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During pregnancy the mammary epithelium and its supporting vasculature rapidly expand to prepare for lactation. To investigate the role of oxygenation and metabolism in these processes the oxygen-responsive component of the hypoxia-inducible factor (HIF)-1 complex, *HIF-1α*, was deleted in the murine mammary gland using the Cre/*lox*P system. Although vascular density remained similar, loss of *HIF-1α* impaired mammary differentiation and lipid metabolism, culminating in lactation failure and changes in milk composition. Reduced expression of classical HIF-1 target genes as well as multiple markers of differentiation were observed. Transplantation experiments confirmed that these developmental defects were epithelial cell-autonomous. Based on these novel results, we suggest that HIF-1α plays a critical role in the differentiation and function of the mammary epithelium.

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Table of Contents

Introduction

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

Under normoxic conditions, HIF-1α protein is rapidly degraded through targeted ubiquitination mediated by direct binding of its oxygen dependent domain (ODD) to the β subunit of von Hippel Lindau (VHL) tumor suppressor protein (reviewed in (Kondo and Kaelin, 2001)). In response to hypoxia, HIF-1α protein accumulates, due to decreased interaction with VHL (Krek, 2000). An increase in HIF-1α protein is first detectable at partial pressures of oxygen equivalent to 6% O_2, and becomes maximal between 0.5-1.0% O_2 (Stroka et al., 2001). In a hypoxic environment, HIF-1 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 in (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 patients with VHL disease, an autosomal, dominantly inherited cancer syndrome. These patients often develop renal clear cell carcinomas that are massively hypervascular, with highly elevated levels of VEGF expression due to constitutive HIF-1 activity (Kondo and Kaelin, 2001).

Recently, HIF-1α has been demonstrated to be up-regulated 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 HIF-1α protein was over-expressed 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 previous that HIF-1α functions as a positive regulator of tumor growth (Ryan et al.,

2000; Seagroves and Johnson, 2002). In a subsequent study, the level of HIF-1α expression in breast tumors was correlated with other prognostic factors. Specifically, in ductal carcinoma *in situ* (DCIS) lesions, relatively high levels of HIF-1α expression were associated with increased proliferation as well as increased expression of VEGF and the estrogen receptor (Bos et al., 2001). On the other hand, HIF-1α expression did not correlate with p53 expression, supporting our own laboratory's observations that p53 expression is independent of the effects of loss of *HIF-1α* on cell growth, metabolism or tumorigenesis (Ryan et al., 2000).

Because tumor cells exist in a more hypoxic environment than normal cells, therapies could be developed that target tumor cells specifically for destruction without serious side effects for normal cells (Brown and Giaccia, 1998), especially if administered in combination with drugs that inhibit angiogenesis. HIF-1α has been demonstrated to regulate genes implicated in both cellular metabolism and angiogenesis. Therefore, an enhanced understanding of the molecular mechanisms of HIF-1α function could potentially result in development of new compounds that control mammary epithelial cell fate, which has significant clinical and therapeutic value. In order to provide a foundation for understanding the role of HIF-1 in mammary tumorigenesis, the function of HIF-1α was investigated during normal mammary gland development.

Several laboratories, including our own, have demonstrated that HIF-1α 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 HIF-1, there were several compelling reasons to study HIF-1α 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 intersucception (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, 2002). 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 since 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 HIF-1 and its subunit HIF-1α 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, HIF-1α may be required to alleviate transient hypoxia through angiogenesis, increased dependence on glycolysis, or regulation of substrates for the production of milk. In order to test this hypothesis, we have specifically removed *HIF-1α* from the mammary epithelium using previously characterized HIF *floxed* mice (Ryan et al., 1998) 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 failure of the dams to provide milk for their pups.

Report Body

a) Summary of research objectives

The specific objectives of this project, described in the original proposal are listed below:

Objective 1: To determine the function of HIF-1α during normal mammary development

The majority of the experiments as described in objective 1 of the proposal have been successfully completed, leading to the preparation of a manuscript for publication.

Objective 2: To determine if deletion of HIF-1α suppresses mammary tumorigenesis

Because the focus of the first year of the grant was to analyze defects in normal mammary gland development (Objective 1), these studies have not yet been initiated. However, we have received approval for the DMBA treatment protocol from Animal Subjects Committee.

b) Results (Please refer to attached manuscript draft for figures)

Objective 1: To determine the function of HIF-1α during normal mammary development

HIF-1α is expressed in normal primary mammary epithelial cells and is required for hypoxia-inducible gene transcription

To determine if HIF-1α is expressed in the normal murine mammary epithelium, mammary epithelial cells (MEC) were purified from wild type mid-pregnant C57BL/6:129-Sv mice, cultured under hypoxic conditions, and the extracts probed for HIF-1α protein. In contrast to previous reports that HIF-1α is undetectable in normal human breast tissue (Zhong et al., 1999), low levels of HIF-1α protein were detectable in nuclear extracts of purified murine MEC cultured at normoxia (21% 0_2). Robust induction of HIF-1α was observed in response to culture under hypoxic conditions (**Figure 1A**).

To determine if HIF-1 is essential for hypoxia-induced transcription in mammary epithelial cells, the expression levels of *PGK*, *Glut-1* and *VEGF* were compared in primary cultures of wild type and *HIF-1α*$^{-/-}$ cells cultured at normoxia or hypoxia by real-time detection PCR (RTD-PCR). **Figure 1B**). Under hypoxia loss of *HIF-1α* resulted in minimal induction of *PGK* mRNA and the hypoxia-inducible expression of both *Glut-1* and total *VEGF* was reduced by at least 50%. These results demonstrated that regulation of hypoxic response acts through HIF-1 in mammary epithelium.

Conditional deletion of HIF-1α in the mouse mammary gland

To determine whether HIF-1 function is required *in vivo* for normal mammary gland development, a conditional gene deletion strategy was employed to delete *HIF-1α* in the mammary epithelium of mice. Mice harboring two *floxed* alleles of the *HIF-1α* locus ((Ryan et al., 1998); *HIF DF*) were bred with *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. The temporal-spatial pattern of Cre recombinase activity in this line of mice (MMTV-Cre, line A) has been extensively described (Wagner et al., 2001). Southern blot analysis comparing wild type and *HIF-1α* deleted mammary tissue indicated that MMTV-Cre consistently targets deletion of *HIF-1α* in at least 50% of MEC (**Figure 1C**). By lactation, a majority of MEC have been targeted for recombination via MMTV-Cre, therefore, the mammary glands isolated from *HIF-1α floxed* Cre+ mice will be referred to as *HIF-1α* null (*HIF-1α*$^{-/-}$). Further, in contrast to reports that this line of MMTV-Cre transgenic mice induces excision in the ovary of mature mice (Wagner et al., 2001), no recombination of the HIF-1α locus could be detected in DNA prepared from whole ovaries of HIF-1α deleted mice either by Southern blotting (**Figure 1C**) or by RTD-PCR.

During pregnancy, deletion of HIF-1α impairs secretory differentiation, but not vascular expansion

In order to pinpoint the stage of mammary gland development at which HIF-1α function may be required, mammary tissue was harvested from mice over the course of gestation. No differences in ductal morphogenesis were noted in nulliparous mice (data not shown). At day 10 of gestation (mid-pregnancy), a stage of development prior to differentiation, no defects in histology were observed in *HIF-1α*$^{-/-}$ glands at either the gross or microscopic level, indicating that HIF-1α is not critical for early rounds of alveolar proliferation (data not shown). Similarly, by day 12 of pregnancy, when secretory differentiation typically begins in most mouse strains, no differences in morphology were noted.

However, by day 15 of pregnancy, well into the period of secretory differentiation, although the glands of either genotype were indistinguishable at the whole mount level (data not shown), histological examination revealed significant abnormalities in the *HIF-1α*$^{-/-}$ glands (**Figure 2A-B**). In particular, the protein and lipid droplets that gave the wild type epithelium a

"lacey" appearance were completely absent in the *HIF-1α*$^{-/-}$ glands. In addition, null alveoli were small, 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, since 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 HIF-1 has been implicated in the control of angiogenesis (Forsythe et al., 1996) we next analyzed the effect of deletion of HIF-1 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 (**Figure 2C-D**). Surprisingly, we observed no gross differences in microvessel patterning, or vascular density in relationship to the epithelium in *HIF-1α*$^{-/-}$ mammary glands.

Expression profiles of HIF-1 targets and markers of differentiation in pregnant mice

The expression of HIF-1 targets was also 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* expression differed significantly between genotypes (**Figure 3A**). However, *Glut-1* 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 (**Figure 3B**). Two of these markers, β–casein, a major milk protein, and alpha-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 oxidase (XO), a redox enzyme (Jarasch et al., 1981), butyrophilin, a hydrophobic glycoprotein found only in differentiated mammary epithelial cells (Banghart et al., 1998), adipocyte differentiation-related protein (ADRP, or adipophilin) (Heid et al., 1996), and perilipin, a marker of the adipose fraction in the mammary gland. Perilipin is normally down-regulated over the course of pregnancy as the adipose fraction shrinks (Blanchette-Mackie et al., 1995). In response to deletion of *HIF-1α*, *β-casein*, *α-lactalbumin*, *ADRP*, and *XO* mRNA levels decreased by over 50%, whereas *butyrophilin* expression remained fairly constant, and there was a failure to down-regulate *perilipin.* This indicates that there were severe deficiencies in markers of milk production.

Loss of HIF-1α blocks secretory activation at the transition to lactation

In null glands at day 18 of gestation, at the cusp of the transition from pregnancy to lactation, large areas of alveoli had failed to differentiate although there were areas of normal development (**Figure 4A-B**). To investigate whether the areas of pathology corresponded with expression of Cre recombinase, and therefore deletion of *HIF-1α*, Cre immunostaining was performed. As can be seen in **Figure 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. In contrast, the adjacent patches of alveoli that were negative for Cre were distended with milk precursors.

Although there were fewer alveoli present per field in the *HIF-1α*$^{-/-}$ glands, it was possible that this was an artifact due to the lack of alveolar cell expansion associated with differentiation. In order to compare cellularity as well as secretory activity, the amount of DNA, RNA and protein produced by these tissues per gram of tissue was quantified. It has been previously demonstrated in the mammary gland that DNA content/g tissue correlates with cellularity (Knight and Peaker, 1982). As can be seen in **Figure 4D**, there was no significant difference in DNA content between genotypes of mammary tissue, suggesting that epithelial cell number is approximately equivalent at this stage of development. In support of these results, no significant differences in the rate of proliferation of epithelial cells were observed at this stage of development (data not shown). However, there was a significant decrease in the amount of RNA produced by *HIF-1α*$^{-/-}$ glands at day 18 of pregnancy. . Finally, there was trend for decreased production of protein in *HIF-1α* null glands, though due to animal-to-animal variability, this difference did not reach statistical significance.

Because mammary epithelium-associated angiogenesis is completed by the end of pregnancy, we next analyzed microvessel density (MVD) of the glands at 18-P. Vessels were visualized by immunostaining with anti-CD31 antibodies, and the density of the vessels calculated by Chalkley counting as described previously (Ryan et al., 2000). As expected based on the results of lectin staining at day 15 of pregnancy, there were no significant differences in MVD of the mammary gland between genotypes (**Figure 4F**).

HIF-1α 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 *HIF-1α*$^{-/-}$ glands exhibited fewer alveoli that contained fewer secretory vesicles and lipid droplets within the lumens (**Figure 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 (data not shown).

Glands were then compared at mid-lactation, a period of copious milk production, following a period of weaning of the suckling pups to allow milk to fill the gland. Several defects were apparent in HIF-1α$^{-/-}$ glands. First, in stark contrast to the wild type glands (**Figure 5C**) that contained large, well-developed, expanded alveoli, large areas of fat were present in the *HIF-1α*$^{-/-}$ glands, and the alveolar lumens were small (**Figure 5D**). Secretory failure was also evident by the shape of the alveoli. In the *HIF-1α*$^{-/-}$ glands, individual epithelial cells containing large nuclei and scant cytoplasm were still distinguishable. In 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 *HIF-1α*$^{-/-}$ glands contained abnormally large droplets of trapped lipid within the epithelial cells. The reduced numbers of alveoli as well as the decrease of retained milk explain the approximately 50% decrease in wet weight of the *HIF-1α* null inguinal mammary glands collected at mid-lactation (**Figure 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 runted compared to 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 **Figure 5F**, the differences 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 *HIF-1α*$^{-/-}$ 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.

Milk volume is reduced and milk composition is altered as a result of deletion of HIF-1α

To determine if milk quality was affected by deletion of *HIF-1α*, milk was collected from mid-lactation dams into a tared vial 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 *HIF-1α*$^{-/-}$ glands. First, the milk was more difficult to collect from these dams, was more viscous and was more difficult to dissolve into the water at collection, suggesting a high fat content. Further, less total volume could be collected from the HIF-1α$^{-/-}$ glands (**Figure 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 (**Figure 6A**). Water content was decreased and the [Na^+] and [Cl^-] were greatly elevated in milk collected from HIF-1α$^{-/-}$ glands relative to milk of wild type glands. These changes reflected an ionic concentration closer to that observed in plasma, and indicate a fundamental failure to properly regulate mammary secretion.

Changes in gene expression at mid-lactation

Expression of HIF-1 targets was also analyzed at mid-lactation. In contrast to mid-pregnancy, *PGK* expression was down-regulated by 67% in mid-lactation *HIF-1α*$^{-/-}$ mice, and there were no significant changes in *Glut-1* expression (**Figure 6B**). The decreased water content and increased [Na] and [Cl] in milk observed in mid-lactation milk are hallmarks of tight junction closure failure (Stelwagen et al., 1999), and, the family of claudin proteins are implicated in regulation of TJ strand composition (Furuse et al., 2001; Morita et al., 1999). Therefore, the expression of mammary epithelial cell-specific TJ claudins was compared in mid-lactation mice. *Claudin 7* and *claudin 8* were specifically chosen because, relative to epithelial cell content, c*laudin 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 null glands (**Figure 6B**), however, expression of *claudin 8* decreased by 60%.

A requirement for HIF-1α in the mammary epithelium

To confirm that the defects observed in *HIF-1α* null glands were epithelial cell autonomous, primary *HIF-1α floxed* mammary epithelial cells were infected with either an adenoviral vector expressing beta-galactosidase (wild type control) or an adenoviral vector expressing Cre recombinase. Infection with Cre induced deletion of *HIF-1α* in over 99% of cells (data not

shown). The wild type and null mammary epithelial cells were then transplanted into the right and left cleared inguinal fat pads, respectively, of 3-week old immunocompromised *RAG-1*$^{-/-}$ host mice, using a technique previously described by Rjinkels et al (Rijnkels and Rosen, 2001). Following outgrowth, the hosts were mated and the transplants harvested from the transplanted females on the date of birth of their litters. Transplanted wild type cells successfully differentiated and secreted milk (Figure 7A, purple granules). However, the *HIF-1α* null outgrowths contained relatively few alveoli; which were small and poorly differentiated with collapsed lumens, and retained lipid droplets in the cytoplasm (**Figure 7B**). In addition, the alveoli were surrounded by increased connective tissue (stained blue). Therefore, the histology of the transplanted null outgrowths recapitulated that observed in intact *HIF-1α* null glands. These results confirmed that the defects resulting from deletion of *HIF-1α* are mammary epithelial cell autonomous, and were not due to defects in the stroma or deletion in other tissues.

Key Research Accomplishments:

- We have confirmed that HIF-1α protein is expressed in purified mammary epithelial cells and is induced by hypoxia
- We have confirmed that the hypoxic response is conserved in mammary epithelial cells
- We have demonstrated a role for HIF-1α in secretory differentiation of alveoli
- We have demonstrated that loss of HIF-1α blocks milk production and impairs milk secretion
- We have demonstrated that loss of HIF-1α impacts milk nutrition
- We have discovered the first mouse model to separate mammary epithelial cell proliferation from differentiation
- We have demonstrated that the angiogenesis that occurs during pregnancy during normal mammary gland development is HIF-1α-independent

-We have prepared a manuscript for submission to Developmental Cell by November 1, 2002.

Reportable Outcomes:

Manuscripts:

HIF-1α is a critical regulator of functional differentiation and secretory activation, but not vascular expansion, in the mouse mammary gland. Tiffany N. Seagroves, Darryl Hadsell, Jim McMananman, Carol Palmer, Debbie Liao, Wayne McNulty, Bryan Welm, Kay-Uwe Wagner[5], Margaret Neville, and Randall S. Johnson. Prepared for submission to Developmental Cell, November 1, 2002.

Abstracts:

* Keystone Conference, Angiogenesis, Banff, Canada, February 2002.
* Gordon Conference on Mammary Gland Biology, Barga, Italy, 2002.

Presentations:

* Department of Defense, Era of Hope meeting, Orlando, FL, 2002.

Funding applied for, based on work supported by this award:

We have used the work presented in this report to apply for a renewal of NIH CA 82515, which if awarded, would be used to supplement costs associated with this project.

Summary and Conclusions

We have demonstrated a requirement for HIF-1-mediated transcription in the mammary epithelium in order to produce and to secrete milk. These results point to a novel role for *HIF-1α* 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 total VEGF expression were noted in response to *HIF-1α* deletion, confirming that the angiogenesis that occurs in the mammary gland during pregnancy is HIF-1-independent.

Although it is possible that the severe block in differentiation observed in the *HIF-1α* null mammary gland prevented the transition to secretory activation, based on the previously described functions of HIF-1 (Semenza, 1999), it is more likely that loss of *HIF-1α* impaired

metabolic activity at the time of highest demand, lactation. In support of this hypothesis, at this stage of development, the normalized expression of *PGK* was reduced by over 67%, whereas *Glut-1* decreased slightly. HIF-1 mediation of glucose metabolism may be necessary to supplement energy production at lactation since synthesis and transport of milk components, as well as tight junction closure, are energy-dependent processes. This function for HIF-1 is also supported by previous observations that increases in glycolytic enzyme activities occur at lactation (Mazurek et al., 1999). Therefore, glycolysis may be necessary to supplement 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, HIF-1-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.

What does this work mean?

Although over expression of HIF-1α has been documented in breast tumors compared to 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 may be useful in determining the complexity of HIF-1α regulation of epithelial cell biology and secretion. Since 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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HIF-1α is a critical regulator of functional differentiation and secretory activation, but not vascular expansion, in the mouse mammary gland

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Summary

During pregnancy the mammary epithelium and its supporting vasculature rapidly expand to prepare for lactation. To investigate the role of oxygenation and metabolism in these processes the oxygen-responsive component of the hypoxia-inducible factor (HIF)-1 complex, *HIF-1α*, was deleted in the murine mammary gland using the Cre/*lox*P system. Although vascular density remained similar, loss of *HIF-1α* impaired mammary differentiation and lipid metabolism, culminating in lactation failure and changes in milk composition. Reduced expression of classical HIF-1 target genes as well as multiple markers of differentiation were observed. Transplantation experiments confirmed that these developmental defects were epithelial cell-autonomous. Based on these novel results, we suggest that HIF-1α plays a critical role in the differentiation and function of the mammary epithelium.

Introduction

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

Under normoxic conditions, HIF-1α protein is rapidly degraded through targeted ubiquitination mediated by direct binding of its oxygen dependent domain (ODD) to the β subunit of von Hippel Lindau (VHL) tumor suppressor protein (reviewed in ((Kondo and Kaelin, 2001)). In response to hypoxia, HIF-1α protein accumulates, due to decreased interaction with VHL (Krek, 2000). An increase in HIF-1α protein is first detectable at partial pressures of oxygen equivalent to 6% O_2, and becomes maximal between 0.5-1.0% O_2 (Stroka et al., 2001). In a hypoxic environment, HIF-1 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 in (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 patients with VHL disease, an autosomal, dominantly-inherited cancer syndrome. These patients often develop renal clear cell carcinomas

that are massively hypervascular, with highly elevated levels of VEGF expression due to constitutive HIF-1 activity (Kondo and Kaelin, 2001).

Recently, HIF-1α has been demonstrated to be up-regulated 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 HIF-1α protein was over-expressed 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 previous that HIF-1α functions as a positive regulator of tumor growth (Ryan et al., 2000; Seagroves and Johnson, 2002). In a subsequent study, the level of HIF-1α expression in breast tumors was correlated with other prognostic factors. Specifically, in ductal carcinoma *in situ* (DCIS) lesions, relatively high levels of HIF-1α expression were associated with increased proliferation as well as increased expression of VEGF and the estrogen receptor (Bos et al., 2001). On the other hand, HIF-1α expression did not correlate with p53 expression, supporting our own laboratory's observations that p53 expression is independent of the effects of loss of *HIF-1α* on cell growth, metabolism or tumorigenesis (Ryan et al., 2000). In order to provide a foundation for understanding the role of HIF-1 in mammary tumorigenesis, the function of HIF-1α was investigated during normal mammary gland development.

Several laboratories, including our own, have demonstrated that HIF-1α 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 HIF-1, there were several compelling reasons to study HIF-1α 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 intersucception (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, 2002). 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 since 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 HIF-1 and its subunit HIF-1α 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, HIF-1α may be required to alleviate transient hypoxia through angiogenesis, increased dependence on glycolysis, or regulation of substrates for the production of milk. In order to test this hypothesis, we have specifically removed *HIF-1α* from the mammary epithelium using previously characterized HIF *floxed* mice (Ryan et al., 1998) 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 failure of the dams to provide milk for their pups.

Results

HIF-1α is expressed in normal primary mammary epithelial cells and is required for hypoxia-inducible gene transcription

To determine if HIF-1α is expressed in the normal murine mammary epithelium, mammary epithelial cells (MEC) were purified from wild type mid-pregnant C57BL/6:129-Sv mice, cultured under hypoxic conditions, and the extracts probed for HIF-1α protein.. In contrast to previous reports that HIF-1α is undetectable in normal human breast tissue (Zhong et al., 1999), low levels of HIF-1α protein were detectable in nuclear extracts of purified murine MEC cultured at normoxia (21% 0_2). Robust induction of HIF-1α was observed in response to culture under hypoxic conditions (**Figure 1A**).

To determine if HIF-1 is essential for hypoxia-induced transcription in mammary epithelial cells, the expression levels of *PGK*, *Glut-1* and *VEGF* were compared in primary cultures of wild type and *HIF-1α*$^{-/-}$ cells cultured at normoxia or hypoxia by real-time detection PCR (RTD-PCR). **Figure 1B**). Under hypoxia loss of *HIF-1α* resulted in minimal induction of *PGK* mRNA and the hypoxia-inducible expression of both *Glut-1* and total *VEGF* was reduced by at least 50%. These results demonstrated that regulation of hypoxic response acts through HIF-1 in mammary epithelium.

Conditional deletion of HIF-1α in the mouse mammary gland

To determine whether HIF-1 function is required *in vivo* for normal mammary gland development, a conditional gene deletion strategy was employed to delete *HIF-1α* in the mammary epithelium of mice. Mice harboring two *floxed* alleles of the *HIF-1α* locus ((Ryan et al., 1998); *HIF DF*) were bred with *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. The temporal-spatial pattern of Cre recombinase activity in this line of mice (MMTV-Cre, line A) has been extensively described (Wagner et al., 2001). Southern blot analysis comparing wild type and *HIF-1α* deleted mammary tissue indicated that MMTV-Cre consistently targets deletion of *HIF-1α* in at least 50% of MEC (**Figure 1C**). By lactation, a majority of MEC have been targeted for recombination via MMTV-Cre, therefore, the mammary glands isolated from *HIF-1α floxed* Cre+ mice will be referred to as *HIF-1α* null (*HIF-1α*$^{-/-}$). Further, in contrast to reports that this line of MMTV-Cre transgenic mice induces excision in the ovary of mature mice (Wagner et al., 2001), no recombination of the HIF-1α locus could be detected in DNA prepared from whole ovaries of HIF-1α deleted mice either by Southern blotting (**Figure 1C**) or by RTD-PCR.

During pregnancy, deletion of HIF-1α impairs secretory differentiation, but not vascular expansion

In order to pinpoint the stage of mammary gland development at which HIF-1α function may be required, mammary tissue was harvested from mice over the course of gestation. No differences in ductal morphogenesis were noted in nulliparous mice (data not shown). At day 10 of gestation (mid-pregnancy), a stage of development prior to differentiation, no defects in histology were observed in *HIF-1α*$^{-/-}$ glands at either the gross or microscopic level, indicating that HIF-1α is not critical for early rounds of alveolar proliferation (data not shown). Similarly, by day 12 of pregnancy, when secretory differentiation typically begins in most mouse strains, no differences in morphology were noted.

However, by day 15 of pregnancy, well into the period of secretory differentiation, although the glands of either genotype were indistinguishable at the whole mount level (data not shown), histological examination revealed significant abnormalities in the *HIF-1α*$^{-/-}$ glands (**Figure 2A-B**). In particular, the protein and lipid droplets that gave the wild type epithelium a "lacey" appearance were completely absent in the *HIF-1α*$^{-/-}$ glands. In addition, null alveoli were small, 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, since 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 HIF-1 has been implicated in the control of angiogenesis (Forsythe et al., 1996) we next analyzed the effect of deletion of HIF-1 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 (**Figure 2C-D**). Surprisingly, we observed no gross differences in microvessel patterning, or vascular density in relationship to the epithelium in *HIF-1α*$^{-/-}$ mammary glands.

Expression profiles of HIF-1 targets and markers of differentiation in pregnant mice

The expression of HIF-1 targets was also 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* expression differed significantly between genotypes (**Figure 3A**). However, *Glut-1* 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 (**Figure 3B**). Two of these markers, β–casein, a major milk protein, and alpha-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 oxidase (XO), a redox enzyme (Jarasch et al., 1981), butyrophilin, a hydrophobic glycoprotein found only in differentiated mammary epithelial cells (Banghart et al., 1998), adipocyte differentiation-related protein (ADRP, or adipophilin) (Heid et al., 1996), and perilipin, a marker of the adipose fraction in the mammary gland. Perilipin is normally down-regulated over the course of pregnancy as the adipose fraction shrinks (Blanchette-Mackie et al., 1995). In response to deletion of *HIF-1α*, *β-casein*, *α-lactalbumin*, *ADRP*, and *XO* mRNA levels decreased by over 50%, whereas *butyrophilin* expression remained fairly constant, and there was a failure to down-regulate *perilipin.* This indicates that there were severe deficiencies in markers of milk production.

Loss of HIF-1α blocks secretory activation at the transition to lactation

In null glands at day 18 of gestation, at the cusp of the transition from pregnancy to lactation, large areas of alveoli had failed to differentiate although there were areas of normal development (**Figure 4A-B**). To investigate whether the areas of pathology corresponded with expression of Cre recombinase, and therefore deletion of *HIF-1α*, Cre immunostaining was performed. As can be seen in **Figure 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. In contrast, the adjacent patches of alveoli that were negative for Cre were distended with milk precursors.

Although there were fewer alveoli present per field in the *HIF-1α*$^{-/-}$ glands, it was possible that this was an artifact due to the lack of alveolar cell expansion associated with differentiation. In order to compare cellularity as well as secretory activity, the amount of DNA, RNA and protein produced by these tissues per gram of tissue was quantified. It has been previously demonstrated in the mammary gland that DNA content/g tissue correlates with cellularity (Knight and Peaker, 1982). As can be seen in **Figure 4D**, there was no significant difference in DNA content between genotypes of mammary tissue, suggesting that epithelial cell number is approximately equivalent at this stage of development. In support of these results, no significant differences in the rate of proliferation of epithelial cells were observed at this stage of development (data not shown). However, there was a significant decrease in the amount of RNA produced by *HIF-1α*$^{-/-}$ glands at day 18 of pregnancy. . Finally, there was trend for decreased production of protein in *HIF-1α* null glands, though due to animal-to-animal variability, this difference did not reach statistical significance.

Because mammary epithelium-associated angiogenesis is completed by the end of pregnancy, we next analyzed microvessel density (MVD) of the glands at 18-P. Vessels were visualized by immunostaining with anti-CD31 antibodies, and the density of the vessels calculated by Chalkley counting as described previously (Ryan et al., 2000). As expected based on the results of lectin staining at day 15 of pregnancy, there were no significant differences in MVD of the mammary gland between genotypes (**Figure 4F**).

HIF-1α 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 *HIF-1α*$^{-/-}$ glands exhibited fewer alveoli that contained fewer

secretory vesicles and lipid droplets within the lumens (**Figure 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 (data not shown).

Glands were then compared at mid-lactation, a period of copious milk production, following a period of weaning of the suckling pups to allow milk to fill the gland. Several defects were apparent in HIF-1$\alpha^{-/-}$ glands. First, in stark contrast to the wild type glands (**Figure 5A**) that contained large, well-developed, expanded alveoli, large areas of fat were present in the *HIF-1*$\alpha^{-/-}$ glands, and the alveolar lumens were small (**Figure 5B**). Secretory failure was also evident by the shape of the alveoli. In the *HIF-1*$\alpha^{-/-}$ glands, individual epithelial cells containing large nuclei and scant cytoplasm were still distinguishable. In 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 *HIF-1*$\alpha^{-/-}$ glands contained abnormally large droplets of trapped lipid within the epithelial cells. The reduced numbers of alveoli as well as the decrease of retained milk explain the approximately 50% decrease in wet weight of the *HIF-1*α null inguinal mammary glands collected at mid-lactation (**Figure 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 runted compared to 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 **Figure 5F**, the differences 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 *HIF-1α*$^{-/-}$ 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.

Milk volume is reduced and milk composition is altered as a result of deletion of HIF-1α

To determine if milk quality was affected by deletion of *HIF-1α*, milk was collected from mid-lactation dams into a tared vial 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 *HIF-1α*$^{-/-}$ glands. First, the milk was more difficult to collect from these dams, was more viscous and was more difficult to dissolve into the water at collection, suggesting a high fat content. Further, less total volume could be collected from the HIF-1α$^{-/-}$ glands (**Figure 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 (**Figure 6A**). Water content was decreased and the [Na^+] and [Cl^-] were greatly elevated in milk collected from HIF-1α$^{-/-}$ glands relative to milk of wild type glands. These changes reflected an ionic concentration closer to that observed in plasma, and indicate a fundamental failure to properly regulate mammary secretion.

Changes in gene expression at mid-lactation

Expression of HIF-1 targets was also analyzed at mid-lactation. In contrast to mid-pregnancy, *PGK* expression was down-regulated by 67% in mid-lactation *HIF-1α*$^{-/-}$ mice, and there were no significant changes in *Glut-1* expression (**Figure 6B**). The decreased water content and increased [Na] and [Cl] in milk observed in mid-lactation milk are hallmarks of tight junction closure failure (Stelwagen et al., 1999), and, the family of claudin proteins are implicated in

regulation of TJ strand composition (Furuse et al., 2001; Morita et al., 1999). Therefore, the expression of mammary epithelial cell-specific TJ claudins was compared in mid-lactation mice. *Claudin 7* and *claudin 8* were specifically chosen because, relative to epithelial cell content, c*laudin 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 null glands (**Figure 6B**), however, expression of *claudin 8* decreased by 60%.

A requirement for HIF-1α in the mammary epithelium

To confirm that the defects observed in *HIF-1α* null glands were epithelial cell autonomous, primary *HIF-1α floxed* mammary epithelial cells were infected with either an adenoviral vector expressing beta-galactosidase (wild type control) or an adenoviral vector expressing Cre recombinase. Infection with Cre induced deletion of *HIF-1α* in over 99% of cells (data not shown). The wild type and null mammary epithelial cells were then transplanted into the right and left cleared inguinal fat pads, respectively, of 3-week old immunocompromised *RAG-1*$^{-/-}$ host mice, using a technique previously described by Rjinkels et al (Rijnkels and Rosen, 2001). Following outgrowth, the hosts were mated and the transplants harvested from the transplanted females on the date of birth of their litters. Transplanted wild type cells successfully differentiated and secreted milk (Figure 7A, purple granules). However, the *HIF-1α* null outgrowths contained relatively few alveoli; which were small and poorly differentiated with collapsed lumens, and retained lipid droplets in the cytoplasm (**Figure 7B**). In addition, the alveoli were surrounded by increased connective tissue (stained blue). Therefore, the histology

of the transplanted null outgrowths recapitulated that observed in intact *HIF-1α* null glands. These results confirmed that the defects resulting from deletion of *HIF-1α* are mammary epithelial cell autonomous, and were not due to defects in the stroma or deletion in other tissues.

Discussion

We have demonstrated a requirement for HIF-1-mediated transcription in the mammary epithelium in order to produce and to secrete milk. These results point to a novel role for *HIF-1α* 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 total VEGF expression were noted in response to *HIF-1α* deletion, confirming that the angiogenesis that occurs in the mammary gland during pregnancy is HIF-1-independent.

Deletion of *HIF-1α* 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 HIF-1α inhibited the expression of markers critical to secretory function, including several milk protein and MLG markers. More importantly, 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 *HIF-1α*. Consistent with this finding, transcriptional activity was reduced by 50% at this stage in response to deletion of *HIF-1α*. Because of the tight association between the presence or absence of *HIF-1α* and the production of milk components in preparation for lactation, *HIF-1α* is a critical regulator of the process of secretory differentiation in the mammary gland.

In addition, it is important to note that 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 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 HIF-1 transcriptional activity, the selective decrease of *Glut-1* expression by 60% during pregnancy may explain both the observed defects in differentiation and lipid metabolism in *HIF-1α*$^{-/-}$ mice. Normally, Glut-1, the exclusive glucose transporter utilized in the mammary epithelium at lactation, is considerably up-regulated during secretory differentiation in order to increase glucose availability (Camps et al., 1994). This is critical, since 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 utilization 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 utilized for the production of fatty acid precursors.

The severity of the defects observed during pregnancy was demonstrated during lactation since dams lacking *HIF-1α* in the mammary epithelium were unable to support nursing pups. Normally, secretory activity peaks at mid-lactation, but, as evident by the histology, the alveoli of these mice contained relatively few milk granules and large lipid droplets, normally secreted 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). Since the claudins are implicated in tight junction strand regulation (Furuse et al., 2002), and claudin 8 expression was down-regulated by 50%, HIF-1 may also play a role in tight junction closure.

Although it is possible that the severe block in differentiation observed in the *HIF-1α* null mammary gland prevented the transition to secretory activation, based on the previously described functions of HIF-1 (Semenza, 1999), it is more likely that loss of *HIF-1α* impaired metabolic activity at the time of highest demand, lactation. In support of this hypothesis, at this stage of development, the normalized expression of *PGK* was reduced by over 67%, whereas *Glut-1* decreased slightly. HIF-1 mediation of glucose metabolism may be necessary to supplement energy production at lactation since synthesis and transport of milk components, as well as tight junction closure, are energy-dependent processes. This function for HIF-1 is also supported by previous observations that increases in glycolytic enzyme activities occur at lactation (Mazurek et al., 1999). Therefore, glycolysis may be necessary to supplement 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, HIF-1-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.

In primary mammary epithelial cell cultures lacking *HIF-1α* that were exposed to hypoxia, VEGF mRNA decreased by approximately 50%. However, we were unable to detect any changes in MVD in the intact mammary gland *in vivo*, either by qualitative analysis of the lectin staining pattern or by Chalkley counts following CD31 immunostaining. In addition, there was no significant decrease in VEGF mRNA expression from *HIF-1α*$^{-/-}$ glands at day 15 of gestation. Since loss of *HIF-1α*did not impact vasculature expansion during pregnancy, we argue that the mechanisms of angiogenesis with respect to normal mammary gland development

are HIF-1α-independent. These results are 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 *HIF-1α* is conditionally deleted (Ryan et al., 2000; Schipani et al., 2001).

Low levels of HIF-1α were detectable in primary mammary epithelial cells cultured at normoxia. Because *HIF-1α* protein was dramatically increased by hypoxic treatment and because loss of *HIF-1α* diminished HIF-1's transcriptional activity, we conclude that the hypoxic response is intact in the normal murine mammary gland. Yet, hypoxia *per se* may not be a stimulus of HIF-1α activity in the mammary gland. Several laboratories have reported that, *in vitro*, HIF-1α protein is also stabilized upon treatment with insulin, insulin-like growth factor I (IGF-I), IGF-II, or activation of HER-2/neu receptor upon addition of heregulin; all potent cell survival factors/mitogens for normal and breast cancer cell lines (Feldser et al., 1999; Laughner et al., 2001; Zettl et al., 1992). Additionally, a positive feedback loop between HIF-1α and IGF-II transcription has been reported in human 293 cells and in mouse embryonic fibroblasts (Feldser et al., 1999). These observations are noteworthy, since IGF-II 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 stimulate HIF-1α protein stability even under aerobic conditions (Lu et al., 2002).

Recently, Le Provost et al. have deleted *HIF-1α*'s partner, *HIF-1β* (*ARNT-1*), from the mammary gland (Le Provost et al., 2002). Deletion of *ARNT-1* 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-1* 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 nor detected recombination of the *HIF-1α* locus in the ovaries of transgenic mice. Further, in comparison to deletion of *ARNT-1*, loss of *HIF-1α* impacted relatively late stages of mammary gland development. These differences are perplexing, since HIF-1α partnering with ARNT is required for HIF-1 activity. It is possible that *ARNT-1* function may be compensated for by other family members that complex with HIF-1α, such as ARNT-2 (Keith et al., 2001) or ARNT-3 (Takahata et al., 1998). Nevertheless, transplantation experiments of *HIF-1α* null epithelium into a wild type fat pad of a RAG-$1^{-/-}$ female host confirmed that the defects associated with deletion of *HIF-1α* were mammary epithelial cell autonomous. Therefore, even if low, but undetectable, levels of recombination of the *HIF-1α* 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 *HIF-1α* specifically inhibited the synthesis of milk components during pregnancy and milk production and secretion at lactation, we argue that HIF-1 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. Further, these defects are manifested independently of regulation of angiogenesis through VEGF.

Although over expression of HIF-1α has been documented in breast tumors compared to 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 HIF-1α regulation of epithelial cell biology and secretion. Since 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.

Materials and Methods

Animals and tissue collection

Animals were housed in an AAALAC-approved facility at UCSD in filter-topped cages and provided with food and water *ad libitum.* All animal experiments were conducted by use of the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice harboring two *floxed* alleles of exon 2 of *HIF-1α*, corresponding to the bHLH DNA-binding domain (Ryan et al., 1998), were bred to MMTV-Cre (line A) transgenic mice (Wagner et al., 2001). Nontransgenic and transgenic *floxed* females were bred with CD-1 males (day plug =day 0). Mammary glands were harvested from mice at day 10 of pregnancy (n=3/genotype), day 15 of pregnancy (n=6/genotype), day 1 of lactation (date of birth, n=4/genotype) or day 10 of lactation (n= >25/genotype). At sacrifice, one inguinal gland was fixed for 6h at RT with 10% neutral buffered formalin (NBF) prior to paraffin embedding and sectioning and staining with H&E. The contralateral inguinal gland and both thoracic glands were flash frozen stored at –80°C for subsequent analyses.

Quantitation of DNA, RNA and protein

A small piece of the 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 (50mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 400mM NaCl, 1mM EDTA in RNAase-free water). Prior to extraction, the ground powder was weighed. DNA was quantitated by Hoechst 33258 staining using a Horiba Micromax fluorometer (excitation, 350 nm; emission 473 nm). To quantitate RNA, homogenates were first treated for 90 min with DNase-I, and the fluorescence intensity quantitated following incubation with Ribogreen Dye (Molecular Probe)

according to manufacturer's instructions. Protein was measured using a Bradford assay (Bio-Rad) according to manufacturer's instructions.

Milk collection and analysis

At birth pups were removed from their natural mothers, randomized and 10-12 pups were placed with each dam. The average pup weight/litter per day was determined until mid-lactation (day 9-11 lactation), when milk and mammary tissues were collected . Milk was collected from weaned dams injected with oxytocin (1.5U i.m. per leg) under gentle vacuum into tared tubes on ice. The total volume of milk collected was the difference between the final weight and the tared weight. The milk was then flash frozen in liquid nitrogen prior to analysis. For each sample, the content of water, fat, nitrogen, lactose, sodium and chloride 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-CIROS^{CCD} (Spectro Analytical Instruments Ins. Fitchburg, MA). To compare wet weight of the lactating glands, both inguinal glands were dissected following milking, flash frozen and weighed .

Primary culture, adenoviral infection and transplantation

Primary MEC were isolated from HIF *floxed* pregnant mice according to Pullan et al. (Pullan and Streuli, 1997). Equal volumes of cells were allowed to spread onto plastic dishes in plating medium (Ham's F12 GlutaMax medium containing 10% FBS, 5μg/ml insulin, 1ug/ul hydrocortisone, 20ng/mL murine epidermal growth factor, 5 ng/ml cholera toxin, 50 ug/ml gentamicin, 100U Penincillin/10 U Streptomycin) for 48h before replacing this medium with growth medium (same as plating, but no cholera toxin; 5%FBS). The next day, the cells were infected overnight with either Adenovirus-beta-galactosidase or Adenovirus-Cre recombinase

(generously provided by Dr. Frank Giordiano, Harvard University) at a multiplicity of infection of 60-65 particles/cell (Rijnkels and Rosen, 2001). The next day, the cells washed several times with PBS and fresh growth medium was added. The cells were allowed to recover from infection for 24-48h prior to transplantation or incubation at hypoxia to analyze gene expression.

For transplantation into *RAG-1* $^{-/-}$ immunocompromised hosts (Jackson Labs), cells were trypsinized, washed, and resuspended to 50,000- 100,000 cells/ul in HBSS. Approximately 10-15ul of cells were injected into the cleared fat pads of 3-week old female *RAG 1*$^{-/-}$ mice with a 50ul Hamilton syringe. After a period of outgrowth of at least 10 weeks, the hosts were mated and the transplanted glands (4R-β-gal-infected; 4L-Cre infected) and endogenous thoracic glands were harvested and fixed in 10% NBF.

To compare mRNA expression of target genes of MEC cultured at hypoxia or to prepare nuclear extracts for western blotting, the medium was changed to growth medium containing 25mM HEPES pH 7.4 at 0 hours. Cells were then left at normoxia or transferred to a hypoxic incubator (0.5% O_2 balanced with N_2) for 24 hours.

Nuclear extract preparation and western blotting

Nuclear extracts (NE) were prepared as previously described (Ryan et al., 1998). HIF-1α protein was detected by western blotting using 60ug 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 incubation in a 1:1000 dilution of anti-mouse HIF-1α antibody (Novus, NB100-123) for 3h at RT, and washed several times in TBS +0.1% Tween (TBST). The blot was then incubated in a 1:10,000 dilution of sheep anti-mouse whole IgG-HRP for 30 min at RT and washed several times in TBST, followed by incubation in ECLPlus substrate (Amersham) prior to exposure to Kodak MR film.

Preparation of RNA and DNA

At harvest, cells were washed twice with cold PBS before being directly extracted with RNAzol B for RNA preparation (Tel-Test, Friendswood, TX) or scraped into buffer containing proteinase K for preparation of genomic DNA followed by phenol:chloroform extraction and ethanol precipitation. 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 reagent and RNA prepared according to manufacturer's directions (Tel Test, cs-500).

Immunostaining

For CD31 staining and Chalkley analysis slides were processed as previously described (Ryan et al., 2000) Briefly, a Chalkley graticle was inserted into the eyepiece of a microscope Slides were blinded and ten 10x power fields were counted twice independently. The mean Chalkley score was determined by averaging the 10 fields, and the grand mean ± SEM presented, split by genotype. Cre immunostaining was performed as previously described (Seagroves and Li, 2002).

Semi quantitative reverse transcription PCR assays (SQ-RT-PCR) Neville lab

Random-primed reverse transcription was carried out on 30 ng of total RNA. The cDNA was amplified using primers to mouse XO, α-lactalbumin, β-casein, ADRP, butyrophilin and β-actin. 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, Foster City, CA), and 2 μl of PCR product. 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 Danes-I treated and directly used to prepare first-strand cDNA from random hexamer primers using the Superscript II Reverse Transcription Kit (Invitrogen). For RTD-PCR 5ng of input cDNA was analyzed in triplicate per primer pair. All reactions were performed using the 2x Taq Master Mix (ABI), 900nM each of the forward and reverse PCR primers and 250nM of a fluorescently-tagged primer pair-specific probe in a total volume of 25ul using default cycling parameters on an ABI Prism 7200 Sequence Detector.

Real time PCR primer/probe sequences

PGK:(F) 5'-CAGGACCATTCCAAACAATCTG-3'
(R) 5'CTGTGGTACTGAGAGCAGCAAGA-3'
(probe) 5'-(6~FAM)TAGCTCGACCCACAGCCTCGGCATAT-(TAMRA)-3'

Glut-1: (F) 5'-ACGAGGAGCACCGTGAAGAT-3'
(R) 5'-GGGCATGTGCTTCCAGTATGT-3'
(probe) 5'-(6~FAM)CAACTGTGCGGCCCCTACGTCTTC-(BHQ)-3'

VEGF-total: (F) 5'-ATCCGCATGATCTGCATGG-3'
(R) 5'-AGTCCCATGAAGTGATCAAGTTCA-3'
(probe) 5'-(6~FAM)TGCCCACGTCAGAGAGCAACATCAC-(BHQ)-3'

Claudin-7: (F) 5'-CGAAGAAGGCCCGAATAGCT-3'
(R) 5'-GCTACCAAGGCAGCAAGACC-3'
probe 5'-(6-FAM)-GCCACAATGAAAACAATGCCTCCAGTCA-(BHQ)-3'

Claudin-8: (F) 5'-TGGTGGATGTGGCCCTAAA-3'
(R) 5'-CGCTGTGGTCCAGCCTATGT-3'
probe: 5'-(6-FAM)-GAGGGCTTCTCCCAGCTCGCG-(BHQ)-3'

K19: (F) 5'-CCCTCCCGAGATTACAACCA-3'
(R) 5'-TGGTGGCACCAAGAATCTTG-3'
(probe) 5'-(6-FAM)-CTTTAAGACCATCGAGGACTTGCGCG-(BHQ)-3'

Normalization of real time PCR assays

In cultured cells, target gene mRNA expression was normalized to 18S ribosomal RNA (Applied Biosystems, cat#4308329). For mammary gland tissue samples were normalized to *cytokeratin 19* (*K19*) to correct for any differences in epithelial cell content between genotypes. To determine the percent deletion of *HIF-1α* in cultured cells or in tissues, genomic was prepared and the DNA content of exon 2 of *HIF-1α* normalized to content of *c-jun*. For all primer sets tested to date, each set approached 100% amplification efficiency, allowing direct comparison of threshold cycle (Ct) to determine relative gene expression (Muller et al., 2002). Briefly, the average threshold cycle (Ct) per sample was averaged by genotype +/- SEM. For each genotype and time point, the percentage of normalized gene expression in the *HIF-1α*$^{-/-}$ samples was expressed relative to wild type samples (wild type, normoxia fold=1) according to standard procedures (Perkin Elmer 7200 manual and (Muller et al., 2002)).

Vasculature labeling

Fluorescein-conjugated lectin from tomato (Vector Laboratories, cat#FL-1171) diluted to 1mg/ml in PBS was injected into the tail vein of un-anesthetized mice 5 min prior to perfusion. Approximately 1 min prior to perfusion, mice were anesthetized, and 1% paraformaldehyde/0.5% glutaraldehyde/PBS was perfused directly in the heart with an 22-gauge needle at the rate of 1mL/min followed by clearing with PBS. Mammary tissues were equilibrated in cold 30% sucrose/PBS for 2h before embedding in OCT compound on dry ice. Thick frozen sections were post-fixed in 4% paraformaldehyde/PBS for 5 min at RT, rinsed in PBS, immersed into 0.5% Triton-X for 10 min at RT, rinsed twice in PBS and incubated with AlexaFluor 595-conjuated phalloidin (Molecular Probes, cat#A123-81) in 1% BSA/PBS for at least 4h at RT, washed 2 x 5min in PBS and then counterstained with DAPI. Images were

collected at low power using a Zeiss confocal microscope; 0.5um serial slices were 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 to *HIF-1α*$^{-/-}$ samples, are indicated with an asterisk.

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Figure legends

Figure 1: Mammary epithelial cells contain functional HIF-1. (**A**) HIF-1α, a triplet present at ~120 kDa, was detected in purified MEC at normoxia and was induced dramatically upon culture at 0.5% oxygen. (**B**) Purified MEC were cultured at either normoxia or hypoxia, harvested, cDNA prepared, real time PCR conducted and expression normalized as described in the materials and methods. No significant differences in gene expression were observed for any gene between *HIF-1α* wild type (bgal) and null (cre) mammary cells cultured under normoxic conditions. At hypoxia, relative to the robust induction of *PGK*, *Glut-1* and *VEGF* observed in wild type cells, induction of all of these mRNAs was decreased by at least 50% in *HIF-1α* null cells. **C**) Genomic DNA was prepared from the mammary glands (mg) and ovaries (ov) of HIF *floxed* (wild type; Cre-) or *floxed* MMTV-Cre+ mice and used for Southern blotting as described in (Ryan et al., 1998). As a control for excision, DNA was prepared from primary mouse embryonic fibroblasts (MEF) infected with Adeno-Cre.

Figure 2: Defects in secretory differentiation, but not vascular expansion, at day 15 of pregnancy. (**A-B**) Paraffin-embedded sections prepared from glands isolated at day 15 of pregnancy were stained with H&E. There was a striking block in differentiation in the *HIF-1α*$^{-/-}$ glands (**B**); the alveolar lumens remained closed. Images were taken at 200x magnification; scale bar 50μm. (**C-D**) Representative images of the patterning of the vasculature in relationship to the mammary epithelium following lectin (green) and phalloidin (red) staining as described in the materials and methods. There were not any qualitative differences in vascular density between wild type (**C**) and *HIF-1α*$^{-/-}$ glands (**D**).

Figure 3: Analysis of markers at day 15 of pregnancy. **A**) RTD-PCR was utilized to compare expression of HIF-1 target genes following normalization to *K19* as described in the materials and methods (wild type, white bars; *HIF-1α*$^{-/-}$, dark grey bars; ± SEM). There were no significant differences in *PGK* or *VEGF* mRNA levels, but *Glut-1* was down-regulated by over 60%. (**B**) The mRNA expression levels of several known markers of mammary epithelial cell differentiation, including *β-casein*, *α-lactalbumin* (α-lac), *butyrophilin*, *XO*, *ADRP* and *perilipin* were analyzed by semi-quantitative RT-PCR. The bar graph indicates the average level of expression per gene normalized to β-actin (wild type, white bars; *HIF-1α*$^{-/-}$ dark grey bars; ± SEM).

Figure 4: Block in secretory differentiation and activation at the transition to lactation. **(A-B)** H&E stained paraffin-embedded sections at day 18 of gestation. Note the uniform size and extent of differentiation in the wild type glands (**A**), versus the mixture of collapsed, non-differentiated as well as differentiated alveoli in the *HIF-1α*$^{-/-}$ glands (**B**). Images were taken at 200x magnification; scale bar 50μm. (**C-D**) Anti-Cre immunostaining was performed to determine the pattern of distribution of Cre (brown, nuclear staining) in the *HIF-1α*$^{-/-}$ gland (**C**, 100x magnification; **D**, 600x magnification). Areas that expressed Cre contained small, relatively undifferentiated alveoli (open-ended arrows), whereas areas negative for Cre (solid arrows) achieved differentiation. (**E**) DNA, RNA, and protein content per gram tissue at day 18 of gestation (wild type, white bars; *HIF-1α*$^{-/-}$, dark grey bars; mean ± SEM). (**F**) Average Chalkley score following anti-CD31 immunostaining to determine MVD at day 18 of gestation, (wild type, white bars; HIF-1α$^{-/-}$, dark grey bars; mean ± SEM).

Figure 5: Impaired secretory function at lactation. (**A-B**) Paraffin-embedded sections from mammary glands harvested on the date of birth were stained with H&E. In comparison to wild type (**A**), glands lacking *HIF-1α* (**B**) contained fewer alveoli, which were less well-differentiated. Images were taken at 100x magnification, scale bar 50μm. (**C-D**). Glands were also harvested at mid-lactation from weaned dams, allowing milk to fill the gland. In wild type mice (C), the accumulation of milk (purple granules) fully distended the alveoli. In addition, the relatively small volume of adipose in the wild type mid-lactation gland should be noted. In contrast, in the *HIF-1α*$^{-/-}$ glands (**D**), accumulation of milk was minimal and large lipid droplets were trapped within the epithelial cells, indicative of secretory failure. Large areas of adipose tissue were still visible in the *HIF-1α*$^{-/-}$ glands. As noted during pregnancy, there was excess connective tissue surrounding the alveoli of the null glands. Images were taken at 200x magnification, scale bar 50μm. (**E**) Wet weight (± SEM) of frozen inguinal glands harvested from lactating dams. (**F**) Representative growth curve of pups nursing wild type and mutant dams. Pronounced defects in pup weight gain were observed by day 3 of lactation that persisted until mid-lactation.

Figure 6: Changes in milk nutrition and gene expression at mid-lactation. (**A**) Analysis of milk volume and ion content in milk collected from wild type (open bars) and *HIF-1α*$^{-/-}$ glands (grey bars) at mid-lactation, mean ± SEM. Significantly less milk could be collected from *HIF-1α*$^{-/-}$ glands than from wild type. In addition, in comparison to wild type mice, the milk contained less water and more sodium and chloride ions. (**C**) RTD-PCR was used to determine the expression of *PGK*, *Glut-1*, *claudin 7* and *claudin 8* in mid-lactation tissue as described in the materials and methods.

Figure 7: The effects of deletion of HIF-1α are mammary epithelial cell autonomous. As described in the materials and methods, primary *HIF-1α floxed* MEC were infected with either Adeno–βgal (**A**) or Adeno-Cre (**B**) and injected into the cleared fat pads of female host mice and allowed to regenerate for 12 weeks. Paraffin-embedded sections harvested from mice on the date of birth (without prior weaning of pups) were stained with Mason's Trichrome; 200x magnification; 50μm scale bar; n=3 mice with 100% outgrowths/ genotype. Adeno-Cre-infected MEC outgrowths recapitulated the phenotype observed in intact *HIF-1α*$^{-/-}$ dams. Note the lack of milk products in the Cre-infected outgrowth, and the presence of large, trapped lipid droplets within the epithelial cells (open arrow). In addition, there was an abnormal thickening of collagen fibers (stained blue, open arrow) around the alveoli in *HIF-1α*$^{-/-}$ glands as observed in intact mice.

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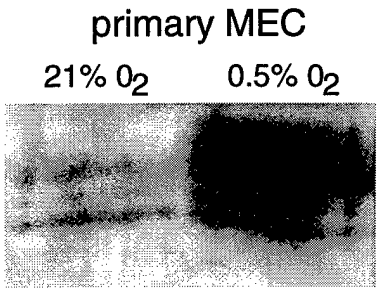
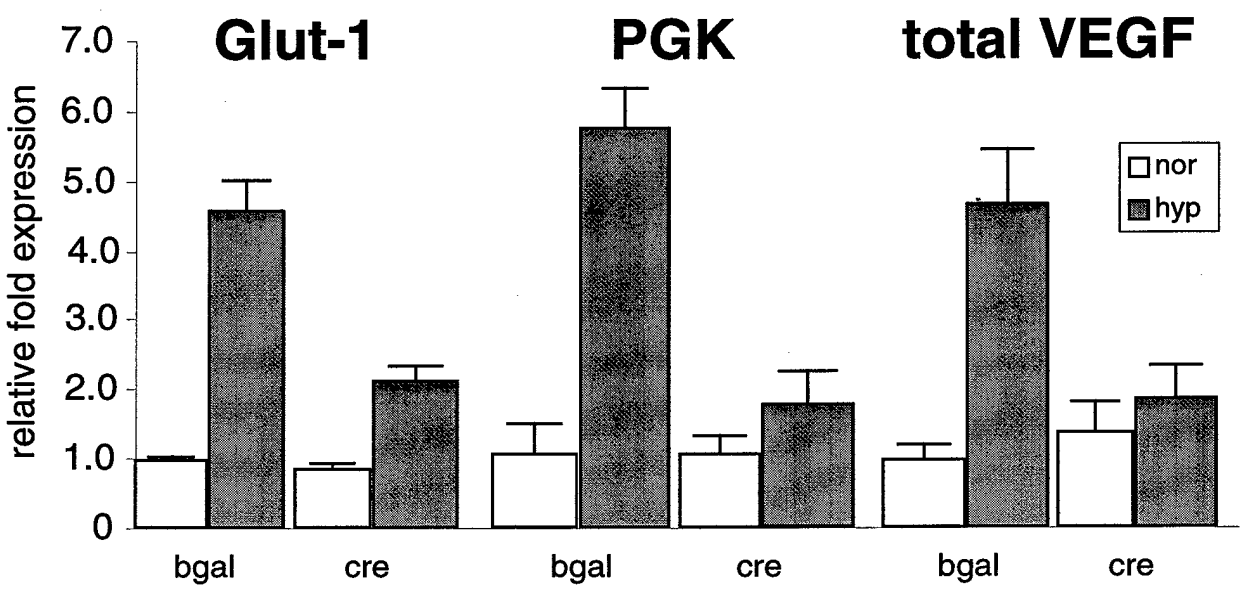
Figure 1

A


primary MEC
21% 0_2
0.5% 0_2

B


Glut-1
PGK
total VEGF
relative fold expression
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
nor
hyp
bgal
cre
bgal
cre
bgal
cre

C

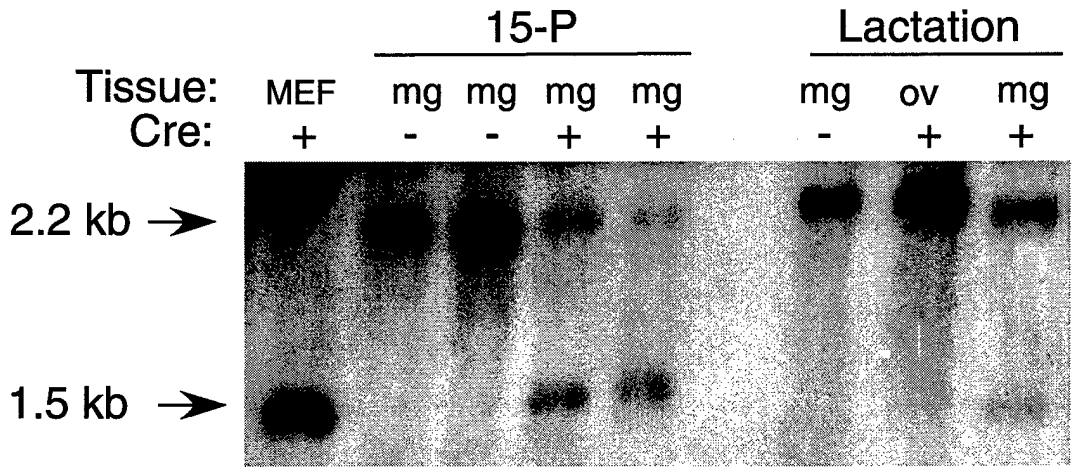

15-P
Lactation
Tissue:
Cre:
MEF
mg
mg
mg
mg
mg
ov
mg
+
-
-
+
+
-
+
+
2.2 kb
1.5 kb

Figure 2

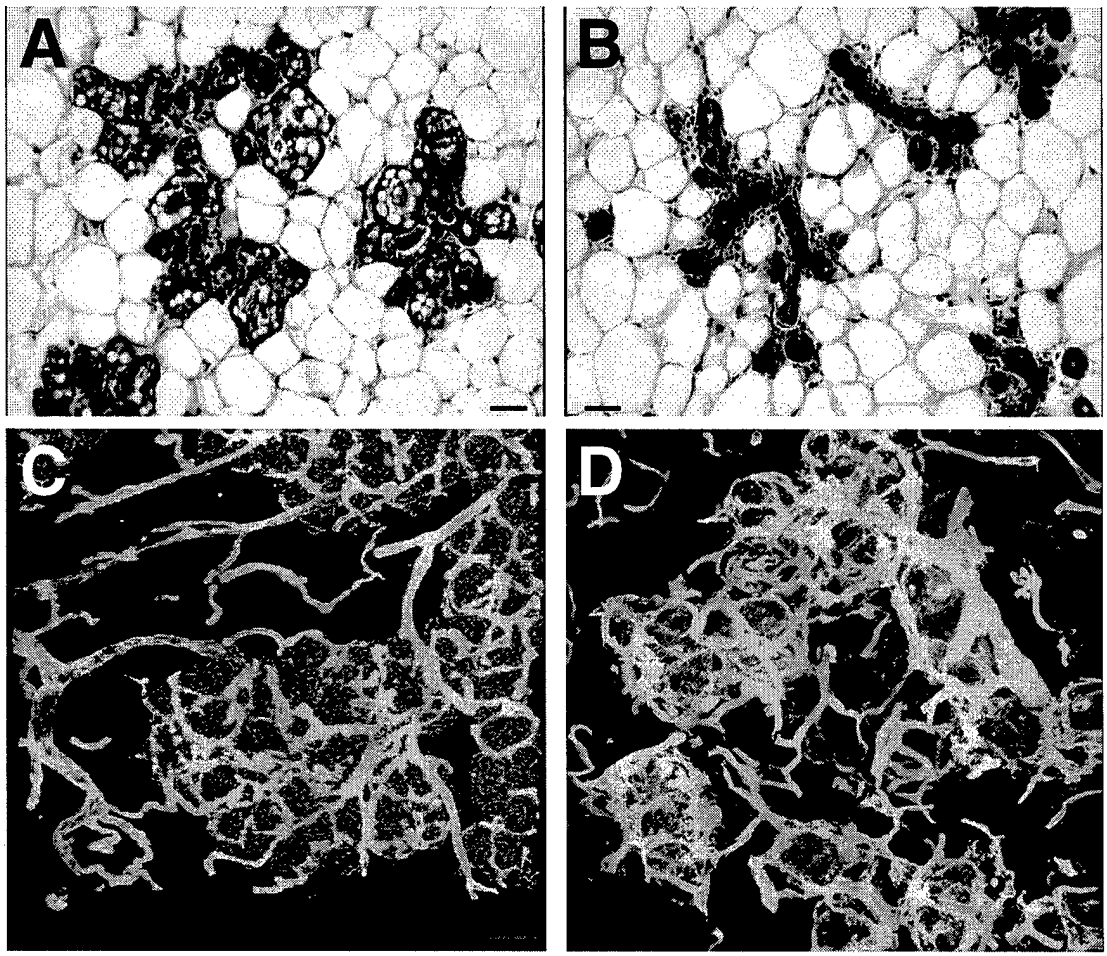


Figure 3

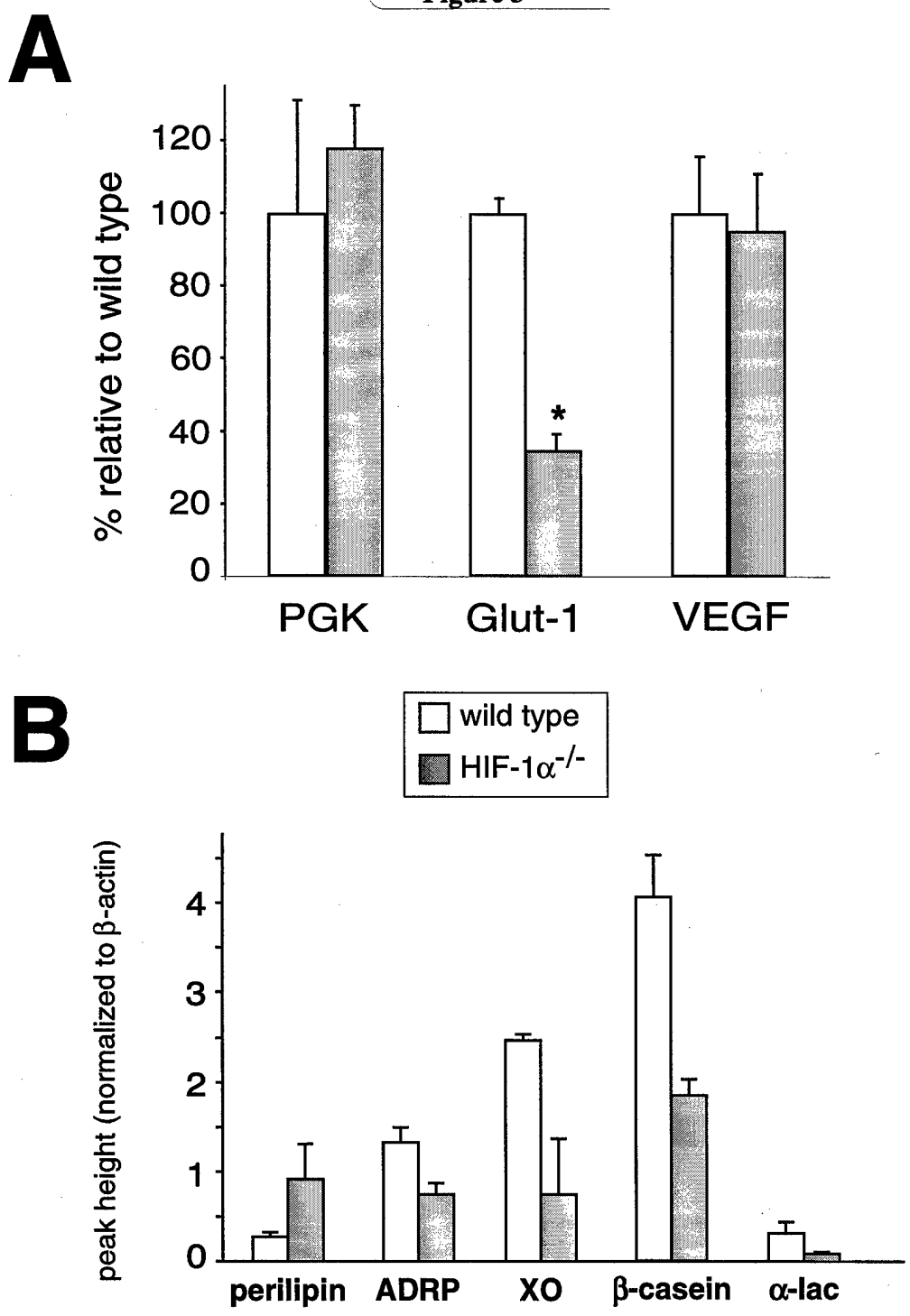
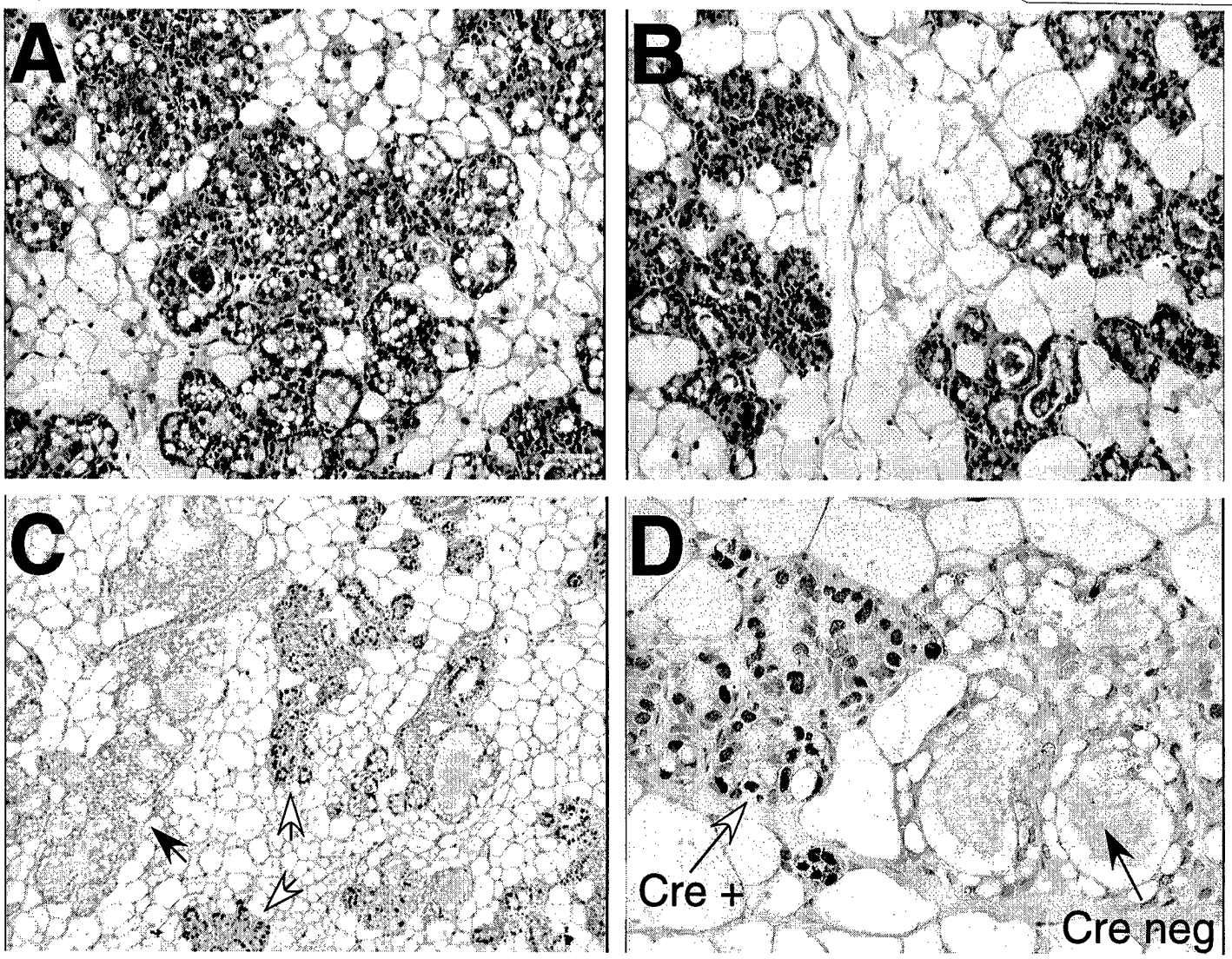
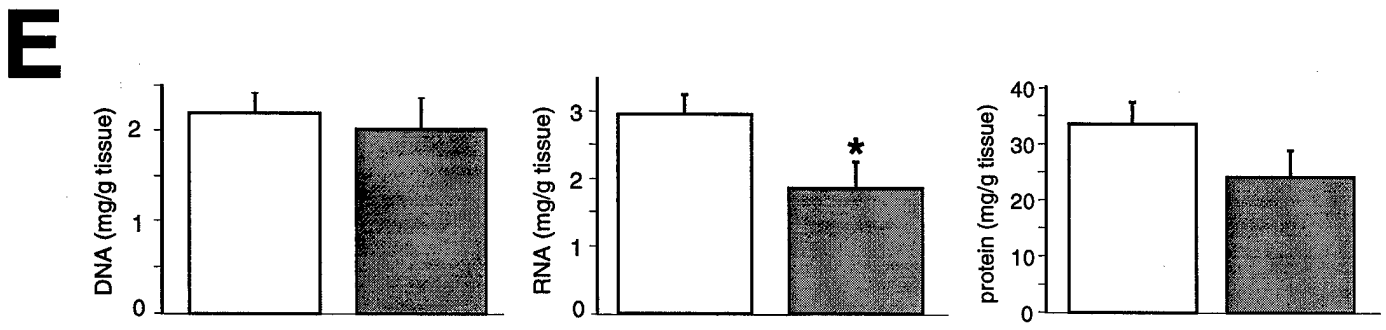

A
% relative to wild type
120
100
80
60
40
20
0
*
PGK
Glut-1
VEGF
B
wild type
HIF-1α-/-
peak height (normalized to β-actin)
4
3
2
1
0
perilipin
ADRP
XO
β-casein
α-lac

Figure 4


A
B
C
D
Cre +
Cre neg


E
DNA (mg/g tissue)
0
1
2
RNA (mg/g tissue)
0
1
2
3
*
protein (mg/g tissue)
0
10
20
30
40

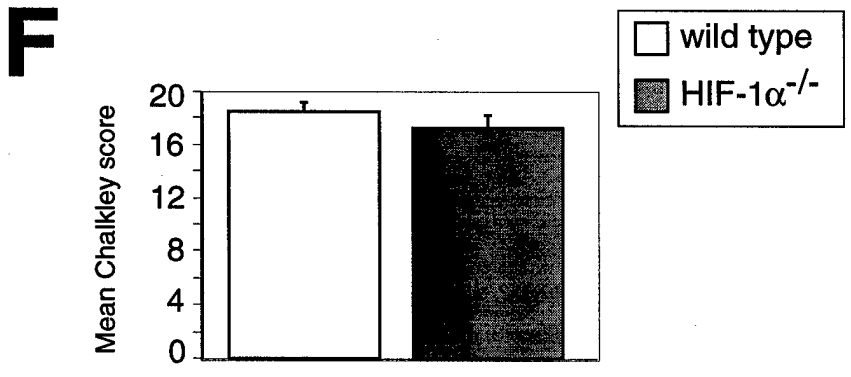
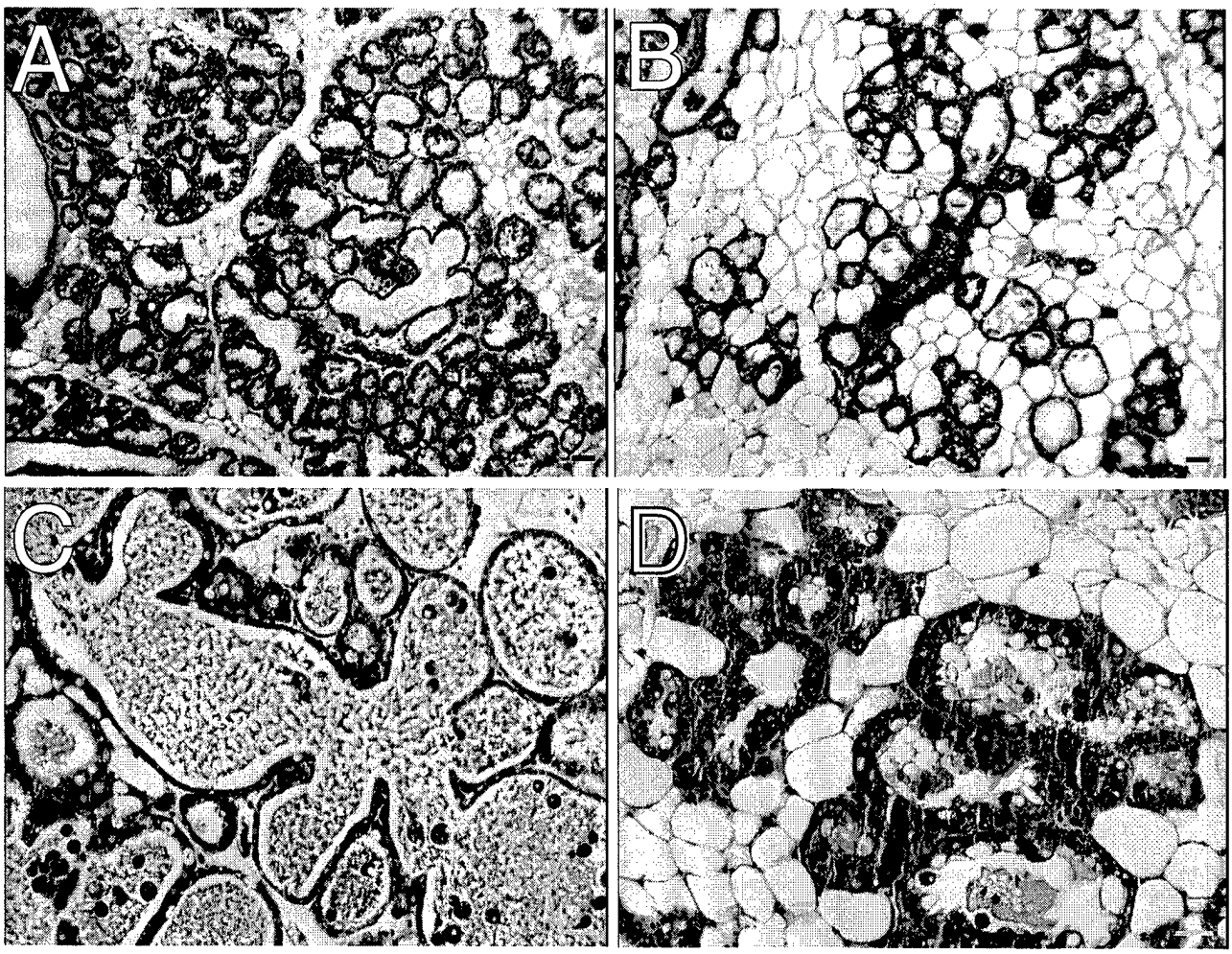
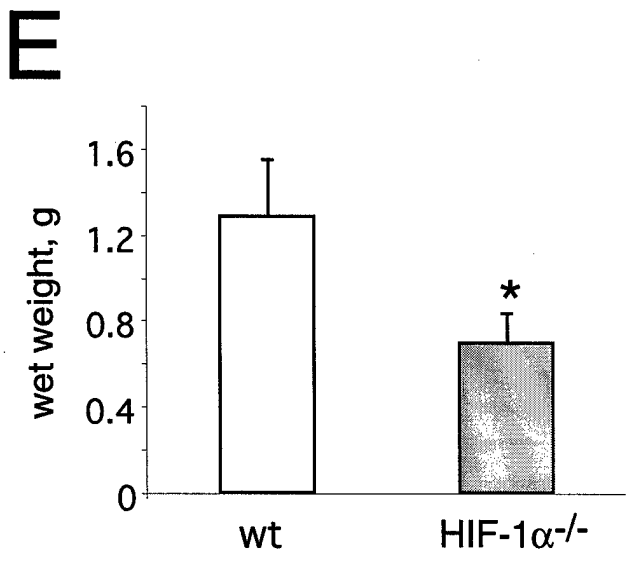

F
wild type
HIF-1α-/-
Mean Chalkley score
0
4
8
12
16
20

Figure 5


A
B
C
D


E
wet weight, g
1.6
1.2
0.8
0.4
0
*
wt
HIF-1α-/-

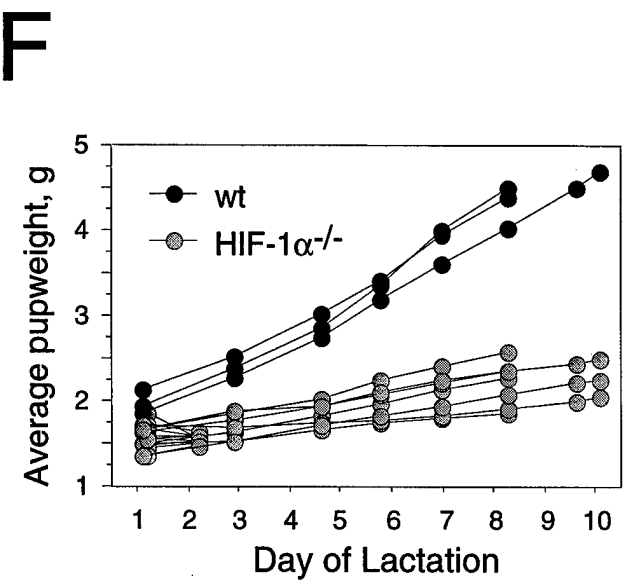
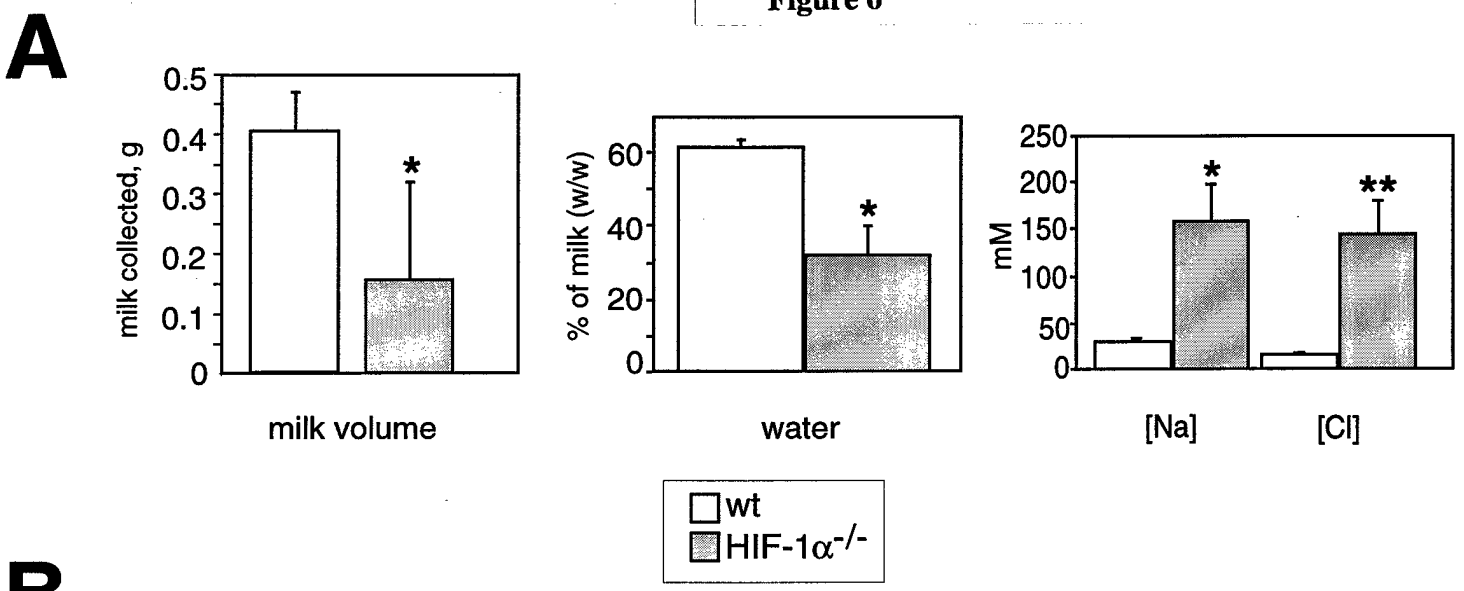

F
Average pupweight, g
5
4
3
2
1
wt
HIF-1α-/-
1 2 3 4 5 6 7 8 9 10
Day of Lactation

Figure 6


A
milk collected, g
0.5
0.4
0.3
0.2
0.1
0
*
milk volume
% of milk (w/w)
60
40
20
0
*
water
mM
250
200
150
100
50
0
*
**
[Na]
[Cl]
wt
HIF-1α-/-

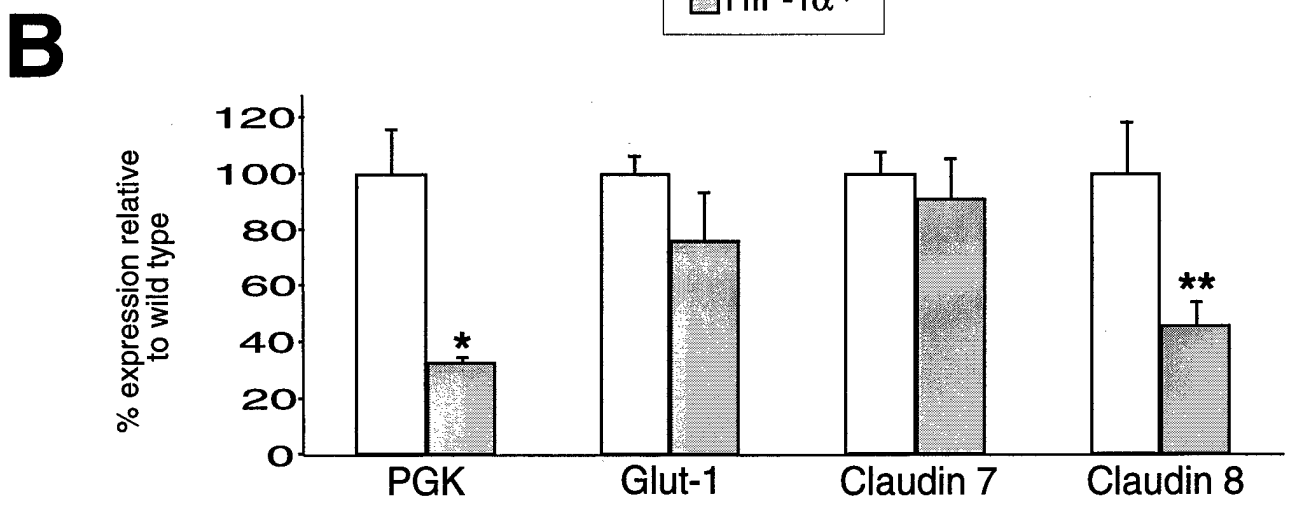

B
% expression relative to wild type
120
100
80
60
40
20
0
*
**
PGK
Glut-1
Claudin 7
Claudin 8

Figure 7