for 30 minutes followed by incubation in ECLPlus substrate (Amersham) prior to exposure to Kodak MR film.

**Preparation of RNA and DNA**

Cells were washed twice with cold PBS before being directly extracted with RNAzol B for total RNA preparation (Tel-Test) or scrapped into buffer containing proteinase K for preparation of genomic DNA. To prepare total RNA from tissues, snap-frozen tissue was pulverized with a mortar and pestle directly in liquid nitrogen and homogenized in chilled RNAzol B and RNA prepared according to manufacturer’s instructions.

**Immunostaining**

For CD31 staining and Chalkley analysis slides were processed as previously described (Ryan et al., 2000). Slides were blinded and ten 10× power fields counted twice independently (n=5 per genotype). Anti-Cre immunostaining was performed as previously described (Seagroves and Li, 2002). For Glut1, antigen retrieval was performed on paraffin wax sections using 1× citrate buffer (DAKO) followed by overnight incubation at room temperature of a 1:200 dilution of anti-Glut1 antibody (Alpha Diagnostics). Staining was visualized via the ABC Elite staining kit (Vector Laboratories) using DAB as a substrate followed by counterstaining with Hematoxylin. For double labeling to detect both Glut1 and Cre on the same paraffin wax sections, Glut1 was first detected as described using DAB substrate. The sections were then re-blocked with 10% goat serum and Cre detected using Vector VIP (purple color) substrate followed by counterstaining with Methyl Green.

**Semi quantitative reverse transcription PCR assays**

Random-primed reverse transcription was carried out on 30 ng of total RNA. The cDNA was amplified using primers to mouse Xor, α-lactalbumin, β-casein, adipophilin (Adfp), butyrophilin and β-actin genes. Samples were prepared for loading onto the Applied Biosystems 310 Genetic Analyzer by mixing 12 µl of formamide, 1 µl of TAMARA size standard (Perkin Elmer Applied Biosystems) and 2 µl of PCR product. The size and amount of PCR product was calculated using GeneScan software (Perkin Elmer Applied Biosystems). Control experiments were performed to define signal linearity for each probe pair.

**Real-time PCR assays**

Two micrograms of total RNA was DNase I treated and directly used to prepare first-strand cDNA from random hexamer primers using the Superscript II Reverse Transcription Kit (Invitrogen). For real-time detection PCR (RTD-PCR), 5 ng of input cDNA was analyzed in triplicate per primer pair per sample and the corresponding threshold cycle (Ct) values expressed as the mean±s.e.m. All reactions were performed using 2× Taq Master Mix (Perkin Elmer Applied Biosystems), 900 nM each of the forward and reverse PCR primers and 250 nM of a fluorescently tagged primer pair-specific probe in a total volume of 25 µl using default cycling parameters on an ABI Prism 7200 Sequence Detector. The following primer and probe sequences were used:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Probe Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGK1</td>
<td>5′-CGAGGGCATTCCTACAACTTCTG-3′</td>
<td>5′-FAM-CAAGAAAACTCTCTGCAGATGTA-3′</td>
<td>5′-GACTGAGGA-3′</td>
</tr>
<tr>
<td>Glut1</td>
<td>5′-ACGGAGGACCGTGAAAGTCTG-3′</td>
<td>5′-6-FAM-GCCACCATCATCCAGATCAGTC-3′</td>
<td>5′-GTTCCAGGAGAATACAT-3′</td>
</tr>
<tr>
<td>Claudin 8</td>
<td>5′-TTGGTGATGTCGCCCTAAA-3′</td>
<td>5′-FAM-CAATCTGGTTCCTCTAGTGAT-3′</td>
<td>5′-GTTCCAGGAGAATACAT-3′</td>
</tr>
<tr>
<td>Ck19</td>
<td>5′-5′-CACTGGTCACAGTATAGTCT-3′</td>
<td>5′-6-FAM-CAATCTGGTTCCTCTAGTGAT-3′</td>
<td>5′-GTTCCAGGAGAATACAT-3′</td>
</tr>
<tr>
<td>Ck19</td>
<td>5′-5′-CACTGGTCACAGTATAGTCT-3′</td>
<td>5′-6-FAM-CAATCTGGTTCCTCTAGTGAT-3′</td>
<td>5′-GTTCCAGGAGAATACAT-3′</td>
</tr>
</tbody>
</table>

**Normalization of real-time PCR assays**

In cultured cells, target gene mRNA expression was first normalized to 18S rRNA and then expressed as a percentage of signal observed in wild-type cells (Hif1a+/+), Adeno-β-gal-infected cultured at normoxia (onefold). For mammary gland tissue samples, expression of each sample was first normalized to cytookeratin 19 (CK19), a gene exclusively expressed in epithelial cells (Nagle et al., 1986), to correct for any differences in epithelial cell content between genotypes prior to comparison of gene induction between wild-type (Hif1a+/+ only; MMTV-Cre negative) and null (Hif1a−/−, MMTV-Cre-positive) glands. Expression of each target in null glands was determined relative to the signal observed in wild type samples (onefold) according to standard procedures (ABI Prism 7200 manual and Muller et al. (Muller et al., 2002)). Each primer set approached 100% amplification efficiency, allowing direct comparison of Ct values to determine relative gene expression (Muller et al., 2002). To determine the efficiency of deletion of Hif1α, genomic DNA was prepared and the expression level of Hif1α was compared with that of a control primer set, Jun.

**Vasculature labeling**

Fluorescein-conjugated lectin from tomato (Vector Laboratories) diluted to 1 mg/ml in PBS was injected into the tail vein of live mice 5 minutes prior to perfusion. One minute prior to perfusion, mice were anesthetized and 1% paraformaldehyde (PFA) 0.5% glutaraldehyde was perfused directly in the heart at a rate of 1 ml per minute followed by clearing with PBS. Mammary tissues were equilibrated in cold 30% sucrose/PBS for 2 hours before embedding in OCT compound on dry ice. Thick frozen sections (40-50 µm) were post-fixed in 4% PFA for 5 minutes, treated with 0.5% Triton-X for 10 minutes, and incubated with AlexaFluor 595-conjugated phalloidin (Molecular Probes) at a 1:250 dilution in 1% BSA/PBS for several hours prior to mounting. A Zeiss confocal microscope was used to capture 0.5 µm serial slices at low power, which were then merged into one plane to visualize the vasculature (green) and actin filaments of the epithelial network (red).

**Statistical analysis**

Statistical significance was determined by an unpaired t-test (P set to <0.05), using StatView 5.0 (SAS). Those samples that achieved statistical significance, comparing wild-type with Hif1a−/− samples, are indicated with an asterisk.
RESULTS

**HIF1α is expressed in primary mammmary epithelial cells and is required for hypoxia-inducible gene transcription**

To determine if HIF1α is expressed in normal murine mammary epithelium, mammary epithelial cells were purified from wild-type mid-pregnant C57BL/6:129-Sv mice, cultured under hypoxic conditions and the extracts probed for HIF1α protein. In contrast to previous reports that HIF1α is undetectable in normal human breast tissue (Zhong et al., 1999), low levels of HIF1α protein were detectable in nuclear extracts of purified murine MEC cultured at normoxia (21% O2). Robust induction of HIF1α was observed in response to culture under hypoxic conditions (Fig. 1A).

To determine if HIF1 is essential for hypoxia-induced transcription in mammary epithelial cells, the expression levels of *Pgk* (Pgkl – Mouse Genome Informatics), *Glut1* (Slc2a1 – Mouse Genome Informatics) and *Vegf* (Vegf – Mouse Genome Informatics) were compared in primary cultures of wild-type and *Hif1a*–/– cells cultured at normoxia or hypoxia by real-time detection PCR (RTD-PCR, Fig. 1B). Under hypoxia loss of *Hif1a* resulted in minimal induction of *Pgk* mRNA and the hypoxia-inducible expression of both *Glut1* and total *Vegf* was reduced by at least 50%. These results demonstrated that regulation of hypoxic response acts through HIF1 in mammary epithelium.

**Conditional deletion of HIF1α in the mouse mammary gland**

To determine whether HIF1 function is required in vivo for normal mammary gland development, a conditional gene deletion strategy was employed to delete *Hif1a* in the mammary epithelium of mice. Mice harboring two ‘floxed’ alleles of exon 2 of the *Hif1a* locus (*Hif1a*floxed) (Ryan et al., 2000) were bred with *Hif1a*floxed mice that expressed Cre under control of the mouse mammary tumor virus (MMTV)-LTR, which targets deletion in the mammary epithelium, but not in the stroma. *Hif1a*floxed progeny negative for MMTV-Cre (referred to as wild type) were compared with *Hif1a*floxed littermates that expressed MMTV-Cre. The temporal-spatial pattern of Cre recombinase activity in this line of mice (line A) has been extensively described (Wagner et al., 2001). Southern blot analysis comparing wild-type and *Hif1a*+/+ mammary tissue indicated that MMTV-Cre consistently targeted deletion of *Hif1a* in at least 50% of the epithelial cells (Fig. 1C). However, because the DNA was prepared from whole tissue, the intensity of recombined allele, which is only present in the epithelial cells, is actually underestimated by Southern blotting. As described in detail by Wagner et al. using ROSA reporter mice crossed to individual lines of MMTV-Cre transgenic mice, by lactation, the majority of the epithelial cells have been targeted for recombination (Wagner et al., 2001). Therefore, based on these previous observations, as well as the data generated by Southern blotting, the mammary glands isolated from *Hif1a*floxed, MMTV-Cre-positive mice will be referred to as *Hif1a* null (*Hif1a*−/−). Furthermore, in contrast to reports that this line of MMTV-Cre transgenic mice induced excision in the ovaries of mature mice (Wagner et al., 2001), no recombination of the *Hif1a* locus could be detected in DNA prepared from whole ovaries of *Hif1a*floxed, MMTV-Cre-positive females either by Southern blotting (Fig. 1C) or by RTD-PCR.

**During pregnancy deletion of HIF1α impairs secretory differentiation, but not vascular expansion**

In order to pinpoint the stage of mammary gland development at which HIF1α function may be required, mammary tissue
HIF1: a critical role in lactation

was harvested from mice over the course of gestation. No differences in ductal morphogenesis were noted between genotypes in nulliparous mice (data not shown). At day 10 of gestation, a stage of development prior to differentiation, no defects in histology were observed in Hif1a−/− glands at either the gross or microscopic level (data not shown), indicating that HIF1α is not crucial for early rounds of alveolar proliferation. Similarly, by day 12 of pregnancy, when secretory differentiation typically begins in most mouse strains, no differences in morphology were noted (data not shown).

However, by day 15 of pregnancy, well into the period of secretory differentiation, although the glands of both genotypes were indistinguishable at the whole-mount level (data not shown), histological examination revealed significant abnormalities in the Hif1a−/− glands (Fig. 2A,B). In particular, the protein and lipid droplets that give the wild-type epithelium a ‘lacy’ appearance were completely absent in the Hif1a−/− glands. In addition, null alveoli were smaller, with reduced lumens, and the surrounding connective tissue that normally regresses as the alveoli mature was more prominent than in wild-type glands. These defects resulted from a block in differentiation, rather than proliferation, as the rates of incorporation of bromodeoxyuridine were equivalent at this stage of development (data not shown).

Because vascular density doubles over the course of rodent mammary development, and HIF1 has been implicated in the control of angiogenesis (Forsythe et al., 1996) we next analyzed the effect of deletion of HIF1 on vasculogenesis. Vessels were visualized by injection of fluorescein-conjugated tomato lectin into the tail veins of live mice at day 15-16 of pregnancy (Fig. 2C,D). Surprisingly, no gross differences in microvessel patterning, or vascular density were observed in Hif1a−/− mammary glands.

Expression profiles of HIF1 targets and markers of differentiation in pregnant mice

The expression of HIF1 targets, normalized to the epithelial cell marker cytokeratin 19 (Ck19; Krt1-19 – Mouse Genome Informatics), was then analyzed in mammary glands of pregnant mice at day 15 of gestation. In contrast to cultured primary cells exposed to hypoxia, neither Pgk nor Vegf mRNA expression differed significantly between genotypes (Fig. 3A). However, Glut1 expression was decreased by 60%. During secretory differentiation, transcription of markers associated with milk production increase sharply; therefore, to further characterize the defects in differentiation in the epithelial cells, a panel of markers associated with production of milk components was compared using semi-quantitative RT-PCR following normalization to β-actin (Fig. 3B). Two of these markers, β-casein and α-lactalbumin (α-lac), are markers of the casein and whey fraction of milk, respectively. In addition, several markers were analyzed that are associated with the milk lipid globule (MLG). These included xanthine oxidoreductase (XOR; XDH – Mouse Genome Informatics), a redox enzyme immunolocalized to the apical plasma membrane of lactating

![Fig. 2. Defects in secretory differentiation, but not vascular expansion, at day 15 of pregnancy. (A,B) Paraffin wax-embedded sections prepared from wild-type (A) or Hif1a−/− (B) glands isolated at day 15 of pregnancy stained with Hematoxylin and Eosin. Scale bar: 50 μm. Note the striking block in differentiation in the Hif1a−/− glands (B). (C,D) Patterning of the vasculature in relationship to the mammary epithelium after lectin (green) and phalloidin (red) staining in wild-type (C) and Hif1a−/− glands (D).](image-url)
alveoli (McManaman et al., 2002), butyrophilin, a hydrophobic glycoprotein found only in differentiated mammary epithelial cells (Banghart et al., 1998), the cytoplasmic, lipid-droplet-associated adipophilin protein, also known as ADFP (Heid et al., 1996), and perilipin, a marker of the adipose fraction in the mammary gland. Perilipin is normally downregulated over the course of pregnancy as the adipose fraction shrinks (Blanchette-Mackie et al., 1995). Interestingly, recently, Adfp has been identified as a novel hypoxia-inducible gene in MCF-7 cells (Saarikoski et al., 2002). In response to deletion of Hif1α, β-casein, α-lac, Adfp and Xor mRNA levels decreased by over 50% (Fig. 3B), whereas butyrophilin expression remained fairly constant (data not shown) and there was a failure to downregulate perilipin. These results indicate that there are severe deficiencies in expression of markers of milk production.

**Loss of HIF1α blocks secretory activation at the transition to lactation**

By day 18 of gestation, at the cusp of the transition from pregnancy to lactation, large areas of alveoli had failed to differentiate in Hif1a–/– glands, although there were areas of normal development (Fig. 4A,B). To investigate whether the areas of pathology corresponded with expression of Cre, and therefore deletion of HIF1α, Cre immunostaining was performed. As can be seen in Fig. 4C,D, even within an individual lobule, there was varying expression of Cre in the alveolar units. More importantly, the alveoli that expressed Cre were clearly those that were collapsed, and appeared to be undifferentiated. By contrast, the adjacent patches of alveoli that were negative for Cre were distended with milk precursors.

To confirm that loss of HIF1α in the mammary epithelium results in epithelial-specific downregulation of Glut1 during pregnancy, both Glut1 and Cre were sequentially detected via multiple antigen labeling on the same tissue sections (Fig. 4E-G). These studies revealed that only the Cre-negative epithelial cells expressed high levels of Glut1. By contrast, epithelial cells that expressed Cre, and, therefore, were null for...
**HIF1α is required for production and secretion of milk during lactation**

The histology of mammary glands on the date of birth (day 1 of lactation) was compared without prior weaning of the litter. The Hif1α+/− glands exhibited fewer alveoli, which contained fewer milk granules, and increased evidence of trapping of lipid droplets within the epithelial cells (Fig. 5A,B). In contrast to day 18 of gestation, when expression of Cre was non-uniform, almost 100% of the epithelial cells expressed Cre recombinase by the onset of lactation (data not shown).

Glands were then compared at mid-lactation, a period of copious milk production, after a period of weaning to allow milk to fill the gland. Several defects were apparent in Hif1α+/− glands. First, in stark contrast to the wild type glands (Fig. 5C) that contained large, well-developed, expanded alveoli, large areas of fat were present in the Hif1α+/− glands, and the alveolar lumens were small (Fig. 5D). Secretory failure was also evident by the shape of the alveoli. In the Hif1α+/− glands, individual epithelial cells containing large nuclei and scant cytoplasm were still distinguishable. By contrast, in wild-type mice, the pressure of accumulated milk resulted in engorged alveoli evident by the flattened appearance of the epithelium in which the nucleus of each cell is no longer visible. Finally, although fat is normally secreted into milk as small milk fat globules, the alveoli in Hif1α+/− glands contained abnormally large droplets of trapped lipid within the epithelial cells. The reduced numbers of alveoli as well as the decrease in retained milk explain the ~50% decrease in wet weight of the Hif1α+/− inguinal mammary glands collected at mid-lactation (Fig. 5E).

Together, these defects resulted in reduced pup growth and viability. Although the pups contained milk in their stomachs, confirming normal suckling behavior, all of the pups were

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**Fig. 5.** Impaired secretory function at lactation. (A,B) Paraffin wax-embedded sections from mammary glands harvested on the date of birth were stained with Hematoxylin and Eosin. Scale bar: 50 μm. In comparison with wild-type (A), glands lacking Hif1α (B) contained fewer alveoli, which were less differentiated. (C,D) Glands were also harvested at mid-lactation from weaned dams, allowing milk (indicated by yellow stars) to fill the gland. Scale bar: 50 μm. In wild-type mice (C), the accumulation of milk fully distended the alveoli, and a relatively small volume of adipose tissue was present. By contrast, in the Hif1α+/− glands (D), accumulation of milk was minimal, large lipid droplets remained trapped within the epithelial cells (white arrow), and large areas of adipose tissue were visible. (E) Wet weight (±s.e.m.) of frozen inguinal glands harvested from lactating dams. (F) Representative growth curve of litters nursing wild-type (black circles) and Hif1α+/− dams (gray circles). Pronounced defects in pup weight gain were observed by day 3 of lactation that persisted until mid-lactation.
runted compared with wild-type controls, and a majority died within 15 days of birth. In order to more fully characterize the differences in pup growth, pups were weighed every day after birth. As evident in Fig. 5F, the differences in weight were observed as early as day 3 of lactation, and were maintained as lactation progressed. The decrease in growth could be reversed if litters that began nursing from Hif1a\(^{-/-}\) glands were fostered to a wild-type dam instead (data not shown), showing that the failure of the pups to grow resided in defects in the mother. Furthermore, to control for potential deleterious effects that the failure of the pups to grow resided in defects in the mother. Furthermore, to control for potential deleterious effects of Cre expression upon mammary gland development, lactating mice that expressed only the MMTV-Cre transgene were also analyzed. No defects in pup weight gain or mammary gland histology were noted in these dams (n=4), confirming that expression of Cre alone does not impair mammary gland development (data not shown).

**Milk volume is reduced and milk composition is altered as a result of deletion of HIF1α**

To determine if milk quality was affected by deletion of Hif1a, milk was collected from mid-lactation dams and analyzed for percentage of nitrogen, fat, water and lactose, as well as sodium and chloride ion concentrations. Several trends were noted in collection of milk from Hif1a\(^{-/-}\) glands. First, the milk was more difficult to collect, was more viscous and was more difficult to dissolve into the water at collection, suggesting a high fat content. Furthermore, less total volume could be collected from the Hif1a\(^{-/-}\) glands (Fig. 6A). No statistical differences were observed in protein content, whereas the amount of lactose and fat varied widely (data not shown). However, highly significant differences in water content and ion content were observed (Fig. 6A). Water content was decreased and the [Na\(^+\)] and [Cl\(^-\)] were greatly elevated in milk from Hif1a\(^{-/-}\) glands relative to milk of wild-type glands. These changes reflected an ionic concentration closer to that observed in plasma, and indicated a fundamental failure to regulate mammary secretion properly.

**Changes in gene expression at mid-lactation**

Expression of HIF1 targets was also analyzed at mid-lactation following normalization to Ck19. In contrast to mid-pregnancy, Pkg expression was downregulated by 67% in mid-lactation Hif1a\(^{-/-}\) glands, but there were no significant changes in Glut1 expression (Fig. 6B). The decreased water content and increased [Na\(^+\)] and [Cl\(^-\)] observed in mid-lactation milk collected from Hif1a\(^{-/-}\) dams are hallmarks of tight junction closure failure (Stelwagen et al., 1999). The family of claudin proteins is implicated in regulation of TJ strand composition (Furuse et al., 2001; Morita et al., 1999); therefore, the expression of mammary epithelial cell-specific claudin proteins was compared in mid-lactation mice. Claudin 7 and claudin 8 were specifically chosen because, relative to epithelial cell content, claudin 8 expression increases as the mammary gland progresses from mid-pregnancy to functional lactation, whereas the expression of claudin 7 remains constant (B. Blackman and M. C. Neville, unpublished observations). As expected, claudin 7 expression remained constant between wild type and Hif1a null glands (Fig. 6C); however, expression of claudin 8 decreased by 60%.

**A requirement for HIF1α in the mammary epithelium**

To confirm that the defects observed in Hif1a null glands were epithelial cell-autonomous, primary Hif1a\(^{+/+}\) MEC were infected with an adenoviral vector expressing either β-galactosidase (control, wild type MEC) or Cre recombinase (generating Hifα\(^{-/-}\) MEC). Infection with Adeno-Cre induced deletion of Hif1a in over 99% of cells (data not shown). The wild-type and Hif1a\(^{-/-}\) MEC were then transplanted into the right and left cleared, inguinal fat pads, respectively, of 3-week-old female immunocompromised Rag1\(^{-/-}\) host mice, using a technique previously described by Rijnkels and Rosen (Rijnkels and Rosen, 2001). After outgrowth of the transplanted cells into the Hif1a wild-type stroma for a period of 12 weeks, the hosts were then mated and the outgrowths harvested from hosts on the date of birth of pups. Transplanted wild-type cells successfully differentiated and secreted milk (Fig. 7A, purple granules). However, the Hif1a\(^{-/-}\) outgrowths contained small, poorly differentiated alveoli with collapsed lumens, and retained lipid droplets in the cytoplasm (Fig. 7B). In addition, the alveoli were surrounded by increased connective tissue (stained blue). Therefore, the histology of the Hif1a\(^{-/-}\) alveoli, which regenerated in the presence of Hif1a wild-type stroma, recapitulated the phenotypes observed in intact Hif1a-null glands, confirming that HIF1α acts in an epithelial cell-autonomous manner to control mammary gland development. Glut1 immunostaining was also performed on paraffin wax-embedded sections from the transplanted outgrowths. As shown in Fig. 7C, expression of Glut1 was uniformly detected in the wild type alveoli. By contrast, in the Hif1a null alveoli (Fig. 7D), expression of Glut1 was decreased and patchy. Therefore, the defects in mammary gland development and physiology resulting from deletion of Hif1a...
Fig. 7. The effects of deletion of HIF1α are mammary epithelial cell autonomous. Primary Hif1a+/+ mammary epithelial cells were infected with either Adeno-βgal or Adeno-Cre and transplanted into the cleared fat pads of female host mice in order to generate wild-type (A) and Hif1a–/– (B) epithelial outgrowths, respectively. Paraffin wax-embedded sections harvested from mice on the date of birth (without prior weaning of pups) were stained with Mason’s Trichrome. Scale bar: 50 μm (n=3 mice with 100% outgrowths/genotype). Outgrowths derived from Hif1a–/– mammary epithelial cells recapitulated the phenotype observed in intact Hif1a–/– glands. Note the lack of milk products (indicated by yellow stars) in the Hif1a–/– outgrowth, and the presence of large, trapped lipid droplets within the epithelial cells (B, right white arrow). In addition, there was an abnormal thickening of collagen fibers (stained blue; B, left white arrow) around the alveoli in these outgrowths compared with wild type (black arrow). (C, D) Glut1 (brown staining) was detected in paraffin wax-embedded sections prepared from transplanted outgrowths. Compared to wild type (C), expression of Glut1 in the Hif1a null outgrowths (D) was less intense and more patchy.

via MMTV-Cre were not due to defects in the stroma or deletion in other tissues.

DISCUSSION

We have demonstrated a requirement for HIF1α-mediated transcription in the mammary epithelium in order to produce and to secrete milk. These results indicate a novel role for HIF1α in the control of the critical transition from secretory differentiation to secretory activation and of the composition and secretion of milk at lactation. In contrast to our expectations, no changes in microvessel patterning, density or VEGF expression were noted in vivo in response to Hif1a deletion, demonstrating that the angiogenesis that occurs in the mammary gland during pregnancy is HIF1α-independent.

Deletion of Hif1a did not impact ductal morphogenesis in nulliparous mice or the proliferation of alveoli during pregnancy. Instead, defects in differentiation were observed by histology beginning at day 15 of gestation. Loss of HIF1α inhibited the expression of markers critical to secretory function, including several milk protein and milk fat globule markers. For example, expression of Xor mRNA was dramatically reduced at day 15 of gestation in Hif1a–/– glands. This result is intriguing in light of a recent report that mice heterozygous for Xor fail to properly secrete lipid into milk at lactation, ultimately resulting in pup death (Vorbach et al., 2002), a phenotype similar to that observed in response to deletion of HIF1α. In addition, expression of Adfp mRNA, which has recently been shown to be induced up to 70-fold by hypoxia in MCF-7 cells (Saarikoski et al., 2002), was also reduced by 50% in glands null for Hif1a.

Furthermore, as observed at day 18 of gestation, the failure of the alveoli to differentiate and to produce milk components corresponded completely with the expression pattern of Cre. Hence, the pronounced block in differentiation is due to loss of Hif1a. Consistent with this finding, transcriptional activity, as measured by RNA content per gram of tissue, was reduced by 50%. Because of the tight association observed between the presence or absence of Hif1a and the production of milk components in preparation for lactation, HIF1α is a critical regulator of the process of secretory differentiation in the mammary gland.

In addition, it is important to note that mammary gland DNA content was similar between genotypes at the end of pregnancy, and that no differences in rates of epithelial cell proliferation were noted at either day 15 or day 18 of gestation (data not shown). Therefore, the observed differences in histology of pregnant mice must have resulted from a failure to accumulate milk products in preparation for lactation. Of note, this mouse model is the first to date to describe defects in differentiation during pregnancy without accompanying changes in mammary epithelial cell proliferation.

With respect to loss of HIF1 function, the selective decrease of Glut1 mRNA expression by 60% at day 15 of gestation, as well as the decreased levels of Glut1 protein noted at day 18 of gestation, may explain both the defects in differentiation and lipid metabolism observed during pregnancy in Hif1a–/– mice. Normally, Glut1, the exclusive glucose transporter used in the mammary epithelium at lactation, is considerably upregulated during secretory differentiation in order to increase glucose availability (Camps et al., 1994). This is crucial because glucose is a required substrate for the production of lactose, the primary carbohydrate in milk. Furthermore, glucose transport has been proposed to be a rate-limiting factor in glucose use in the mammary gland (Threadgold and Kuhn,
1984). And, in rodents and other animals lacking the acetate-based fatty acid synthetic pathway, glucose is also used for the production of fatty acid precursors. Therefore, loss of HIF1α function during pregnancy may deprive the gland of the glucose it needs to differentiate.

The severity of the defects observed during pregnancy was demonstrated during lactation since dams lacking HIF1α in the mammary epithelium were unable to support nursing pups. Normally, secretory activity peaks at mid-lactation, but, as evident by histology, the HIF1α null alveoli contained relatively few milk granules, and large lipid droplets that are normally secreted into milk as micro-droplets remained trapped within the epithelial cells. As a consequence of defective secretion, the glands yielded less milk volume and milk nutrition was poor. In addition, the sodium and chloride content of milk was elevated, resembling concentrations observed in plasma. These changes are hallmarks of mastitis, in which the normally closed tight junctions become permeable (Nguyen and Neville, 1998). As the claudin proteins are implicated in tight junction strand regulation (Furuse et al., 2002), and claudin 8 expression was downregulated by 50%, HIF1α may also play a role in tight junction closure.

Although it is possible that the severe block in differentiation observed in the HIF1α null mammary gland prevented the transition to secretory activation, based on the previously described functions of HIF1α (Semenza, 1999), it is more likely that loss of HIF1α impaired metabolic activity at the time of highest demand: lactation. In support of this hypothesis, at mid-lactation, the normalized expression of PGK was reduced by over 67% in the HIF1α null glands.

During peak lactation, HIF1α mediation of glycolytic activity may be necessary to supplement energy production since synthesis and transport of milk components, as well as tight junction closure, are energy-dependent processes. This proposed function for HIF1α in the mammary epithelium is also supported by previous observations that increases in glycolytic enzyme activities occur at lactation (Mazurek et al., 1999). Therefore, glycolysis is likely to be necessary to maintain energy production at lactation. Interestingly, previous studies have shown that inhibitors of glucose metabolism interfere with lactation. Administration of 2-deoxyglucose, which inhibits glucose-6-phosphate metabolism in the lactating rat mammary gland, reduced lactose synthesis, as well as protein synthesis and secretion (Sasaki and Keenan, 1978). Thus, HIF1α-dependent regulation of glucose metabolism may be necessary for achieving differentiation during pregnancy as well as the high metabolic rate in the mammary gland at lactation. Based on our observations of HIF1α transcriptional activity, it is possible that during pregnancy, when energy demands are lower than for lactation, successful differentiation may depend more upon Glut1 function than that of PGK. Conversely, during lactation, regulation of PGK may become more critical. The mechanisms underlying the differential impact of deletion of HIF1α on Glut1 and PGK mRNA expression during pregnancy versus mid-lactation are not clear, and will require further investigation.

In Hif1α−/− epithelial cell cultures exposed to hypoxia, VEGF mRNA decreased by ~50%. However, we were unable to detect any changes in MVD in the intact mammary gland in vivo, either by qualitative analysis of the vasculature or by Chalkley counts after CD31 immunostaining. In addition, there was no significant decrease in VEGF mRNA expression in Hif1α−/− glands at day 15 of gestation. As loss of HIF1α did not impact vasculature expansion, the mechanisms regulating angiogenesis during normal mammary gland development must be HIF1α-independent. The absence of an effect on angiogenesis in response to deletion of HIF1α is in agreement with previous results obtained by our laboratory, which showed that the MVD of both developing bone as well as fibrosarcomas remain equivalent to wild type when HIF1α is conditionally deleted (Ryan et al., 2000; Schiapani et al., 2001).

Low levels of HIF1α were detectable in primary mammary epithelial cells cultured at normoxia. HIF1α stability was dramatically increased by hypoxic treatment and loss of HIF1α diminished HIF1α transcriptional activity; therefore, the hypoxic response is intact in normal mammary epithelium. Interestingly, it has been shown that HIF2α, which is structurally related to HIF1α, may also induce expression of HRE-based reporter constructs in a hypoxia-dependent manner (Wiesener et al., 1998); therefore, it is possible that HIF2α may play some role in mammary gland development. However, although both Hif1α and Hif2α transcripts are expressed throughout mammary gland development, and neither gene shows marked fluctuation in expression levels, Hif2α mRNA is expressed at levels approximately one-tenth of those of Hif1α (M.N., unpublished observations). In addition, we argue that as expression of HIF1 targets was reduced to basal levels in hypoxically stimulated primary cells lacking HIF1α, HIF2α plays little, if any, role in transcriptional regulation of HIF target genes in the mammary gland. Furthermore, because HIF2α was unable to compensate for loss of HIF1α during pregnancy and lactation in vivo, it is not likely that HIF2α is a critical mediator of mammary gland development.

Although hypoxia is the classic inducer of HIF1α stability, we have been unable to document the presence of hypoxia in Hif1α wild-type and null mammary tissue using reagents known to detect DNA adducts created by hypoxia, such nitroimidazole (EP5) (Evans et al., 1996). Although waves of hypoxia were readily detectable in mammary tumors, no areas of hypoxia could be detected in the normal mammary gland at any stage of development (data not shown). However, detection of adducts created by hypoxia via immunostaining may not be as sensitive in normal tissue, which can more readily adapt to hypoxia than a rapidly dividing tumor with a necrotic center.

Alternatively, hypoxia per se may not be a stimulus of HIF1α activity in the mammary gland. Several laboratories have reported that in vitro HIF1α protein is also stabilized upon treatment with insulin, insulin-like growth factor 1 (IGF1), IGF2 or activation of HER2/neu receptor upon addition of heregulin – all potent cell survival factors/mitogens for normal and breast cancer cell lines (Fieldser et al., 1999; Laughner et al., 2001; Zettl et al., 1992). Additionally, a positive feedback loop between HIF1α and IGF2 transcription has been reported in human 293 cells and in mouse embryonic fibroblasts (Fieldser et al., 1999). These observations are noteworthy, as IGF2 may function as a local, paracrine mitogen in developing alveoli (Wood et al., 2000). Furthermore, it has been demonstrated that the end products of glycolysis itself, pyruvate and lactate, can induce HIF1α protein stability even under aerobic conditions (Lu et al., 2002).

Recently, Le Provost et al. have deleted the partner of Hif1α, Arnt, in the mammary gland (Le Provost et al., 2002). Deletion
of Arnt blocked early alveolar development and impaired fertility (Le Provost et al., 2002). Based on these results, as well as transplantation of transgenic tissues into the cleared fat pads of host mice, it was argued that deletion of Arnt affects mammary gland development through uncharacterized, indirect effects in the ovary, although there were no differences in circulating estrogen and progesterone levels (Le Provost et al., 2002). We have not noted any differences in ovarian histology or detected recombination of the Hif1a locus in the ovaries of transgenic mice. Furthermore, in comparison with deletion of Arnt, loss of Hif1a impacted relatively late stages of mammary gland development. These differences are perplexing, as HIF1α partnering with ARNT is required for HIF1 activity. It is possible that Arnt function may be compensated for by other family members that complex with HIF1α, such as ARNT2 (Keith et al., 2001) or ARNT3 (ARNTL – Mouse Genome Informatics) (Takahata et al., 1998). Nevertheless, transplantation of Hif1a−/− epithelium into Rag1−/− female hosts revealed that the defects associated with deletion of Hif1a were mammary epithelial cell autonomous. Therefore, even if low, but undetectable, levels of recombination of the Hif1a locus were present in the ovaries in this line of transgenic mice, they have no impact on the phenotype in the mammary gland.

Because deletion of Hif1a specifically inhibited the synthesis of milk components during pregnancy and milk production and secretion at lactation, we argue that HIF1 activity is essential for the transition from pregnancy to functional lactation, and is also required for the maintenance of normal lactation and production of milk. Furthermore, these defects are manifested independently of regulation of angiogenesis through VEGF.

Although overexpression of HIF1α has been documented in breast tumors compared with normal tissues (Bos et al., 2001; Zhong et al., 1999), it is not clear if this contributes to tumorigenesis or is an effect of hypoxia induced by rapid proliferation. Future experiments to compare the gene expression profiles of normal mammary tissue versus mammary tumors will be useful in determining the complexity of HIF1α regulation of epithelial cell biology and secretion. As there are significant differences in how primary cells and tumor cells respond to hypoxia (Brown and Giaccia, 1998), the differential pathways that regulate these processes may prove to be excellent targets for tumor-specific, hypoxia-responsive therapeutic drugs.

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