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It has long been known that nude females display lactation defects, necessitating that nude colonies be maintained with heterozygous females which are phenotypically normal. Here we have shown that the lactation defect is due to intrinsic defects in the mammary epithelium, since the estrogen and progesterone levels are comparable between nude and wild-type mice. Nude glands also show defects in the initial development of the epithelial network of ducts when the tissue is undergoing a period of intense proliferation. In addition, the nude mouse mammary glands fail to develop normally during pregnancy suggesting that Whn is necessary for the mammary epithelial cells to correctly differentiate. These data strongly implicate pleiotropic roles for Whn in the development of mammary glands.

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Introduction

The nude phenotype results from inactivating mutations in a single gene, designated *whn* (winged helix nude) or *hfh 11* (hepatocyte nuclear factor 3/ forkhead homolog 11) (Kurooka et al., 1996; Nehls et al., 1996; Nehls et al., 1994; Segre et al., 1995), and recently renamed Foxn1 (Forkhead Box n1) (Kaestner et al., 2000). Well-characterized features of the nude mutation are the lack of visible hair, abnormal formation of the epidermis, and the absence of a thymus (Sundberg, 1994). In addition to these defects, female nude mice, though fertile, fail to lactate sufficiently to nourish their pups (Eaton et al., 1975; Hegan, 1979; Holmes and Mason, 1974; Militzer and Schwalenstocker, 1996; Morgan, 1977). While the expression and function of Whn in the epidermis and thymus have been extensively studied, little is known about the defect in mammary gland function. We have shown that nude mice have defects in the initial development of the epithelial network of ducts when the tissue is undergoing a period of intense proliferation. In addition, nude mouse mammary glands fail to develop normally during pregnancy suggesting that Whn is necessary for the mammary epithelial cells to correctly differentiate. These data are consistent with the previous work from our laboratory showing that Whn has roles in both proliferation and differentiation of keratinocytes in the skin (Lee et al., 1999; Prowse et al., 1999).

Body

Task 1. Determine the temporal and spatial expression of Whn during mammary gland development

Nude mammary glands display defects in development

The development of mammary glands mainly occurs after puberty and during pregnancy. At birth, the mammary fat pads are present and contain a few simple ducts, consisting of myoepithelial and epithelial cells, proximal to the nipple. At the onset of puberty, highly proliferative structures called terminal end buds (TEB) appear at the end of the ducts, and the growth rate of the epithelium accelerates to form a ductal network extending to the limits of the fat pad. During pregnancy, the epithelium further divides and branches to form lobulo-alveoli, which form the secretory apparatus. Following the birth of the litter, the alveoli display empty lumens as the suckling pups remove the milk (Daniel and Silberstein, 1987; Hennighausen and Robinson, 1998; Pitelka et al., 1973; Russo et al., 1989).

We have investigated the structure of nude mouse mammary glands during development. Whole mounts were prepared from nude mice and their wild-type littermates at 4, 7 and 10 weeks of age, and epithelial structures were identified by staining with carmine alum. Figure 1 (Appendix 1) shows that the development of the epithelium in the nude mice is retarded in comparison to their wild-type littermates. By 7 weeks of age, the epithelial development of the wild-type mice is complete, whereas development in the nude mice is not complete until 10 weeks. Although the epithelium populates the entire mammary fat pad in both the nude and wild-type mice, the degree of branching is significantly reduced

in the nude.

The purpose of the mammary gland is to produce milk to nourish offspring, and to this end the gland undergoes a large amount of growth and modification during pregnancy--the epithelium proliferates and differentiates to give rise to a highly secretory lobulo-alveolar structure. Figure 2 (Appendix 1) shows sections of mammary glands taken from nude and wild-type mice on the day that they gave birth to their pups. Comparison of the samples shows that nude mammary glands do form secretory lobulo-alveolar structures, but that they fail to function properly. The nude gland possesses small lobulo-alveoli that remain full of secretions despite the presence of pups attempting to suckle. These results suggest a defect in the late stages of mammary gland differentiation.

Abnormalities in nude mammary glands are not due to changes in hormonal levels

The development of the mammary glands is highly regulated by the coordinate action of a number of hormones, such as estrogen, progesterone, prolactin, oxytocin, and members of TGF-β family (Hennighausen and Robinson, 1998). Experiments from gene knockout mice show that estrogen is critical for the ductal growth of mammary glands (Korach et al., 1996), and that progesterone is important for both the ductal outgrowth and the lobulo-alveolar development (Humphreys et al., 1997; Lydon et al., 1995). In order to rule out the possibility that the phenotype of the nude mammary glands is due to changes in hormonal levels, we investigated the levels of estradiol and progesterone during virgin development and pregnancy. Table 1 (Appendix 1) shows that at no time point tested is there any significant difference in the levels of these two hormones between the wild-type and nude mice. These data show that the defects in nude mammary gland development are due to an intrinsic defect in the gland, and not a secondary consequence of reduced hormone levels.

Defects in nude mammary glands can be rescued by *inv-whn* transgene

In the course of experiments to investigate the roles of Whn in the epidermis, transgenic mice were generated in which *whn* was placed under the control of the involucrin promoter (*inv*) (Prowse et al., 1999). Involucrin, a component of the cornified envelope, is present in many stratified epithelia, such as epidermis, hair follicles, and ureter (de Viragh et al., 1994; Rice and Green, 1979; Walts et al., 1985). Since involucrin has not been shown to be expressed in the mammary gland, it came as a surprise that the expression of this transgene on the nude background rescued the lactation defects. Transgene expression in the mammary glands was identified by RT-PCR. Figure 3 (Appendix 1) shows that both the *inv-whn* transgene and endogenous involucrin are expressed in the mammary gland both during lactation and in mature non-pregnant mice. Taken together with the lack of detected changes in hormone levels, these data indicate that the abnormalities in the nude mammary glands are due to an intrinsic mammary epithelium defect.

Key research accomplishments

- Identified the developmental defects of nude mammary glands
- Illustrated that the abnormalities of nude mammary glands are due to an intrinsic epithelial defect

Reportable outcomes

- Rescue of nude mouse lactation defect by expression of Whn transgene in mammary glands
- Poster presentation at Cutaneous Biology Research Center retreat, April, 2001

Conclusions

Whn is clearly required for both the initial development of the mammary gland epithelial network, and the subsequent changes seen during pregnancy, parturition, and lactation. The onset of mammary gland development in nude mice is significantly delayed. Even though the ductal network eventually grows to the limits of the fat pad, the degree of branching of the network is often dramatically decreased in the nude glands compared to wild-type. At late pregnancy, nude glands are consistently 1/2 to 1/3 the size of the wild-type glands. The decreased size of the nude gland could be due to a combination of the decreased amount of branching during virgin development, as well as a decreased rate of proliferation of the epithelium during pregnancy. While we have not directly tested the proliferative capacity of the nude mammary epithelium, nude keratinocytes have been shown to have decreased proliferation potential when compared to their wild-type counterparts (Brissette et al., 1996). Though the nude mammary glands form secretory lobulo-alveoli, they fail to lactate, suggesting that the nude mammary epithelial cells also possess defects in differentiation.

We have shown that Whn is directly involved in the growth and differentiation of mammary epithelial cells, since the major hormones responsible for mammary gland development were found to be comparable between nude and wild-type mice. Furthermore, the nude mouse lactation defect can be rescued by the expression of a *whn* transgene in mammary epithelial cells. Taken together, these data demonstrate an essential role for Whn in the development and maturation of the mammary gland.

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Appendix 1

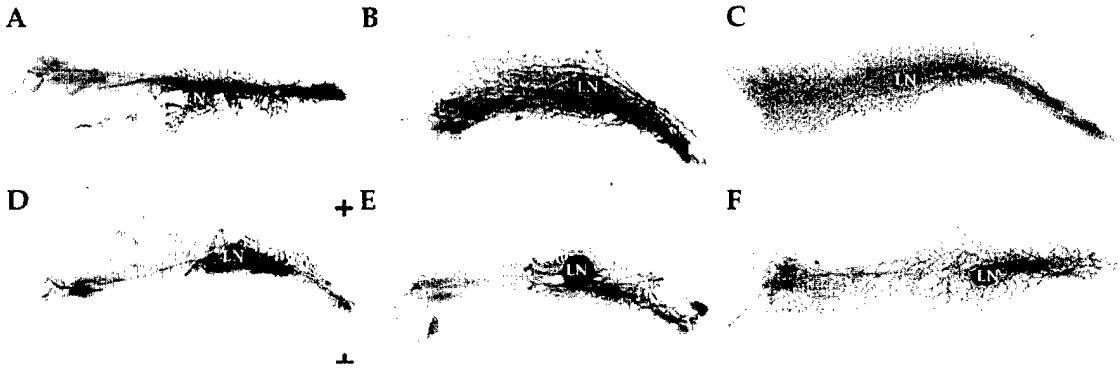

Figure 1. Ductal development in nude mice is delayed, and the degree of branching signifiantly reduced compared to wild-type littermates. Whole mounts of the number 4 inguinal gland were prepared from wild-type (A-C) and nude (D-F) virgin mice at 4 weeks (A, D), 7 weeks (B, E) and 10 weeks (C, F) of age. While ductal development is delayed in nude mice compared with their wild-type counterparts, by 10 weeks the epithelium has reached the margins of the fat pad. However, the degree of ductal branching is lower in the nude mice. Whole mounts are oriented with the nipple towards the right, and LN indicates the position of the lymph node.

Figure 2. The morphology of Nude mammary glands is aberrant following birth of the pups. Mammary tissue was taken from nude and wild-type mothers a few hours after parturition. Tissue was fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. A. Wild-type mammary tissue, showing the appearance of a normal postpartum mammary gland. The empty lumens (*) show that the milk is removed by suckling pups. B. Nude mammary tissue at the same magnification illustrates the reduced size and abnormal appearance of the lobulo-alveoli. The lumens (*) are still full of milk.

Table 1 Hormone levels in nude and wild-type mice [a]

Hormone tested	Age of the mice [b]	wild-type	Nude	*p* value [c]
Estradiol (pg/mL)	virgin	95.6 ± 36.7 [d] n = 8	100.5 ± 35.8 n = 10	0.78
	pregnant	121 ± 26.3 n = 5	120 ± 24.7 n = 5	0.95
Progesterone (ng/mL)	virgin	12.2 ± 12.5 n = 10	5.35 ± 7.44 n = 12	0.12
	pregnant	64.2 ± 33.3 n = 5	23.3 ± 21.5 n = 5	0.05

a, Serum samples were collected from nude mice and their heterozygous littermates at indicated time points and tested for the two hormones. Heterozygous are used as wild-type control since they are phenotypically normal.
b, Virgin mice are between the age of 4 to 8 weeks, which is the time that mammary glands are undergoing robust branching. Pregnant mice are between 14.5 to 18.5 days of pregnancy, when numerous lobulo-alveoli are forming.
c, Single factor anova analysis was done for the data from each time point between nude and wild-type mice with Microsoft Excel. Significant difference is determined to be existing between the two groups when $p < 0.05$. No significant difference is seen at the given time points for the two hormones tested.
d, Average ± standard error of mean, n = number of samples collected

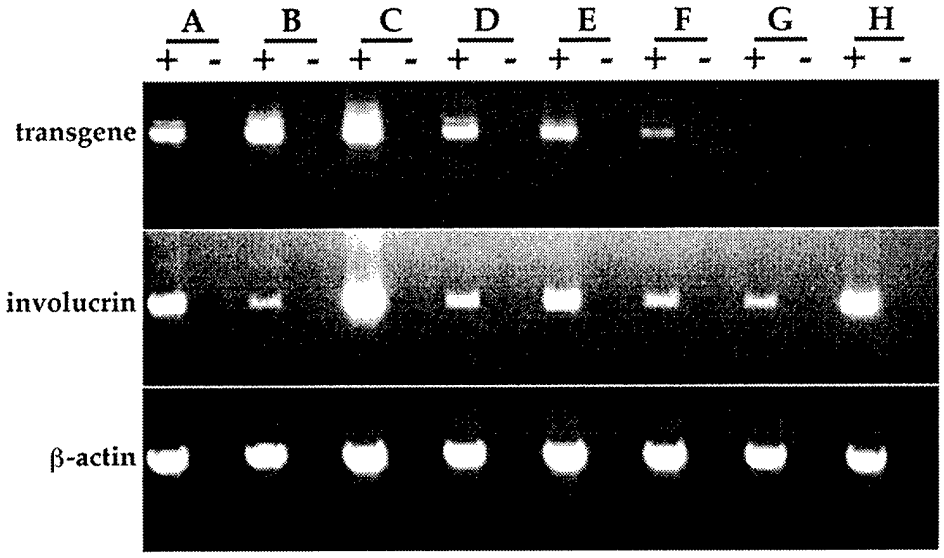

Figure 3. Involucrin and the inv-whn transgene are expressed in the mammary gland. RNA was isolated from mature non-pregnant nude mice carrying the inv-whn transgene (A-E), a lactating mouse expressing the inv-whn transgene (F), a mature non-pregnant wild-type (G) and a mature non-pregnant nude (H). RT-PCR indicated the expression of the inv-whn transgene in the mammary tissue of all mice positive for the transgene. The involucrin transcript was detected in the mammary tissue of all mice regardless of the presence of the transgene, indicating that involucrin is ordinarily expressed in the mammary gland. Lanes with "+" are experiments with reverse transcriptase, and lanes with "-" are negative controls with no reverse transcriptase. The β-actin transcript was used as a positive control.