LACTOGENESIS INDUCTION IN TRANSGENIC VIRGIN PIGS AS A MODEL FOR IDENTIFYING TRANSGENE EXPRESSION AND RECOMBINANT PROTEIN PRODUCTION

by

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LACTOGENESIS INDUCTION IN TRANSGENIC VIRGIN PIGS AS A MODEL FOR IDENTIFYING TRANSGENE EXPRESSION AND RECOMBINANT PROTEIN PRODUCTION

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(ABSTRACT)

The porcine mammary gland can be used for the production of recombinant proteins by directing a transgene to the mammary gland with a milk protein gene promoter. In order to determine whether or not the protein will be expressed, the animals must be maintained at least through their first lactation. An experiment was performed to determine if hormonal induction of lactogenesis in transgenic virgin pigs could be used as a method for identifying those gilts that are likely to express the recombinant protein during a natural lactation. Mammary development and lactogenesis were induced by administration of subcutaneous implants designed to release 7.1 mg of estradiol-17β and 18 mg of progesterone daily for 21 d. Histological analysis of tissue samples before and after the treatment period indicated that mammary secretory tissue underwent dramatic proliferation resulting in a greater degree of alveolar and individual epithelial cell differentiation. The presence of β–lactoglobulin mRNA was detected in high levels in post-implant tissue samples, and minimally detected in samples cultured in media supplemented with insulin, hydrocortisone, and prolactin. However, protein expression was only detected in the post-implant samples, indicating that β-lactoglobulin was not maintained well by in vitro culture. The transgene mRNA, recombinant human fibrinogen (Aα chain), was detected in all analyzed samples at varying levels. However, the corresponding protein was not detected in any sample,

under either reduced or nonreduced conditions. These results indicate that lactogenesis was successfully induced using the hormonal implants. Also, the transgene was activated by the hormonal induction in vivo and in vitro, but the corresponding protein could not be detected. This study indicates that induction of lactogenesis can be used to detect the presence of transgene mRNA in mammary tissue of gilts. However, we cannot conclusively demonstrate that this procedure can be used to identify those gilts that are likely to express the recombinant protein during a natural lactation.

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Everyone knows the saying, "You learn something new every day." Nothing truer could be said about this endeavor. Starting out with very limited knowledge of the types of procedures that would be required by the project, I was more than a little wary. But thanks to a number of people, I have learned a tremendous amount about various aspects of research and from some of you, life in general.

One of the most important things that this project taught me was something that most people don't realize: PIGS ARE GREAT! Before graduate school, if someone had said that I'd be spending my life at the Swine Center, I would have told them they were crazy. But I have come to appreciate the world of the pig, and I love them now. I think little Scooter was the one that changed my mind for good.

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Introduction

Aside from the deposition of adipose tissue and extension of ductular epithelium, the mammary gland is relatively quiescent from birth until puberty. Beginning with the onset of ovarian activity, mammary gland development increases with each recurring estrous cycle. There is a dramatic and rapid increase in mammary growth and morphological development during gestation. Gestational development of the mammary gland can be attributed to the simultaneously high levels of estrogen (E) and progesterone (P_4). However, E and P_4 are incapable of stimulating mammary development in hypophysectomized guinea pigs, mice, and rats (Lyons and Pencharz, 1936; Gomez et al., 1937a; Leonard and Reece, 1942). These studies led to the hypothesis that prolactin is intricately involved in the endocrine control of mammogenesis.

Other studies have implicated glucocorticoids as essential for milk protein synthesis within the mammary gland epithelium and lactogenesis (Terry et al., 1977; Delouis et al., 1980). Using the knowledge of endocrine control of mammary gland development, lactogenesis or lactation has been artificially induced in heifers, goats, ewes, and gilts (Folley and Malpress, 1943, 1944; Hammond and Day, 1944; Parks and Glover, 1944; Folley et al., 1945; Shamay et al, 1992). Procedures for induction of lactation vary among species as to the specific hormone regimen, duration, and method of delivery.

The porcine mammary gland has been shown to be an effective bioreactor for the production of recombinant proteins (Velander et al., 1992a; Morcol et al., 1994). These proteins are targeted to the mammary gland by attaching the appropriate cDNAs or genomic sequences to the promoter region of a milk protein gene endogenous to mice, murine whey acidic protein (mWAP). The promoter activates the transgene at the appropriate time during the pig's gestation

and lactation. One of the proteins currently being produced in transgenic livestock is recombinant human fibrinogen (rhFib).

Human fibrinogen is a complex plasma glycoprotein that is involved in the final stages of blood coagulation. It is synthesized in the liver as a dimer with a molecular weight of 340 kDa. Fibrinogen is made up of two subunits linked by three disulfide bonds. Each subunit contains three polypeptide chains, Aα (66 kDa), Bβ (54 kDa), γ (48 kDa). These chains are linked by disulfide bridges to form α-helices (Doolittle, 1984; Roy et al., 1991). The clinical need for rhFib in the United States well exceeds the quantity available by harvesting the protein from donated human plasma (Paleyanda et al., 1991, as cited by Butler, 1997). In addition, the use of transgenic animals that produce rhFib in their milk eliminates the risk of contamination with disease vectors that accompanies purification of human blood products.

As described by Butler et al. (1997), there are several steps necessary in order to use transgenic swine for the production of rhFib. The first requirement is the formation of DNA constructs using milk protein promoters such as mWAP that will express cDNA or genomic hFib. The constructs must then be microinjected into the pronucleus of the pig zygote. Next, each of the sequences encoding for the three chains (Aα, Bβ, γ) must be cointegrated into a domain of the chromosome that will be transcribed. Van Cott et al. (1997) have shown that in order for the transgene expression to be stable from generation to generation, a single cointegration site and associated transgene copy number is desirable.

Animals are tested for the presence of the three transgenes in tail tissue shortly after birth. However, in order to determine if rhFib will be expressed in the milk of the transgenic pigs, the animals will have to be supported at least through their first lactation. By using hormonal implants, mammary development can be induced in prepubertal gilts and tissue cultures can be

tested for the presence of milk protein genes as well as the actual recombinant protein production (Shamay et al., 1992). One cannot determine the precise level of protein production that would be expressed during a natural lactation, but the presence of the transgene and protein can be detected. This procedure could prove valuable for large-scale pharmaceutical production of rhFib or another transgene. The objective of this study was to determine if induction of lactogenesis can be used to identify transgene expression and recombinant protein production in transgenic virgin pigs.

Literature Review

Mammary Development

Anatomy of the Mammary Gland

Glandular tissue of the mammary gland is classified as either parenchyma (secretory) or stroma. Milk is synthesized in epithelial cells arranged in a single layer lining the lumen of each alveolus. The multiple alveoli of a lobe are drained by a duct system towards the body surface. Other tissue present, e.g. connective tissue, adipose tissue, and skin, make up the stroma (Mepham, 1983). Myoepithelial cells are located between the epithelial cell and the basement membrane of each alveolus. The cells are spindle-shaped and contract in response to oxytocin. This contraction compresses the lumen of the alveolus, forcing milk into the duct system for removal.

Phases of Development

Mammary glands originate in the fetus as raised areas of ectoderm, which will separate to form mammary buds in paired locations analogous to the glands of the mature animal. After undergoing a resting period, the epithelial cells of the mammary buds proliferate, resulting in the formation of one or more primary sprouts. Secondary sprouts then develop from the primary sprout and form lumena. A slightly branched tubular gland is developed by parturition (Cowie et al., 1980).

Following a period of relative inactivity from birth until puberty, the mammary gland begins a phase of active development associated with the onset of ovarian activity. The degree of development varies considerably among species. There is a dramatic and rapid increase in mammary growth and morphological development, or mammogenesis, during gestation. This is

due to the concurrent release of E and P_4. The early stages of gestation are characterized by elongation and branching of mammary ducts. Mammary gland development during mid-gestation involves the formation of alveoli, and during late gestation, final proliferation and differentiation of the mammary epithelial cells, known as lactogenesis, occurs (Akers, 1994).

Endocrine Control

Results of early research on the endocrine regulation of mammary development indicated that for complete alveolar development to occur, treatment with E and P_4 was essential in most species (Folley, 1947). Studies have concluded that E is responsible for the stimulation of ductal growth and P_4 stimulates lobulo-alveolar development (Gardner and Hill, 1936; Gardner and Van Wagenen, 1938). However, in some species, each steroid hormone may be sufficient alone. For example, Folley et al. (1940, 1941) demonstrated that topical treatment with 1% diethylstilbestrol ointment results in an udder capable of secreting copious amounts of milk in the goat. Percutaneous treatment of rabbit mammary gland with estrone or diethylstilbesterol resulted in gland development (Lewis and Turner, 1942). Similar results have been reported in the bovine (Hammond and Day, 1944; Parkes and Glover, 1944). Also, in the monkey, E seems to be capable of causing ductal and lobulo-alveolar development (Gardner and Van Wagenen, 1938).

Gardner and Hill (1936) demonstrated that high doses of P_4 resulted in mammary development in mice. Further studies have shown this to be the case with monkeys (Hartman and Speert, 1941) as well. Since much greater levels of P_4 are needed when administered alone, E seems to be playing a synergistic role. Haslam (1987) reported that E increases the concentration of P_4 receptors, and therefore decreases the levels of P_4 necessary to observe an

effect. This may explain the observed synergism of E and P_4 on lobulo-alveolar development in the mouse (Nandi, 1958).

The presence and location of P_4 receptors is another key component in mammary gland development. Using P_4 receptor knockout mice and tissue transplantation, Humphreys et al. (1997) reported that normal lobulo-alveolar development was prevented by the absence of P_4 receptors in transplanted epithelium, but not in recipient stroma. Also, if P_4 receptors were present in the epithelium but not in the recipient stroma, ductal development was markedly reduced. They concluded that P_4 receptors in the epithelium are necessary for lobulo-alveolar development, and P_4 receptors in the stroma are essential for ductal development.

Studies have shown that E and P_4 are incapable of stimulating mammogenesis in hypophysectomized mice (Gomez et al., 1937a), rats (Leonard and Reece, 1942), and guinea pigs (Lyons and Pencharz, 1936). These studies led to the belief that a compound from the anterior pituitary is necessary for E and P_4 to have their stimulatory effects on the mammary gland. Treatment of hypophysectomized male guinea pigs with a pituitary implant from an E-treated donor resulted in extensive alveolar development (Gomez et al., 1937b). Gardner and White (1941) reported moderately extensive mammary growth in hypophysectomized mice when treated with E and prolactin (PRL). They hypothesized that PRL sensitizes the mammary gland to the action of ovarian steroid hormones, but did not rule out a direct effect of PRL on the mammary epithelium.

Further experiments have confirmed the importance of PRL in mammary gland development using hypophysectomized non-pregnant rats (Talwalker and Meites, 1961) and goats (Cowie et al., 1966). Studies have also shown that if PRL was suppressed by bromocriptine, E and P_4 treatment was ineffective in inducing mammary development in heifers

and ewes (Delouis et al., 1980; Schams et al., 1984). Prolactin has been implicated in the differentiation of mammary epithelial cells (Akers, 1994) and has been shown to increase mRNA synthesis of milk protein genes, namely caseins (Rosen et al., 1975; Terry et al., 1977; Matusik and Rosen, 1978). The addition of PRL to rat mammary gland explant cultures resulted in the rapid accumulation of casein mRNA, as measured by hybridization with a cDNA probe. This effect was potentiated by hydrocortisone and prevented by P_4 (Matusik and Rosen, 1978).

Glucocorticoids also play a role in regulation of mammary gland development. The structural differentiation of alveolar cells is dependent upon glucocorticoids (Akers, 1994). Also, glucocorticoids synergize with PRL to potentiate its effects on lactogenesis (Delouis et al., 1980). Mouse mammary gland primed with estradiol-17β (E_2) and P_4 in vivo exhibits no casein synthesis when cultured in medium containing insulin, PRL, growth hormone, E, and P_4. However, when this tissue was subsequently cultured in medium supplemented with PRL and cortisol, the presence of casein was detected by radioimmunoassay (Terry et al., 1977). This suggests a regulatory role for glucocorticoids on casein synthesis by the mammary alveolar cells.

Mammary cell ultrastructure

Mammary secretory epithelium consists of single layered cuboidal to columnar alveolar cells, which are morphologically and functionally polarized. A basal network of myoepithelial cells surrounds the alveolar cells. This entire epithelium is enclosed by a basement membrane that provides a boundary between the parenchymal and stromal compartments (Pitelka and Hamamoto, 1983).

A nonlactating alveolar cell appears quite different histologically from a lactating alveolar cell. The cells found in mid-gestation can be identified by their sparse cytoplasm with few free ribosomes, a few strands of rough endoplasmic reticulum, scattered mitochondria, a few

scattered vesicles, and large lipid droplets (Akers, 1994). The nuclei of these cells are usually irregularly shaped and occupy the majority of the cell. The alveolar cells undergo quite substantial and dramatic differentiation to prepare for pending lactation. This differentiation is primarily under the control of prolactin and glucocorticoids.

During the cellular differentiation prior to lactation, the cell nucleus becomes rounded and basally positioned in the cell. The majority of the cellular area is dedicated to the synthesis and secretion of milk proteins. Rough endoplasmic reticulum occupies most of the cytoplasmic volume. Secretory vesicles are present at the apical surface of the cell (Pitelka and Hamamoto, 1983). Enlarged lipid droplets are found near the apical surface, awaiting secretion into the alveolar lumen (Akers, 1994). Cross et al. (1958) reported histological changes in sow mammary tissue as farrowing approached. They noted an increase in alveolar lumen size and an increase in the number of fat globules ready for secretion by individual cells.

Overall tissue morphology changes as the mammary gland prepares for lactation. Howe et al. (1975) investigated the histology of dairy cows hormonally induced to lactate with E_2, P_4, and dexamethasone. On Day 18 of hormone administration, the tissue samples were characterized by numerous small alveoli with small lumenal areas and a large stromal area. By Day 21, the alveoli were larger and more irregularly shaped. The amount of stromal tissue was decreased. Tissue from Day 23 was classified as having more uniformly developed alveoli with increased lumenal area and a greatly decreased stromal area.

Kensinger et al. (1982) investigated the histological changes in gilt mammary tissue during pregnancy and lactogenesis. It was evident that as the gland developed, the alveolar epithelium proliferated and became tightly packed. The amount of surrounding stromal and adipose tissue greatly diminished as well.

Milk Composition

Although milk composition varies greatly across species in regards to the ratio of constituents, there are a few components that are typical in the milk of most placental mammals: caseins, whey proteins, lactose, and lipids. Caseins are phosphoproteins that will precipitate when the milk is acidified. These proteins are extremely hydrophobic resulting in the formation of micelles in milk. Casein micelles are a rich source of amino acids and permit large quantities of calcium and phosphorus to be transported in milk. There are different types of caseins, designated as α, β, κ, and γ (Davies et al., 1983). The caseins differ in their primary structure, meaning the number and type of amino acid residues making up the polypeptide chain of the protein.

Another class of proteins found in milk is the whey proteins. They can be distinguished from caseins because they do not precipitate when the milk is acidified. The two major whey proteins are α-lactalbumin and β-lactoglobulin. No specific role has been found for β-lactoglobulin, but α-lactalbumin is involved in lactose synthesis. When α-lactalbumin is combined with the Golgi enzyme galactosyltransferase, lactose synthetase is formed (Davies et al., 1983; Akers, 1994).

Artificial Induction of Lactation

Using the knowledge of endocrine control of mammogenesis, lactation or lactogenesis has been artificially induced in heifers, goats, ewes, and gilts. Early studies reported successful lactation in heifers and goats with various techniques of E administration: oral synthetic estrogens, subcutaneous implants, or single injections of different esters (Folley and Malpress, 1943, 1944; Hammond, Jr. and Day, 1944; Parks and Glover, 1944; Folley et al., 1945). Hancock et al. (1954) induced lactation in Jersey heifers using a five-month period of

diethylstilbestrol and P_4 administration. However, these protocols involve long periods of hormone administration, which make them less practical. Smith and Schanbacher (1973) reported successful lactation in cows treated with injections of E_2 and P_4 for 7 d, but individual milk yields and percent of cows induced were quite variable.

Injections of dexamethasone, a synthetic glucocorticoid, in conjunction with the ovarian steroid treatment, have been shown to decrease the animal variability, but do not have an effect on individual milk yield (Fulkerson and McDowell, 1975; Chakriyarat et al., 1978). Collier et al. (1977a) hypothesized that PRL is the limiting factor in lactation induction in cattle, and that low PRL levels are most likely the cause of the variation in milk yield seen in previous studies. Using 19 nonpregnant, nonlactating cows, Collier et al. (1977a) administered a 7 d treatment of E_2 and P_4, dexamethasone on d 18 to 20, and reserpine on either d 13 to 16 or d 8, 10, 12, and 14. Reserpine injections were shown to release PRL, lasting for several hours (Bauman et al., 1977).

From the study described above, Collier and associates concluded that reserpine administration greatly reduced animal variation in milk production and increased the overall success rate of the induction procedure. However, in a conflicting study, Peel et al. (1979) found no benefit of reserpine administration on milk yields of lactation induced dairy cows.

Other experiments have successfully employed alternative methods of steroid delivery, e.g., intravaginal sponges, for lactation induction (Davis et al., 1983). Head et al. (1982) compared the effects of a 7 d hormone administration period with a 21 d protocol. They reported higher milk yields in cows receiving E_2 and P_4 for 21 d. These results conflict with Peel et al. (1979) who found that prolonging the hormone administration period did not have a positive effect on milk yield.

Studies, using slightly different protocols, have shown that lactation induction is also possible in ewes. Fulkerson and McDowell (1974) administered estradiol benzoate and P_4 every 3 d for 60 d to induce mammogenesis in 12 crossbred ewes. Subsequent injection of either dexamethasone once daily for 6 d or estradiol benzoate and P_4 twice daily for 6 d resulted in further gland development and milk yields similar to naturally lactating ewes from the same flock. However, ewes not receiving a second phase of hormone administration produced lesser quantities of milk.

A similar study showed that lactation could be induced in ewes using a 30 d hormone administration period instead of a 60 d period. Although milk yields in this study were lower than milk yields reported in previous studies, they were still adequate for the Border Leicester X Merino crossbreed used (Fulkerson et al., 1975). A group of ewes receiving estradiol benzoate and P_4 as the second phase of treatment was also given ergocryptine, a proven inhibitor of PRL secretion. These ewes failed to lactate, suggesting that estradiol benzoate and P_4 induce lactation by influencing the release of PRL.

These protocols described involve substantially long periods of hormone administration. Head et al. (1980) investigated the possibility of increasing the dosages given in order to decrease the length of the treatment period. Smith and Schanbacher (1973) demonstrated that increased dosages were effective in cows (.1 mg E_2/kg BW/d, .25 mg P_4/kg BW/d). Head et al. (1980) reported successful lactation induction in ewes with a 7 d treatment of E_2 and P_4 (.5 mg and 1.25 mg/kg BW/d, respectively). However, milk yields and percent of ewes induced to lactate increased significantly in the group receiving additional injections of hydrocortisone.

An E_2 and P_4 administration protocol has been adapted for use in swine as well. Shamay et al. (1992) successfully used slow-release pellets implanted in the flank of five-month-old gilts

to induce mammary development and lactogenesis. These pellets were designed to release E_2 and P_4 for 21 d.

Mammary explant cultures

Mammary explant culture has been used as a method of more precise identification of specific hormone effects or multiple hormone interactive effects on alveolar cell differentiation and the onset of lactogenesis. Studies have been conducted to determine the optimum hormone milieu for extended culture of mammary tissue. Elias (1959) recognized the importance of insulin in the culture media. Insulin was reported as necessary for DNA synthesis and cell division in vitro. Rivera and Bern (1961) identified the primary effect of insulin in culture as being its ability to maintain the alveolar structure during the culture period. They also suggested that cortisol enhances this effect of insulin. Stockdale and Topper (1966) found that mammary epithelial cells of virgin and mid-gestation mice, in the presence of insulin, initiate DNA synthesis and divide.

The changes in cell function associated with lactogenesis are directly related to cell proliferation. Mammary epithelial cells must divide, in the presence of insulin, before casein synthesis is possible. However, culture in insulin alone fails to support casein synthesis. The hormones hydrocortisone and PRL are necessary for this cell function (Stockdale and Topper, 1966). This study supported the results of Juergens et al. (1965), in which they reported that insulin, hydrocortisone, and PRL synergize to stimulate casein synthesis in mice mammary explant cultures. This triple hormone combination was also required for whey protein synthesis in culture (Lockwood et al., 1966). Forsyth (1971) suggested that PRL-linked RNA synthesis was key for the transformation of non-secretory to secretory cells.

Collier et al. (1977b) investigated the hormone milieu necessary for culture of mammary explants of pregnant cows. Their results indicated that insulin was necessary for cell survival. Also, the combination of insulin and hydrocortisone resulted in cytological alterations of the alveolar cells, but casein synthesis was not evident unless PRL was present in the culture media. Further studies with the bovine have shown successful explant culture in the presence of insulin, hydrocortisone, and PRL. McFadden et al. (1988) reported that bull mammary tissue explants were capable of secreting milk proteins in culture. McFadden and associates (1989) also showed that secretion of milk proteins was possible by prepubertal heifer mammary explants when cultured in media supplemented with insulin, hydrocortisone, and PRL.

Jerry et al. (1989) demonstrated that cultured porcine mammary tissue from gilts in late gestation was capable of secreting milk proteins as well. The triple hormone combination of insulin, hydrocortisone, and PRL was necessary and PRL elicited a dose-dependent increase in metabolic activity and an overall increase in fatty acid synthesis. Overall, these studies of mammary explant cultures show that, across species, culture media must be supplemented with insulin, hydrocortisone, and PRL in order for cellular proliferation and differentiation to occur, accompanied by milk protein synthesis and secretion.

Transgenic animals as bioreactors

Recombinant proteins have historically been produced in vitro using cell culture technology. However, these methods are extremely costly and not capable of producing the large quantities of certain proteins that are needed. An alternate means of producing these recombinant proteins is the use of transgenic animals as bioreactors. The proteins can be targeted to a specific tissue within the animal to facilitate their collection.

Studies have shown that it is possible to target the synthesis of the recombinant protein to the mammary gland of transgenic mice and livestock (Clark et al., 1989; Shamay et al., 1991; Wall et al., 1991; Velander et al., 1992b; Morcol et al., 1994). This allows for secretion of the protein into milk, and therefore, easy collection for further processing. The cDNA or genomic sequence corresponding to the recombinant protein can be directed to the mammary gland by attaching it to a milk protein gene promoter.

Whey acidic protein (WAP) is an abundant milk protein endogenous to mice (Piletz et al., 1981; Hennighausen and Sippel, 1982), but not to swine or other livestock. The gene encoding for WAP is activated in the mammary gland and regulated by the hormonal and developmental signals of pregnancy (Hobbs et al., 1982; Pittius et al, 1988). It was demonstrated that WAP can be expressed as a recombinant protein in the milk of swine (Shamay et al., 1991; Wall et al., 1991). These studies led to the use of the regulatory region of the gene as a promoter for other transgenes.

Transgenic research has been conducted using a wide variety of animals including mice, rabbits, cows, sheep, goats, and pigs (Clark et al., 1989; Shamay et al., 1991; Velander et al., 1992a,b; Riego et al., 1993; Houdebine, 1995; Wall et al., 1997). Although dairy animals such as the cow, sheep, and goat produce larger quantities of milk daily than swine, the use of swine is more efficient for the production of transgenic offspring. First, the number of ova collected for microinjection per gilt is 2-3 times greater than possible from a ruminant (Wall et al., 1991). Also, the number of fetuses supported during one gestation in swine is much greater than a cow or ewe. Finally, the generation interval of swine is approximately 11 months, considerably less than that of cattle or sheep. This makes the use of swine as animal bioreactors more cost-effective and requires fewer animals.

There are several steps necessary in order to use transgenic swine for the production of recombinant proteins (Butler et al., 1997). The first requirement is the formation of DNA constructs using milk protein promoters such as mWAP that will express the cDNA or genomic sequence of the transgene. The constructs must then be microinjected into the pronucleus of the pig embryo. Van Cott et al. (1997) have shown that in order for the transgene expression to be stable from generation to generation, a single cointegration site and associated transgene copy number is desirable.

Animals are tested for the presence of the transgene in tail tissue shortly after birth. However, in order to determine if the recombinant protein will be expressed in the milk, the pigs have to be maintained at least through their first lactation. As described earlier, mammary development and lactogenesis can be induced in gilts. Tissue cultures can then be tested for the presence of milk protein genes as well as the actual recombinant protein production (Shamay et al., 1992). One cannot determine the precise level of protein production that will occur in the milk during a natural lactation, but the presence of the transgene and protein can be detected. This procedure could prove valuable for large-scale pharmaceutical production of recombinant proteins by allowing earlier identification of animals that will express the protein.

Fibrinogen

One of the proteins currently being evaluated in transgenic mice and livestock is recombinant human fibrinogen (rhFib). Human fibrinogen is a complex plasma glycoprotein that is involved in the final stages of blood coagulation. It is synthesized in the liver as a dimer with a molecular weight of 340 kDa. Fibrinogen is made up of two subunits linked by three disulfide bonds. Each subunit contains three polypeptide chains, Aα (66 kDa), Bβ (54 kDa), γ

(48 kDa). These chains are linked by disulfide bridges to form α-helices (Doolittle, 1984; Roy et al., 1991).

The estimated clinical need in the United States for rhFib greatly exceeds the amount that can be harvested from donated human plasma (Paleyanda et al., 1991 as cited by Butler, 1997). Also, using proteins obtained from human plasma entails the potential risk of contamination with infectious agents such as HIV and hepatitis C. Transgenic animals are now being considered as a possible source for rhFib. Butler et al. (1997) reported that the mammary gland of transgenic mice is capable of secreting fully assembled rhFib and some individual rhFib chains. They also reported that individual rhFib chains have been secreted by the mammary gland of transgenic swine. The production and secretion of individual chains and fully assembled rhFib secretion by transgenic swine is currently being investigated.

Materials and Methods

Animals

Lactogenesis was induced in five (n=2) and ten (n=2) month-old transgenic gilts, and four (n=2) and seven (n=2) month-old non-transgenic gilts. Details of the animals used are outlined in Table 1. All of the transgenic animals are genetically distinct in the Aα chain, possibly having different integration sites and number of copies of the transgene. Mammary biopsies were also taken from a lactating non-transgenic sow for milk protein controls.

Table 1. Animals.

Gilt	Age	Status	Transgene Construct
97-1	5 months	Transgenic	Fib Aα, γ
97-3	5 months	Transgenic	Fib Aα, Bβ
19-10	10 months	Transgenic	Fib Aα, Bβ, γ
19-12	10 months	Transgenic	Fib Aα, Bβ, γ
35-8	4 months	Non-transgenic	-----
40-6	4 months	Non-transgenic	-----
4-5	7 months	Non-transgenic	-----
5-4	7 months	Non-transgenic	-----

Hormonal implants and mammary biopsy

Gilts were administered sodium thiopental (100 mg/ml) until a surgical plane of anesthesia was achieved. During the surgical procedures, the gilts were maintained on an inhalant anesthetic mixture of NO, O_2, and Halothane. Tissue from several mammary glands was recovered immediately prior to inserting three hormone implants into each pig. The

implants were slow-release pellets containing E_2 (150 mg) and P_4 (200 mg; Innovative Research of America, Toledo, OH) and were placed subcutaneously in the left flank.

Each gilt received one pellet of E_2, designed to release 7.1 mg/d for 21 d. The gilts also received two pellets of P_4, each designed to release 9.5 mg/d for 21 d. The pellets were made of biodegradable material and therefore were not removed after the treatment period. Four weeks after implantation, tissues from several mammary glands was surgically recovered from each gilt, using the anesthesia procedure detailed above. This lactogenesis induction protocol was adapted from Shamay et al. (1992).

Blood sample collection / Radioimmunoassays

Vena caval blood samples were collected from each gilt on d 0, 7, 14, and 21. Progesterone and estradiol concentrations in these samples were measured by radioimmunoassay kits (Diagnostics Products Corporation, Los Angeles, CA). The intra-assay coefficients of variation were 3.9% and 5.0% for progesterone and estradiol, respectively. These data were used to confirm adequate release of hormones from the implants.

Histology

Tissue samples recovered before and after hormonal implantation were fixed in Histochoice (AMRESCO, Solon, OH) and embedded in H-7100 Technovit (Energy Beam Sciences, Inc., Agawam, MA). Tissue samples were sectioned by microtomy and stained with Azure II. Mammary alveolar development in response to *in vivo* induction was evaluated subjectively by light microscopy.

Explant cultures

Tissue samples recovered after hormonal implantation were placed into medium M-199 containing an antibiotic / antimycotic solution (GIBCO, Grand Island, NY). The solution consisted of 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone. The media was supplemented with insulin (I; 1 μg/ml), hydrocortisone (H; .5 μg/ml), porcine prolactin (pP, 1 μg/ml), and ovine prolactin (oP; 1 μg/ml) in the following combinations: 1) I, 2) IH, 3) IHpP, 4) IHoP. Explants were cultured at 37°C in 95% air: 5% CO_2 for 4 d. Media were changed at 48 h. The media at d 4 were collected and stored at -4°C until Western analysis.

DNA, RNA, and protein isolation

Tissue samples were placed in liquid nitrogen and stored at -90°C until processing. Tri Reagent (.5 ml; Molecular Research Center Inc., Cincinnati, OH) was added to each sample. The tissue was homogenized using a Kinematica polytron homogenizer (Brinkman Instruments, Westbury, NY). After homogenization, 0.5 ml of Tri-reagent was added and the procedure followed the manufacturer's protocol for isolation of RNA and protein. For DNA isolation, tissue samples were digested in 840 μl of lysing solution overnight at 55°C. After incubation, 250 μl of phenol/chloroform mixture (125 μl phenol, 120 μl chloroform, 5 μl isoamylalcohol) was added to each lysate. The samples were shaken for 10 s on a Mini-bead shaker and then centrifuged for 10 min at 9,000 x g. The supernatant was transferred to a new micro-centrifuge tube. Five hundred μl of isopropyl alcohol was added and samples were inverted gently until a DNA clot formation. The DNA clot was washed twice with 80% ethanol and resuspended in 150 μl of Tris-EDTA for spectrophotometry.

DNA and RNA quantitation / Gel electrophoresis

Total DNA and RNA of each sample (preimplant, postimplant, I, IH, IHpP, IHoP) for each gilt was measured by spectrophotometry at an absorbance wavelength of 260 nM. Electrophoresis was performed on each RNA sample to determine the RNA integrity, using 1.2% agarose and 2.5% formaldehyde gels. Ethidium bromide stain was loaded in each well of the gels.

Slot blot analyses

The presence of RNA encoding for rhFib Aα chain and β-lactoglobulin in each sample was detected by slot blot analysis. Briefly, samples were added to 25 µl of denaturation solution, consisting of 64% formamide, 8.5% formaledhyde, and 0.66X MOPS running buffer. The samples were then stored at 50° C for 15 min. After incubation, 200 µl of 2.5% formaldehyde was added to the samples. The solution was applied to a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) which blotted the samples onto a Magna Charge nylon membrane (Material Separations Inc., Westboro, MA).

After blotting, the sample wells were washed with deionized water. The membrane was then crosslinked by UV radiation with a UV crosslinker model FB-UVXL-1000 (Fisher Scientific, Pittsburgh, PA). The membranes were probed using the 1.0 kbp Kpn I fragment of the rhFib Aα cDNA. Additional membranes were probed using the full length cDNA of porcine β-lactoglobulin, kindly provided by Craig W. Beattie (University of Minnesota, St. Paul, MN). Signal quantification from autoradiographic film was determined by densitometry.

Dot blot analysis

Media from explant cultures were pooled within treatment per gilt. Dot blot analysis was used to test for the presence of secreted rhFib in the culture media. Briefly, 100 µl of sample was loaded into each well and transferred by vacuum to an Immobilon AV membrane (Millipore Corporation, Bedford, MA). The membranes were blocked overnight in TBSTC (20 mM Tris, 50 mM NaCl, 0.05% Tween 20, 0.5% bovine casein, pH 7.2). The membranes were then incubated in TBSTC for 1 h with the primary antibody ADI anti-hFib (American Diagnostica Inc.; 1:500 dilution). The blots were rinsed with deionized water, followed by another 1 h incubation in TBSTC with the secondary antibody Sigma A9169 (Sigma, St. Louis, MO; 1:2000 dilution). The blots were rinsed again and developed using the metal enhanced DAB immunostaining kit (Pierce, Rockford, IL) according to manufacturer's protocol.

Western analysis

The isolated protein fractions of the tissue samples were separated by electrophoresis using gradient SDS gels (8-16%) from Novex (San Diego, CA). The proteins were transferred to polydivinylidene difluoride membranes (Bio-Rad, Hercules, CA) as described by the manufacturer. The membranes were blocked with TBSTC using a rocker platform. Blots were probed using a primary antibody for 1 h at 37°C. Primary antibodies used were ADI rabbit polyclonal anti-hFib (American Diagnostica Inc.), and PFB polyclonal anti-porcine whey (Pel-freeze Biologicals, Rodgers, AK).

The membranes were rinsed in TBST for 5 min, followed by probing with a second antibody and subsequent incubation for 1 h at 37°C. Secondary antibodies used were Sigma A9169 for the fibrinogen blots and A6154 for the whey blots (Sigma, St. Louis, MO) . The blots

were then rinsed three times in deionized water and developed using the metal enhanced DAB immunostaining kit (Pierce, Rockford, IL) according to manufacturer's protocol.

Statistical analyses

Plasma concentrations of E and P_4 were analyzed by repeated measures ANOVA using the General Linear Models procedure of SAS. Also, fibrinogen concentrations in culture media were evaluated by ANOVA using the GLM procedure. The model included status i.e., transgenic or non-transgenic, pig nested within status, treatment (I, IH, IHpP, IHoP), and the status by treatment interaction as independent variables. Status was tested by pig nested within status (SAS, 1994).

Results

Concentrations of estradiol and progesterone

No estradiol was detected in blood samples taken on the day of pellet implantation. Estradiol concentrations significantly increased over time (P= .029), with concentrations remaining elevated throughout the 21 d treatment period. Figure 1 shows the average concentrations of E_2 of all gilts. Minimal concentrations of P_4 (< 3 ng/ml) were detected in blood samples taken on the day of pellet implantation, with the exception of three gilts. Due to these increased initial concentrations, P_4 did not statistically significantly increase over time (P=.247). However, P_4 levels of individual gilts were elevated sufficiently. Figure 2 shows the average concentrations of P_4 for all gilts, grouped as low initial P_4 or high initial P_4. These results indicate that the pellets adequately released E_2 and P_4 over 21 d.

Histology

Tissue sections from pre-treatment mammary glands were characterized by large amounts of stromal tissue relative to parenchymal tissue. The alveoli were undeveloped, with irregularly shaped lumena present in some epithelial cell clusters. Overall, the individual epithelial cells were relatively undifferentiated. The nuclei of the cells were often irregularly shaped and occupied the majority of the cell. The cytoplasm of the epithelial cells stained moderately light, indicating minimal secretory capacity.

The hormonal implants stimulated apparent parenchymal tissue proliferation and ductal and lobulo-alveolar development. Tissue sections from post-treatment mammary glands showed dramatic relative increases in parenchymal tissue and decreases in stromal tissue. The alveoli were characterized by a higher degree of structural differentiation, with a greater number of

formed lumena. Some apparent secretions were present in some alveolar lumena, and often appeared to compress the epithelial cells. The epithelial cells also showed a higher degree of apparent structural differentiation. The nuclei became more rounded and basally located within the cells. The epithelium showed greater secretory capacity, as determined by increased cytoplasmic staining compared to pre-treatment epithelium. These results indicate that mammogenesis and limited lactogenesis were induced by the E_2 and P_4 implants.

Tissue sections representative of each gilt and treatment are depicted in Figures 3 through 18. Figure 3 shows a pre-implant tissue section from transgenic gilt 19-10. The tissue is made up mainly of adipose tissue, with very little parenchyma. The alveolar cells are unorganized with little lumen formation. The cytoplasm of the cells is stained lightly, indicating little secretory activity. Figure 4 depicts post-implant tissue from the same gilt. There was a dramatic increase in the amount of parenchymal tissue present, accompanied by a decrease in adipose tissue. The epithelium is more organized than before treatment, exhibiting a greater number of developed alveolar lumena. Also, the darker stained alveolar cell cytoplasm is indicative of greater secretory activity.

Pre-implant tissue from transgenic gilt 19-12 is represented in Figure 5. There is a large amount of stromal tissue relative to parenchyma. The tissue is unorganized, and the alveolar cell nuclei are randomly positioned within the cells. In Figure 6, the post-implant tissue is characterized by dramatic parenchymal proliferation, with highly developed alveolar lumena. The epithelium is organized and the cells have taken on a somewhat more polarized appearance. Some secretions can be seen in the alveolar lumena, seemingly compressing the alveolar cells.

Figures 7 and 8 depict pre- and post-implant tissue sections from transgenic gilt 97-1, respectively. The pre-implant tissue section shows loosely packed parenchymal tissue with a

moderate amount of alveolar development. However, the alveolar epithelium is still relatively unorganized. The post-implant tissue section is characterized by more tightly packed parenchyma with greater organization of alveoli. Some secretions can be seen in the alveolar lumena as well.

Pre-implant tissue from transgenic gilt 97-3 is shown in Figure 9. There is comparatively little parenchymal tissue versus adipose and connective tissue. The alveoli are underdeveloped. The dark stained material in the lumena is a result of the dehydration and staining process. The post-implant tissue section in Figure 10 shows dramatic proliferation of parenchymal tissue, a greater number of developed lumena, with a large amount of secretions noted.

Figure 11 depicts pre-implant tissue from non-transgenic gilt 40-6. The tissue is characterized by abnormally large alveoli that are few in number and underdeveloped. The basement membrane of the alveoli is distorted, taking on a scalloped appearance. Also, the individual alveolar cells are uncharacteristically rounded at the apical surface. The cause of this aberrant appearance is unknown. After treatment, tissue from this gilt was comparable to the other gilts in the experiment (Figure 12). The alveoli increased in number and the tissue became tightly packed. The basement membrane was no longer distorted. The epithelial cells were developed and organized, with secretions noted in the lumena.

Tissue from the remaining control gilts followed a pattern of development similar to the transgenic gilts as a result of the induction procedure. Figures 13 and 14 show tissue sections from non-transgenic gilt 35-8. The pre-implant section shows a moderate amount of unorganized parenchyma. There was dramatic parenchymal proliferation and epithelial organization after treatment. Figure 15 depicts a pre-implant tissue section from non-transgenic gilt 4-5. The tissue is characterized by a large amount of stromal tissue relative to parenchyma.

The epithelium present is scattered and disorganized. Figure 16 shows post-implant parenchymal proliferation and development. Alveoli are more organized, with some secretions noted in the lumena.

Pre-implant tissue from non-transgenic gilt 5-4 is shown in Figure 17. There is a small amount of loosely packed parenchymal tissue within a large amount of stromal tissue. The epithelial cells have formed lumena but the cells themselves are undeveloped, with very little cytoplasm. In post-implant tissue from the same gilt (Figure 18), there is a large amount of parenchymal tissue with a greater degree of organization and development. However, there are comparatively fewer developed lumena than post-implant sections from other gilts.

Figure 19 depicts another post-implant tissue section from non-transgenic gilt 35-8. This section shows a longitudinal view of a lobule emptying into a larger duct. Individual alveoli can be distinguished and the epithelial cells are organized.

DNA and RNA quantitation / Formaldehyde gels

DNA isolated from each sample was quantified by spectrophotometry. Results for pre- and post-hormonal treatment samples are shown in Table 2. An increased concentration of DNA indicates cellular proliferation with the assumption that cellular DNA concentrations are essentially constant. The concentrations of DNA measured in this study are conflicting. Cellular proliferation after induction was indicated in four of the eight gilts, exhibiting 33%, 49%, 427%, and 654% increases. However, based on the histological findings, total DNA should have been greater in the post-implant samples for all gilts. This discrepancy can most likely be attributed to incomplete isolation of DNA from the sample.

Total RNA isolated from each sample was similarly measured. Results for all samples are graphically depicted in Figures 20 and 21. Concentrations of RNA were quite variable

among gilts and across treatments. However, total RNA concentrations in post-implant samples were consistently greater than in pre-implant samples, indicating gene activation as a result of the induction. Cultured samples demonstrated a large variation in total RNA concentrations. This variation can possibly be attributed to incomplete isolation of RNA from the sample or inadequate culture conditions to maintain tissue viability. Also, the culture conditions may have been able to support certain cellular functions and not others, e. g., RNA transcription and protein synthesis.

Formaldehyde agarose gels were used to determine the integrity of each RNA sample. Except for four samples, the 18S and 20S bands were distinct with minimal smearing, indicating that the samples were not degraded. A new sample of RNA was isolated for two of the samples that appeared degraded. A second sample was not available for the remaining six degraded samples, hence, those samples were not analyzed further.

RNA Slot blot analyses

Slot blot analysis was used to determine the presence of mRNA for rhFib Aα chain and porcine β-lactoglobulin, an endogenous milk protein. Results from the rhFib blots are depicted in Figure 22, expressed as a percent of the human liver Fib standard. The mRNA for the transgene was detected in all of the samples. However, the transgene did not respond to the explant culture system as expected. Gene activation should increase with addition of each hormone to the culture media. Thus, mRNA levels should be greatest in tissue samples cultured in one of the two types of PRL.

A possible reason for the failure of the culture system to maintain RNA levels of the transgene may be the use of the mWAP promoter region. This sequence is endogenous to mice, and is controlled by the hormonal milieu present in the mouse during gestation and lactation. It

is possible that ovine and porcine PRL are inadequate in stimulating the production of mRNA, whereas murine PRL may be effective.

Results from the β-lactoglobulin blots are depicted in Figures 23 and 24. β-lactoglobulin mRNA was detected in all post-implant samples. However, β-lactoglobulin mRNA was not maintained well in the explant culture samples. Figure 25 shows the rhFib Aα chain slot blot analysis for Gilt 19-12. In this case, the mRNA levels responded to in vivo induction and in vitro culture as expected. The post-implant sample contained a greater amount of rhFib Aα chain mRNA. The cultured samples demonstrated increased levels of mRNA with each additional hormone supplement, with the highest levels found in the triple hormone combination using porcine PRL.

Protein Dot blot analysis

Dot blot analysis was performed to detect rhFib secreted into the explant culture media. The average values for the transgenic and non-transgenic gilts are shown in Table 3. There were no significant differences between the transgenic and non-transgenic gilts (P=.326). These results indicate that rhFib was not secreted into the media by the explant cultures. However, there was a substantial amount of crossreactivity between rhFib and endogenous porcine fibrinogen. Also, although not statistically significant, there was a trend evident across culture treatments. Concentrations of rhFib increased with each additional hormone added to the culture media.

Western analyses

Figures 26 and 27 show Western blots for rhFib for a non-transgenic (35-8) and transgenic (97-3) gilt, respectively. These gilts were chosen because of their consistent total

protein expression across treatments, as determined by silver staining of a Western blot. For the rhFib blots, proteins were separated under non-reducing conditions so that rhFib would be expected to migrate to a molecular weight of 340 kDa. No bands representing the recombinant protein were detected in either of these blots. Western analysis for rhFib was performed a second time under non-reducing conditions using the post-implant and IHpP cultured samples from these gilts (Figure 28). Again, no recombinant protein was detected.

Figure 29 represents Western analysis for rhFib under reducing conditions, which breaks disulfide bonds. This results in the migration of the individual chains of rhFib, Aα to 66 kDa, Bβ to 54 kDa, and γ to 48 kDa. No bands corresponding to these proteins were detected. A reduced Western blot for the remaining cultured samples from all of the transgenic gilts is shown in Figure 30. No rhFib was detected in any of the samples.

Western analysis was also performed to detect the presence of endogenous milk proteins in the tissue samples as a result of the induction procedure (Figure 31). The same blot from Figure 29 was probed again with the anti-pig whey antibody. A distinct band was detected at approximately 20 kDa in the non-transgenic pig whey, lactating sow, and post-implant samples. This band most likely represents the milk protein β-lactoglobulin. However, the protein was not detected in the cultured samples. This finding agrees with the results of the β-lactoglobulin mRNA blots, in which the mRNA was not sustained well in culture.

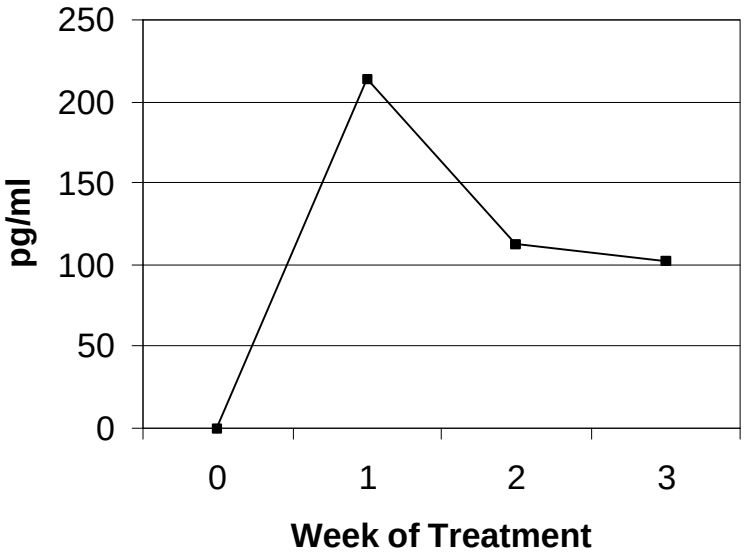


Figure 1. Average serum estradiol concentrations in vena caval blood samples. Estradiol concentrations were significantly increased over time (P=.03).

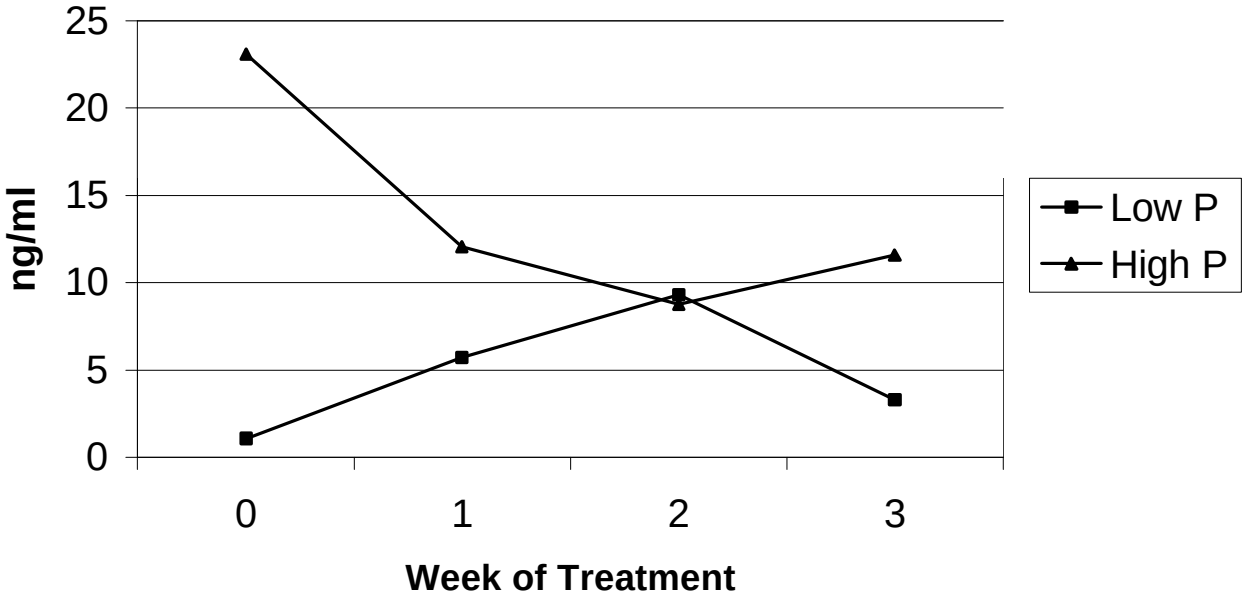


Figure 2. Average serum progesterone concentrations in vena caval blood samples. Gilts were grouped according to initial P_4 concentrations. Due to the elevated initial concentrations measured in 3 gilts, P_4 did not significantly increase over time (P=.25). Low P: Gilts with initial P_4 conc. < 3 ng/ml (n=5). High P: Gilts with initial P_4 conc. > 14 ng/ml (n=3).

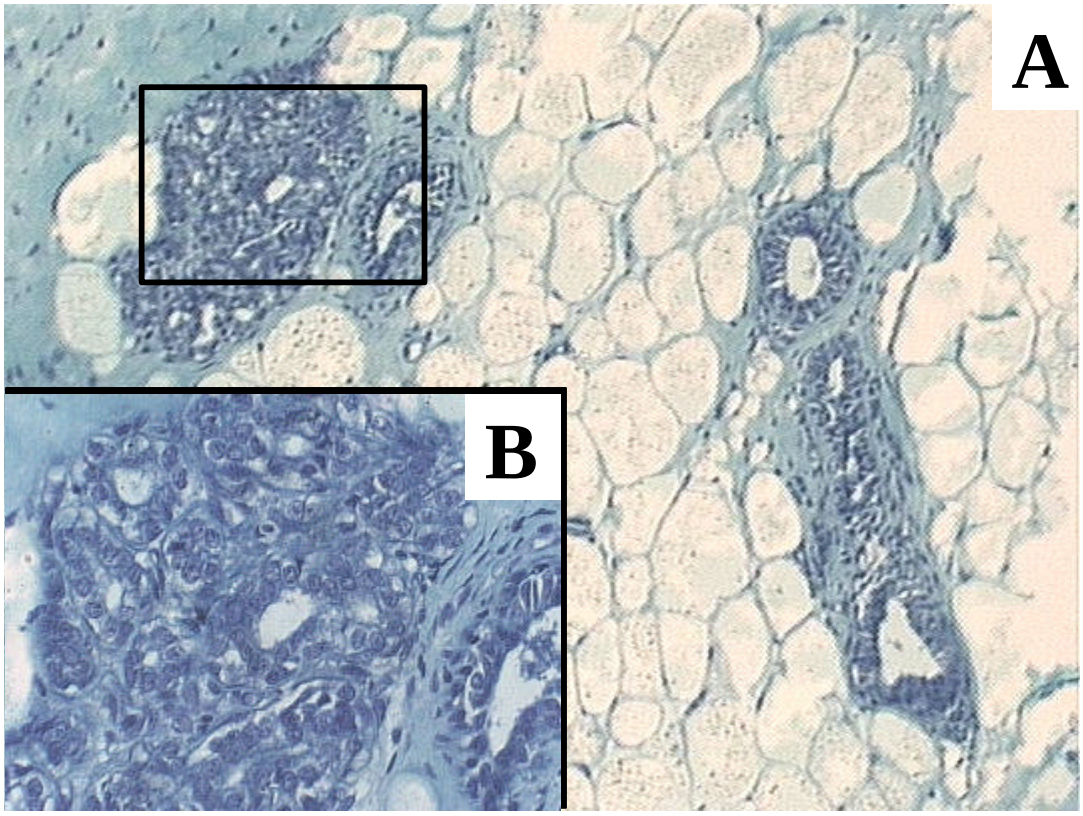


Figure 3A: Transgenic gilt 19-10 pre-implant tissue section. Note large amount of adipose tissue, very little parenchyma. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Epithelial cells are unorganized and exhibit light cytoplasmic staining (magnification 40x).

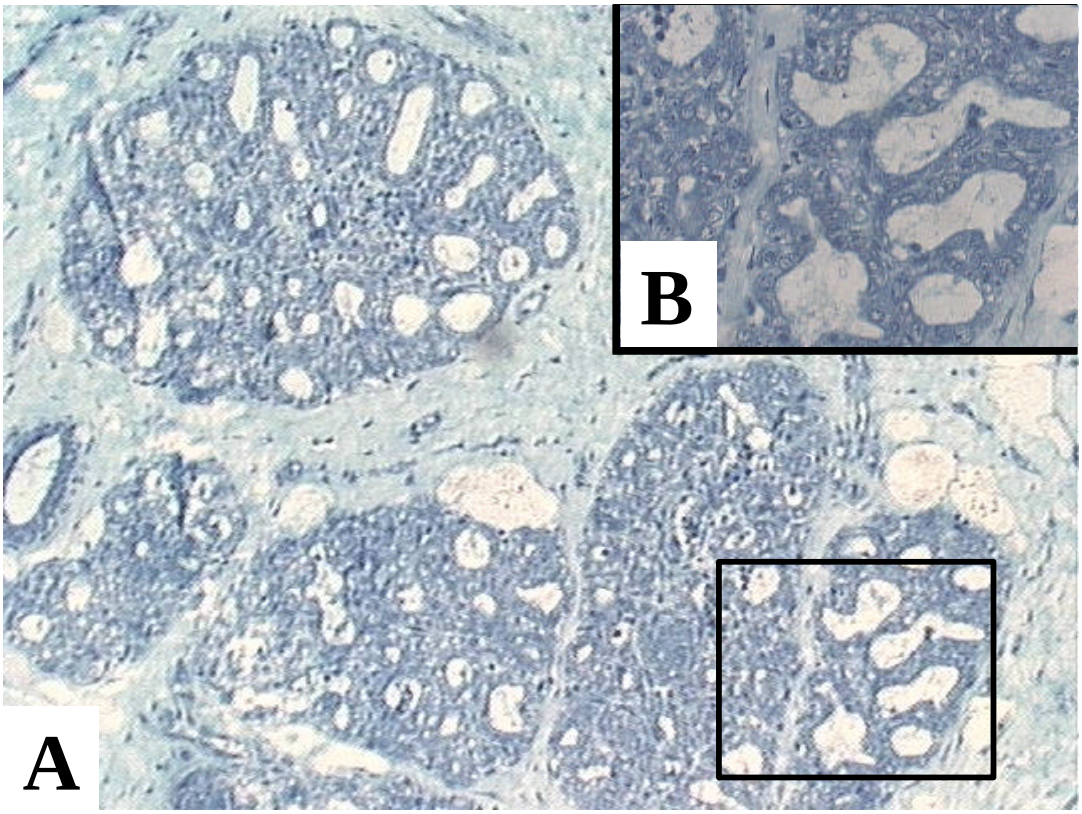


Figure 4A: Transgenic gilt 19-10 post-implant tissue section. Note increased amount of parenchyma. Greater number of developed alveolar lumena. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Tissue is more organized. Darker stained epithelial cell cytoplasm indicates secretory activity. Secretions can be seen in lumena. (magnification 40x).

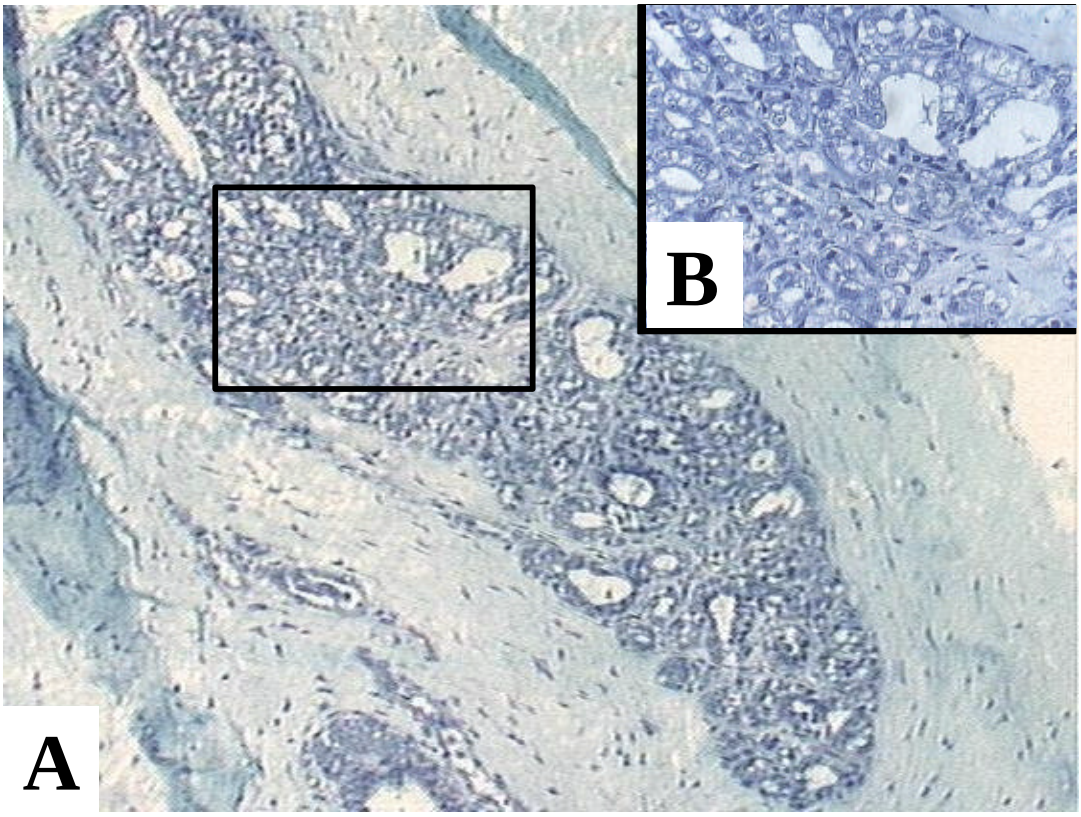


Figure 5A: Transgenic gilt 19-12 pre-implant tissue section. Note large amount of stroma versus parenchyma. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note lack of organization and light cytoplasmic staining of epithelial cells. (magnification 40x).

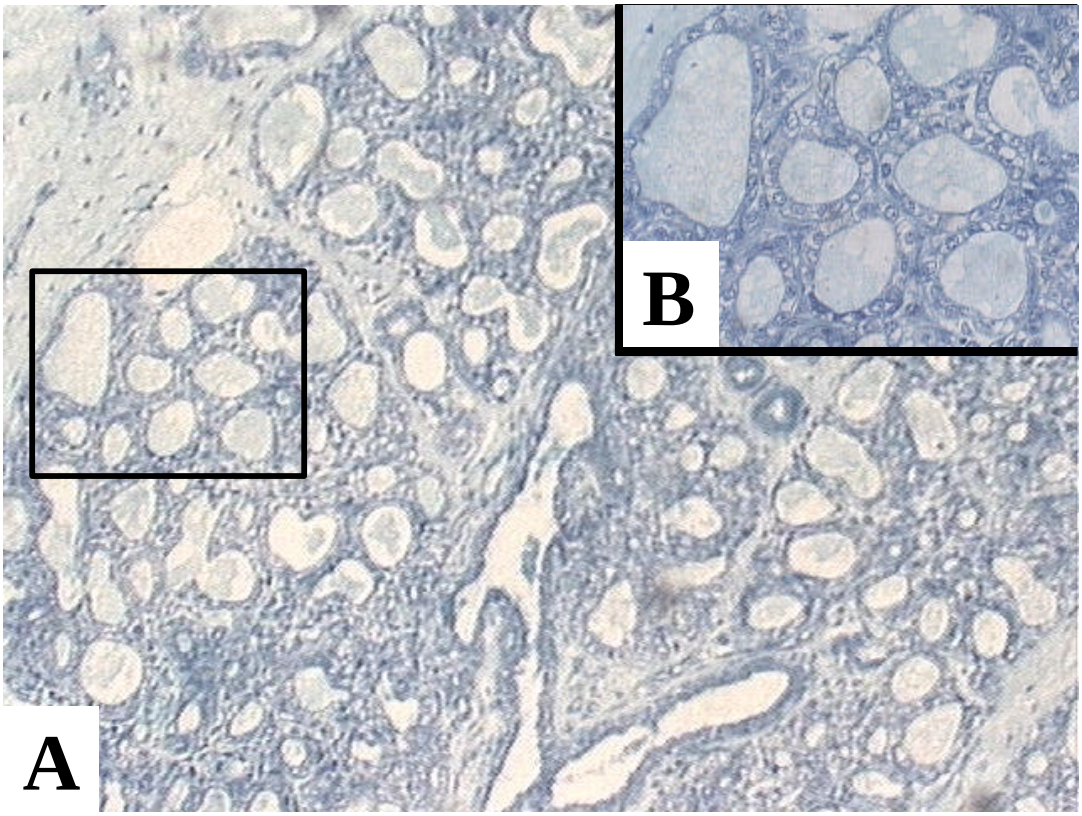


Figure 6A: Transgenic gilt 19-12 post-implant tissue section. Dramatic increase in parenchyma and developed alveoli (magnification 10x). **B:** Detailed inset of area in A outlined by black. Organized epithelium with large amount of secretions noted in lumena. (magnification 40x).

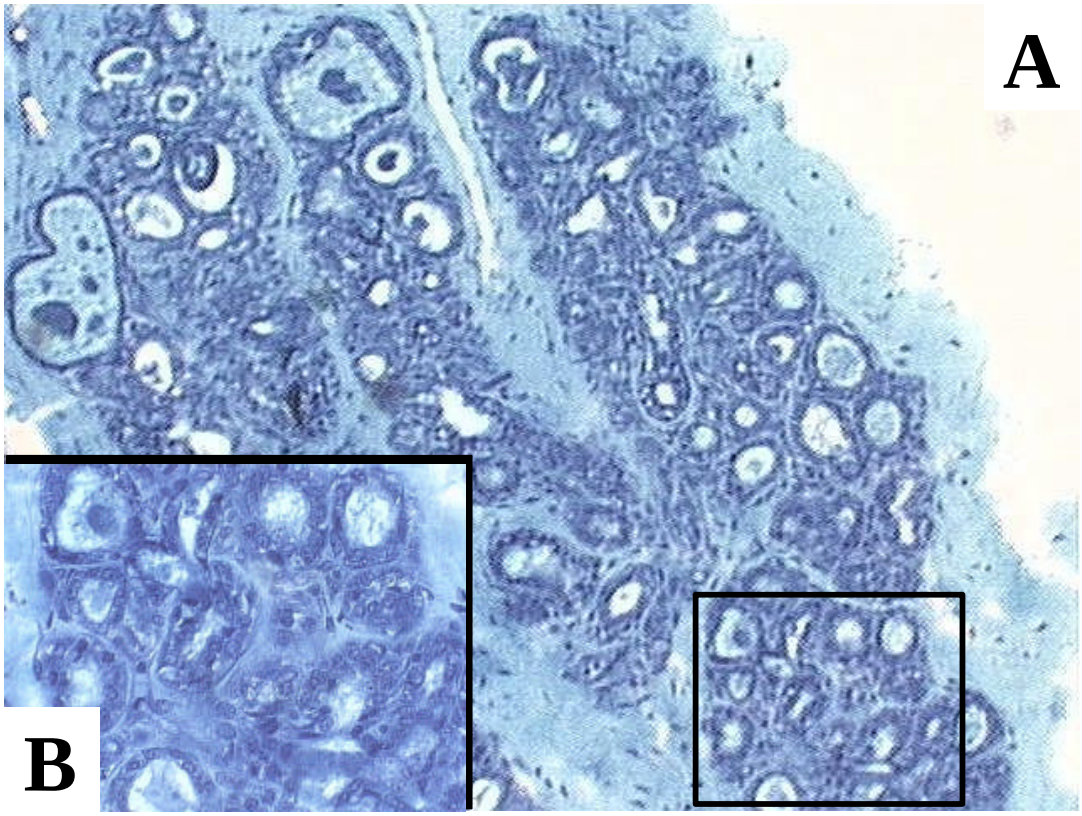


Figure 7A: Transgenic gilt 97-1 pre-implant tissue section. Note loosely packed parenchyma. Moderate alveolar development. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note disorganization of epithelial cells. (magnification 40x).

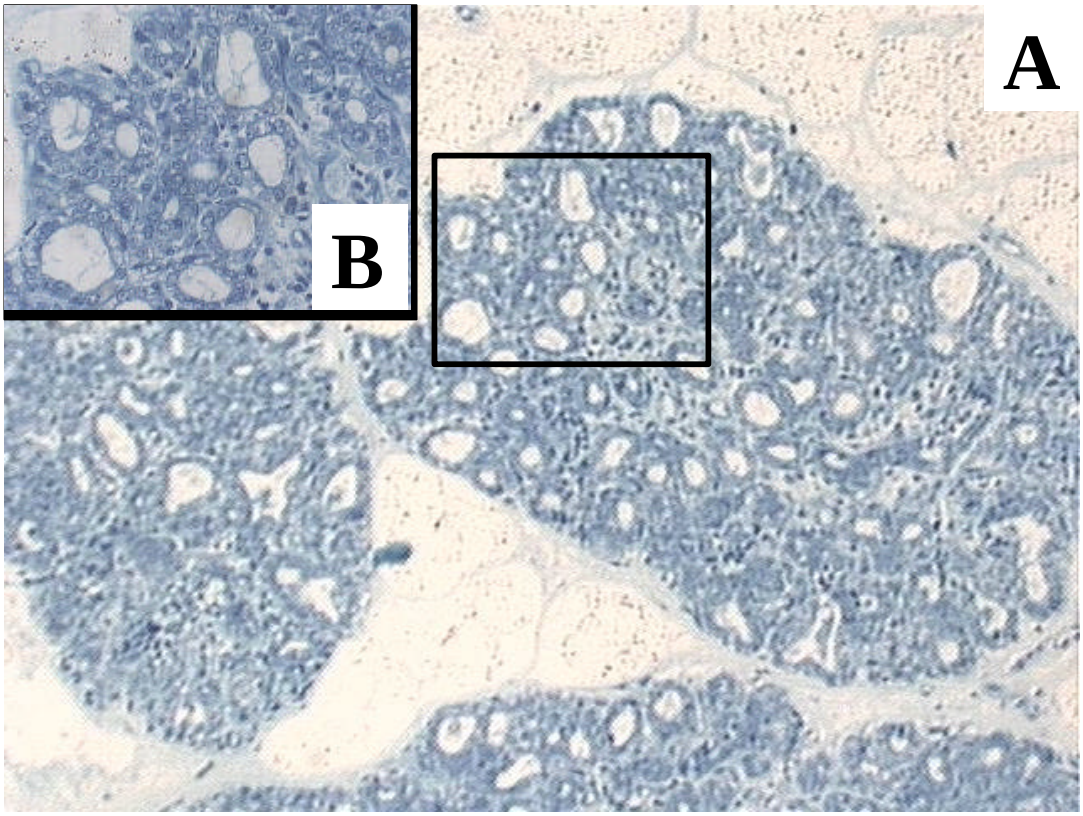


Figure 8A: Transgenic gilt 97-1 post-implant tissue section. Note proliferation of parenchyma. Increased alveolar development. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Greater tissue organization. Secretions can be seen in lumena. (magnification 40x).

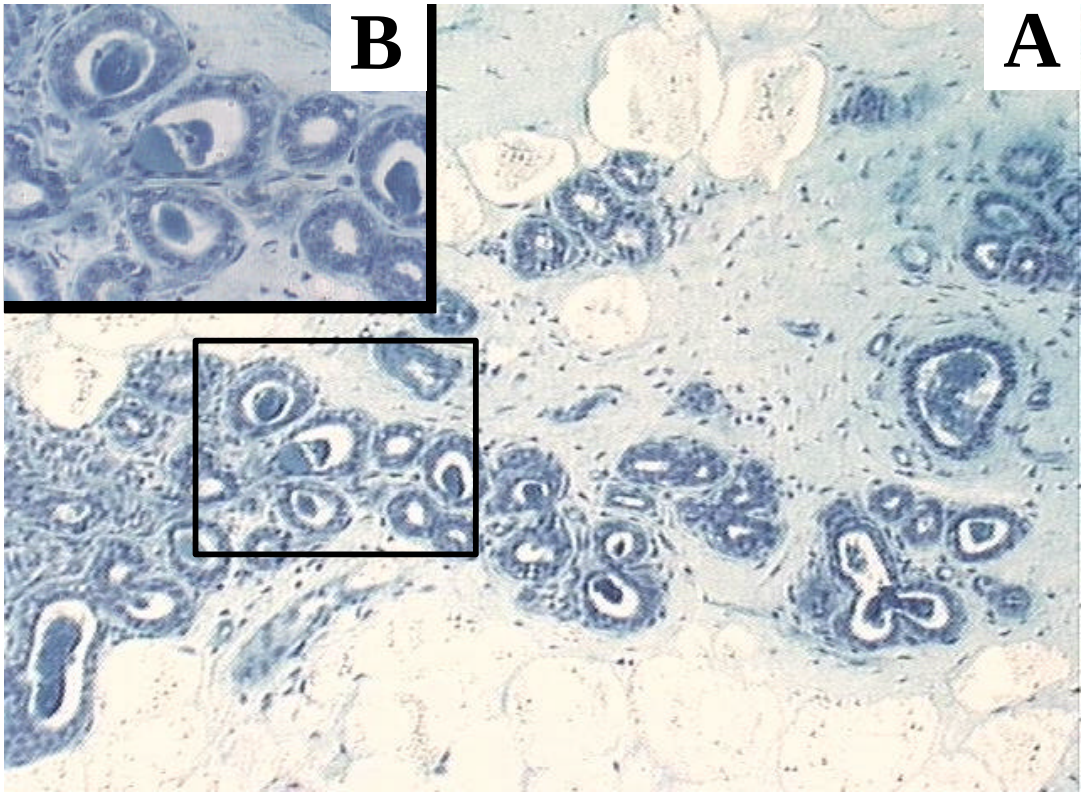


Figure 9A: Transgenic gilt 97-3 pre-implant tissue section. Note large amount of stroma. Alveoli are underdeveloped. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Sparse epithelial cells. (magnification 40x).

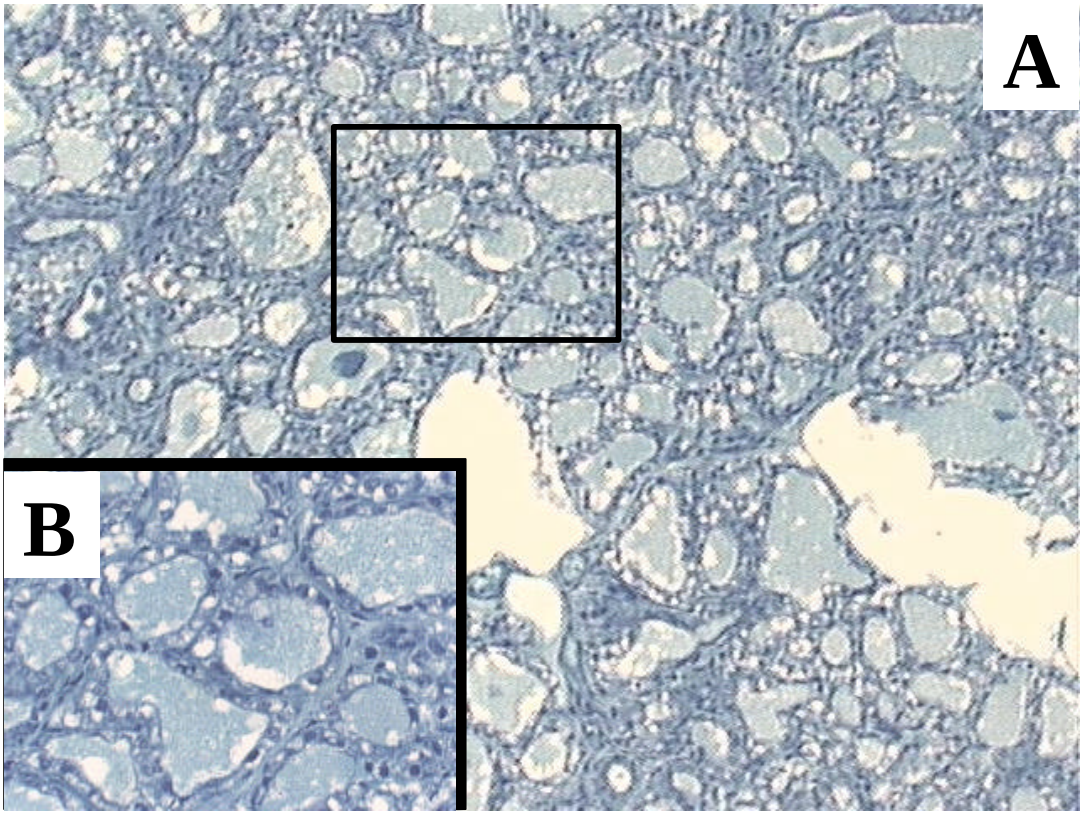


Figure 10A: Transgenic gilt 97-3 post-implant tissue section. Note dramatic proliferation of parenchyma. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note organization of epithelium and large amount of secretions in lumena. (magnification 40x).

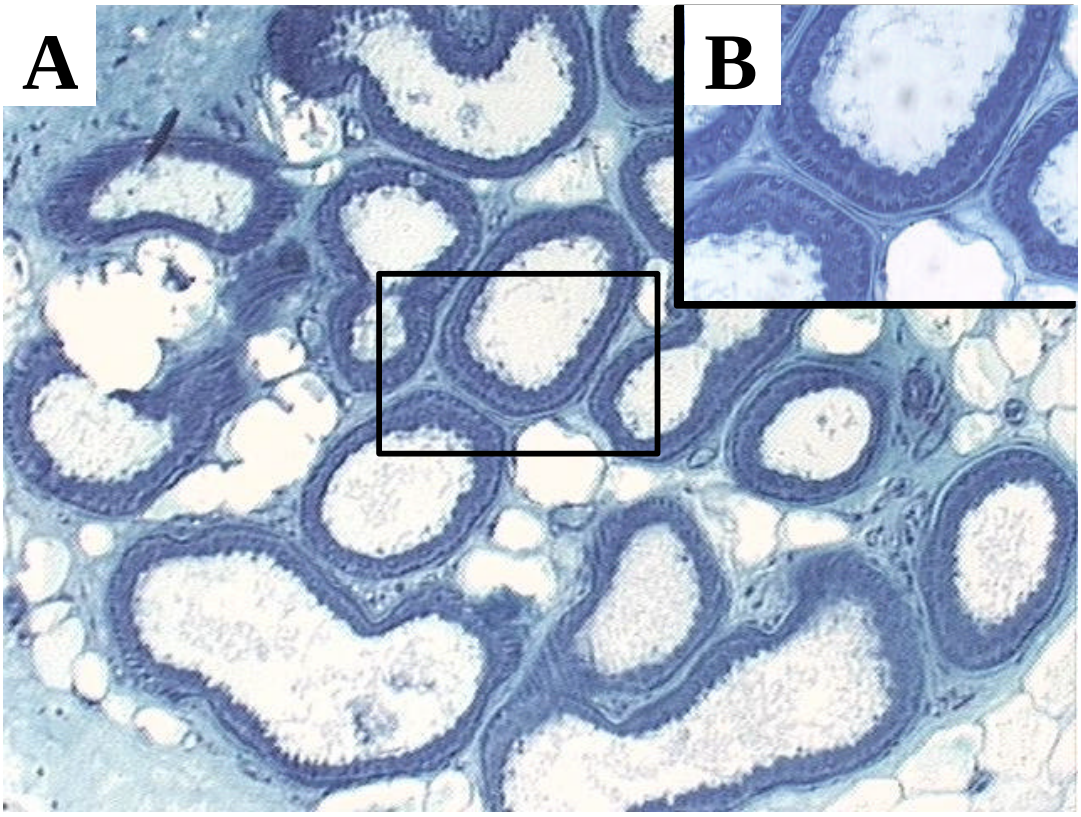


Figure 11A: Control gilt 40-6 pre-implant tissue section. Large alveoli but few in number and undeveloped. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note distorted basement membrane and oddly developed epithelial cells. (magnification 40x).

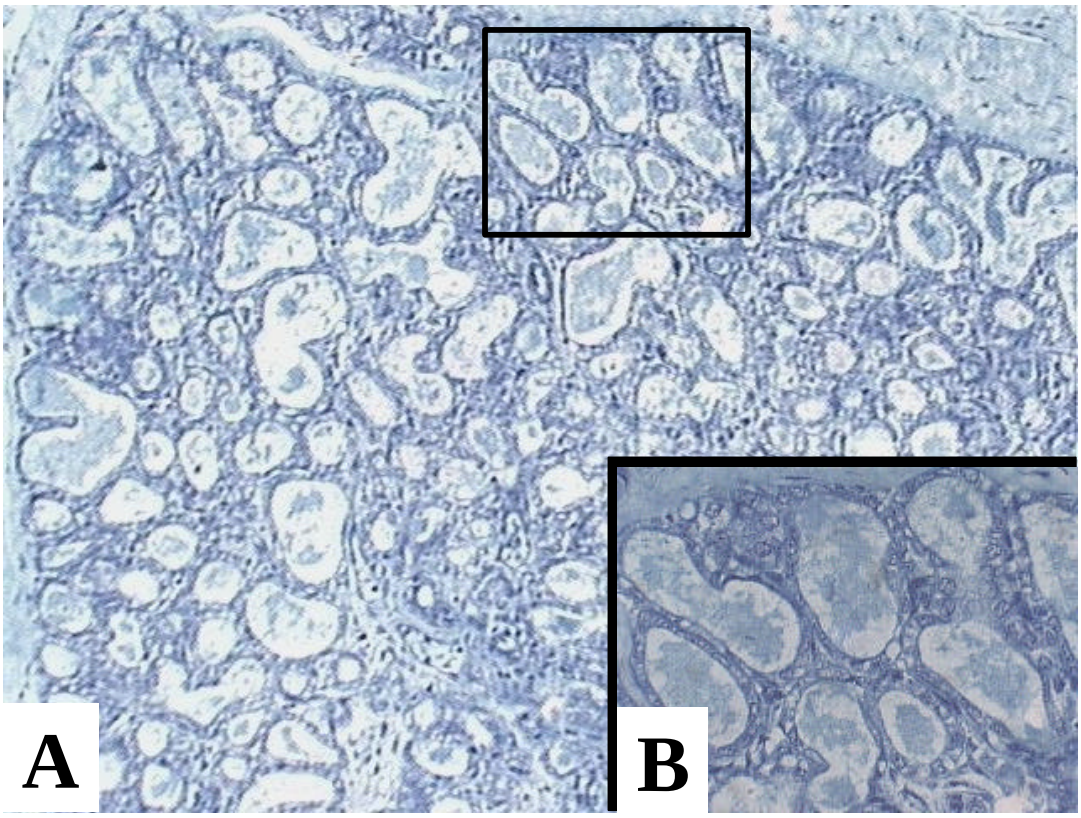


Figure 12A: Control gilt 40-6 post-implant tissue section. Dramatic increase in parenchyma tissue. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Basement membrane no longer distorted. Epithelial cells are developed and organized. Secretions noted in lumena. (magnification 40x).

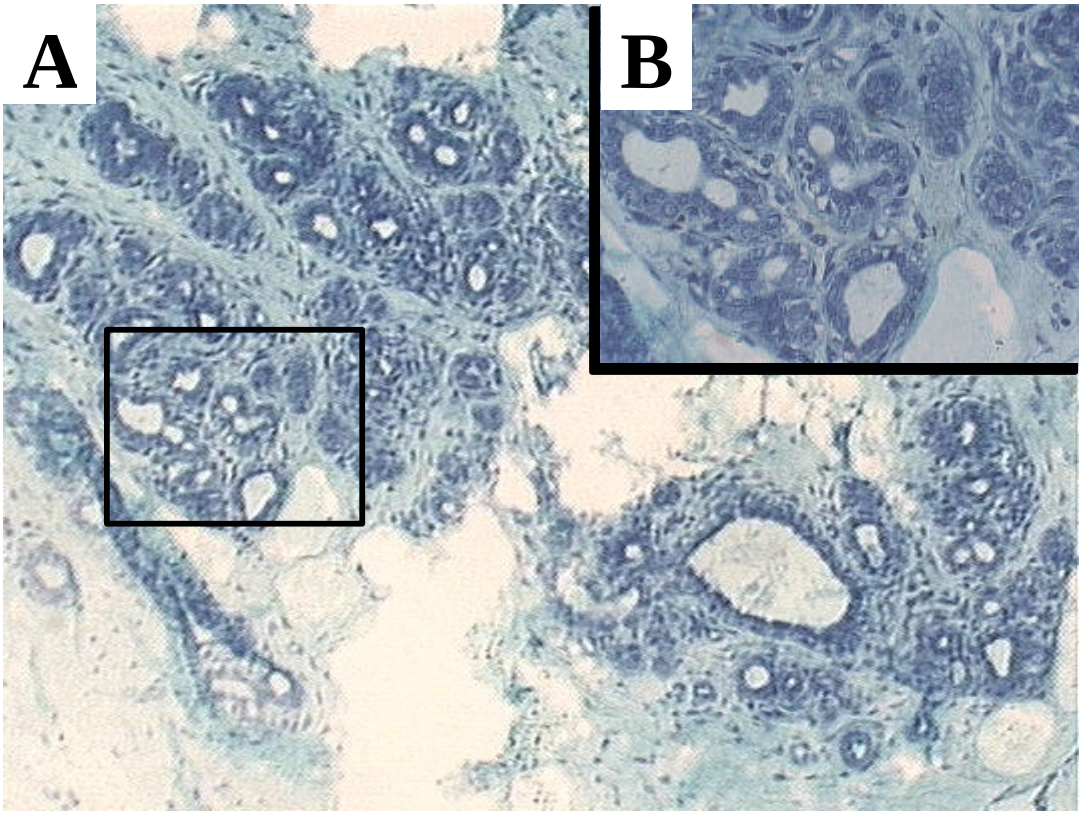


Figure 13A: Control gilt 35-8 pre-implant tissue section. Moderate amount of parenchyma but unorganized. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Epithelial cells are undeveloped and unorganized. (magnification 40x).

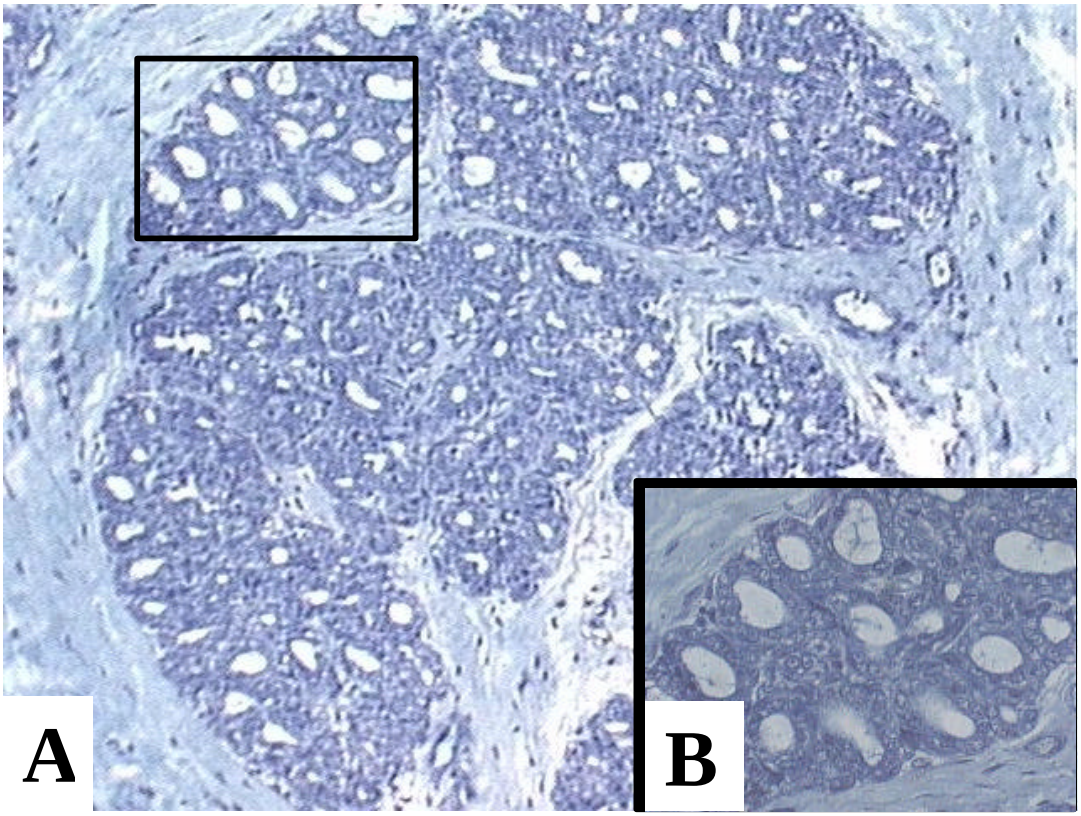


Figure 14A: Control gilt 35-8 post-implant tissue section. Dramatic parenchyma proliferation. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Epithelial cells are more organized. (magnification 40x).

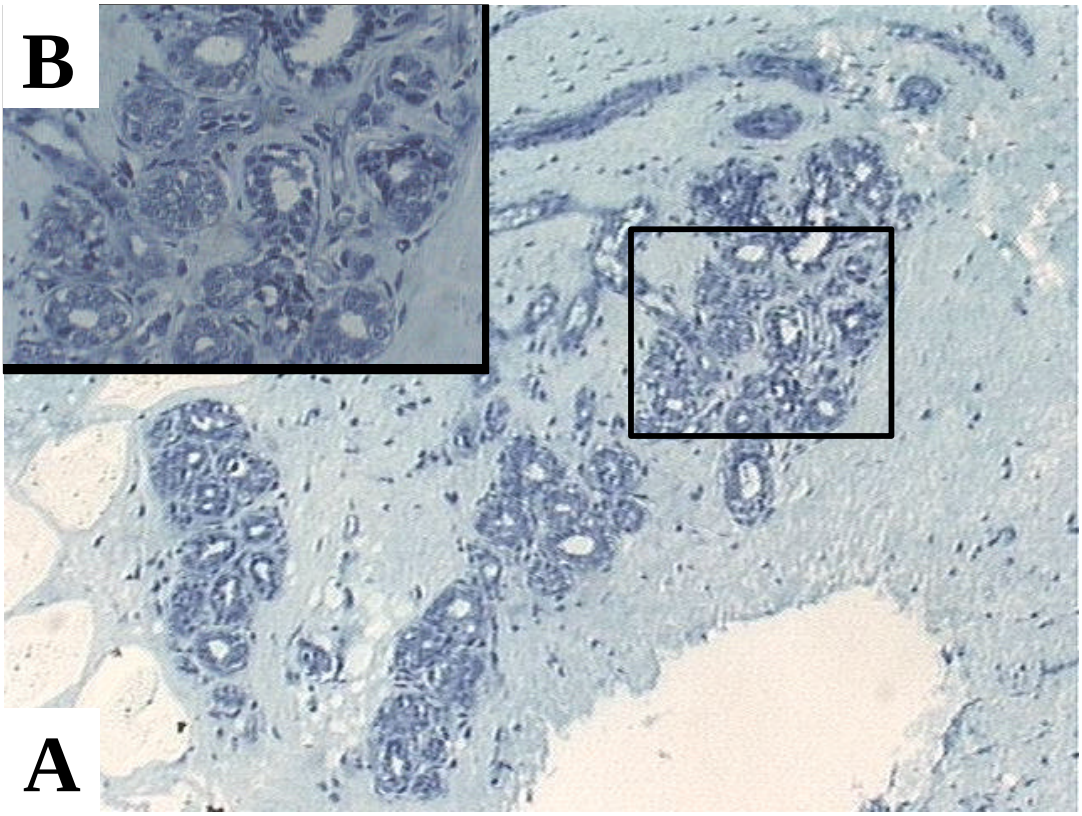


Figure 15A: Control gilt 4-5 preimplant tissue section. Note large amount of stroma versus parenchyma. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note disorganization of epithelium (magnification 40x).

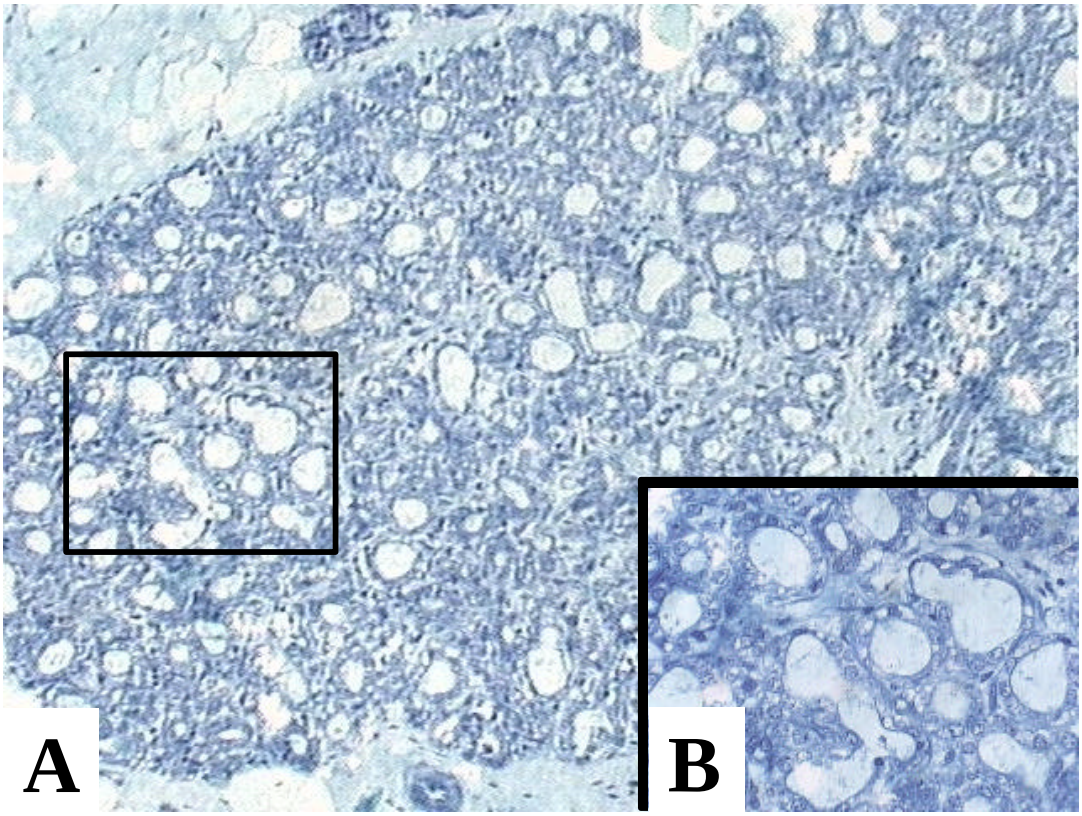


Figure 16A: Control gilt 4-5 post-implant tissue section. Note dramatic proliferation of parenchyma. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Epithelial cells are organized, with some secretions noted in lumena. (magnification 40x).

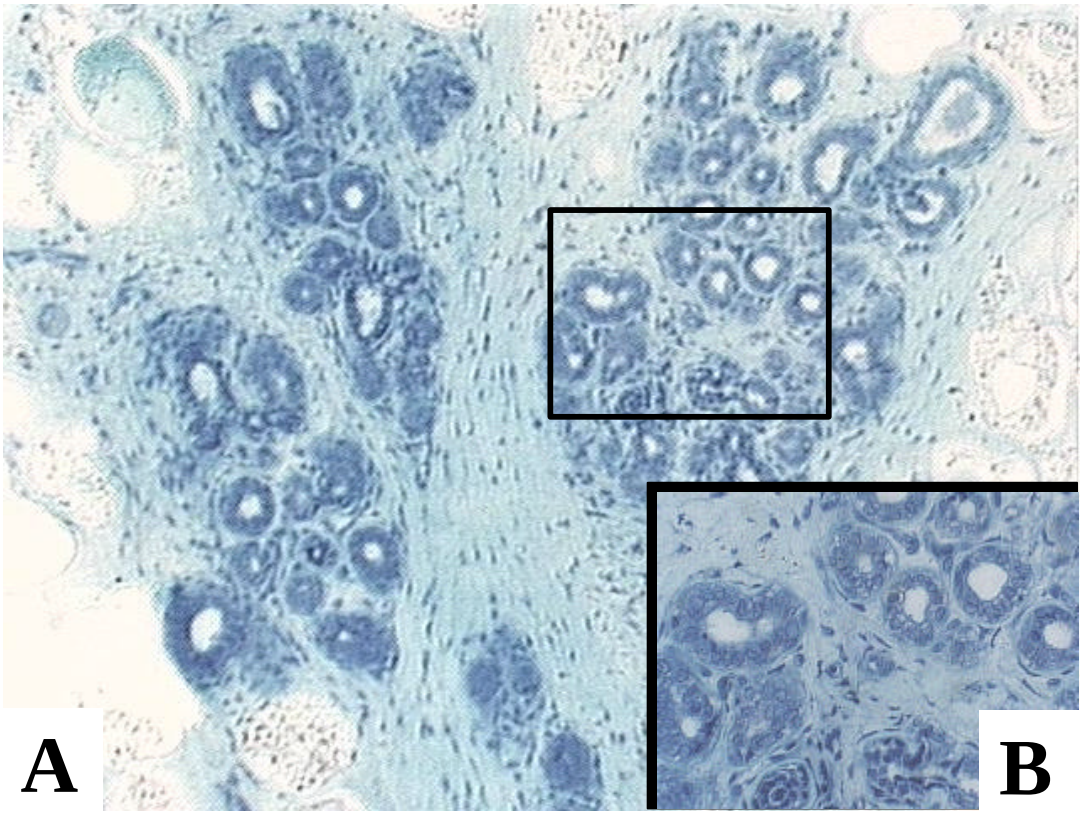


Figure 17A: Control gilt 5-4 preimplant tissue section. Note large amount of stroma tissue, small amount of loosely packed parenchyma (magnification 10x). **B:** Detailed inset of area in A outlined by black. Note undeveloped epithelial cells. (magnification 40x).

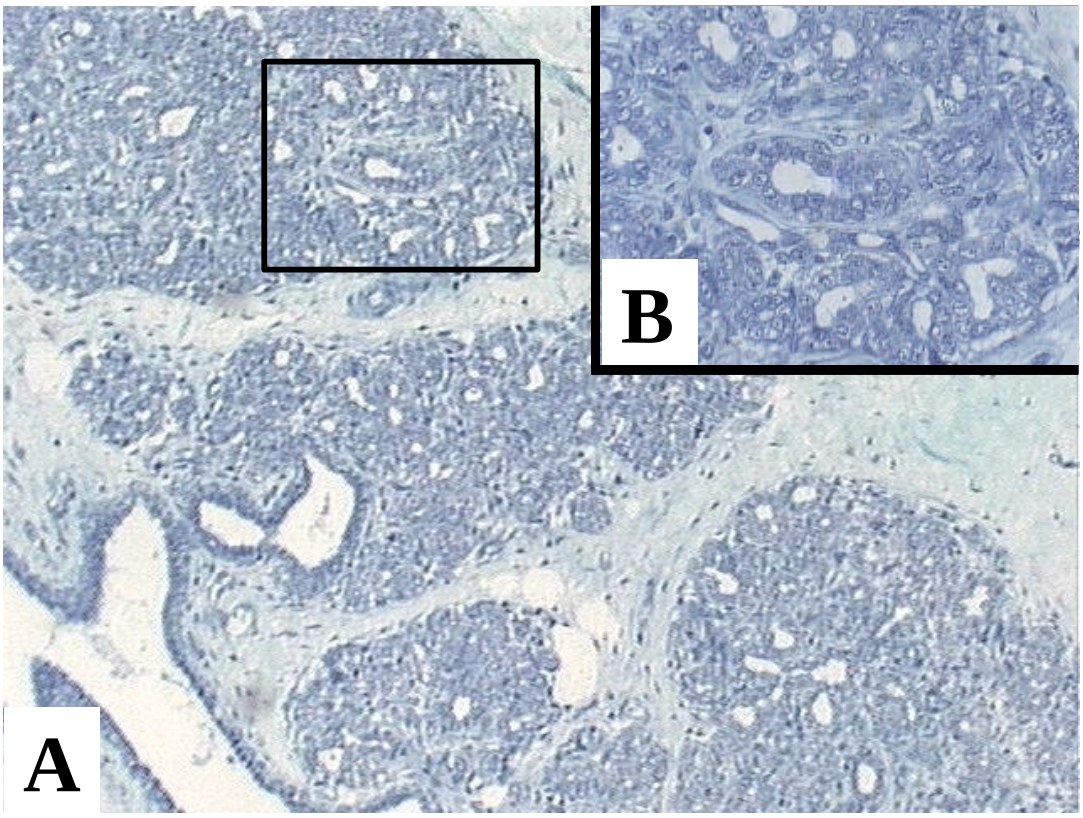
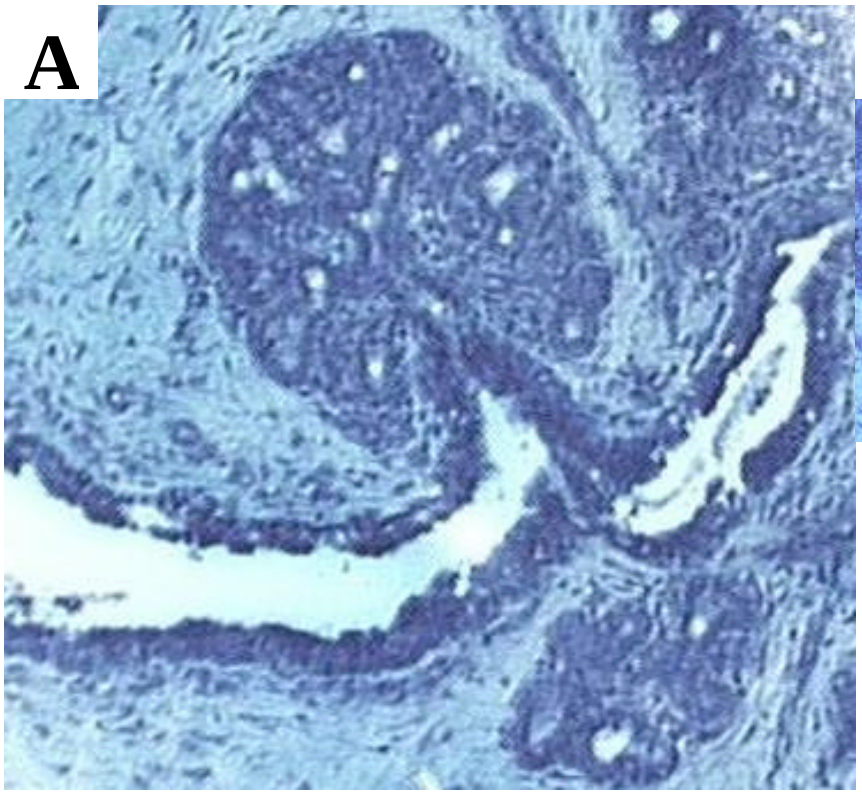


Figure 18A: Control gilt 5-4 post-implant tissue section. Note large amount of parenchyma and larger duct in lower left corner. (magnification 10x). **B:** Detailed inset of area in A outlined by black. Greater organization of epithelium. (magnification 40x).



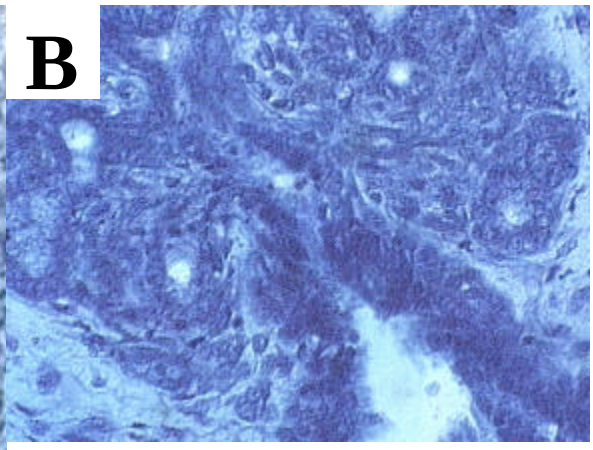


Figure 19A: Gilt 35-8 post-implant tissue section. Note lobule emptying into larger duct. Epithelial cells are organized. Small amount of secretions can be seen. (magnification 10x) **B:** Detail of area. (magnification 40x).

Table 2. Total DNA for pre-and post-implant samples[a]

Gilt ID	Sample	DNA (μg/mg tissue)	Gilt ID	Sample	DNA (μg/mg tissue)
19-10	pre	0.378	40-6	pre	0.489
19-10	post	0.503	40-6	post	0.308
19-12	pre	0.180	35-8	pre	0.097
19-12	post	0.949	35-8	post	0.732
97-1	pre	0.794	4-5	pre	0.361
97-1	post	0.424	4-5	post	0.539
97-3	pre	0.297	5-4	pre	0.278
97-3	post	0.085	5-4	post	0.041

[a] Increased DNA concentrations indicate cellular proliferation, with the assumption that individual cellular DNA is relatively constant. These results indicate that cellular proliferation occurred in only four of the eight gilts, conflicting with the histological evaluation. The discrepancy can most likely be attributed to inadequate isolation of DNA from the samples.

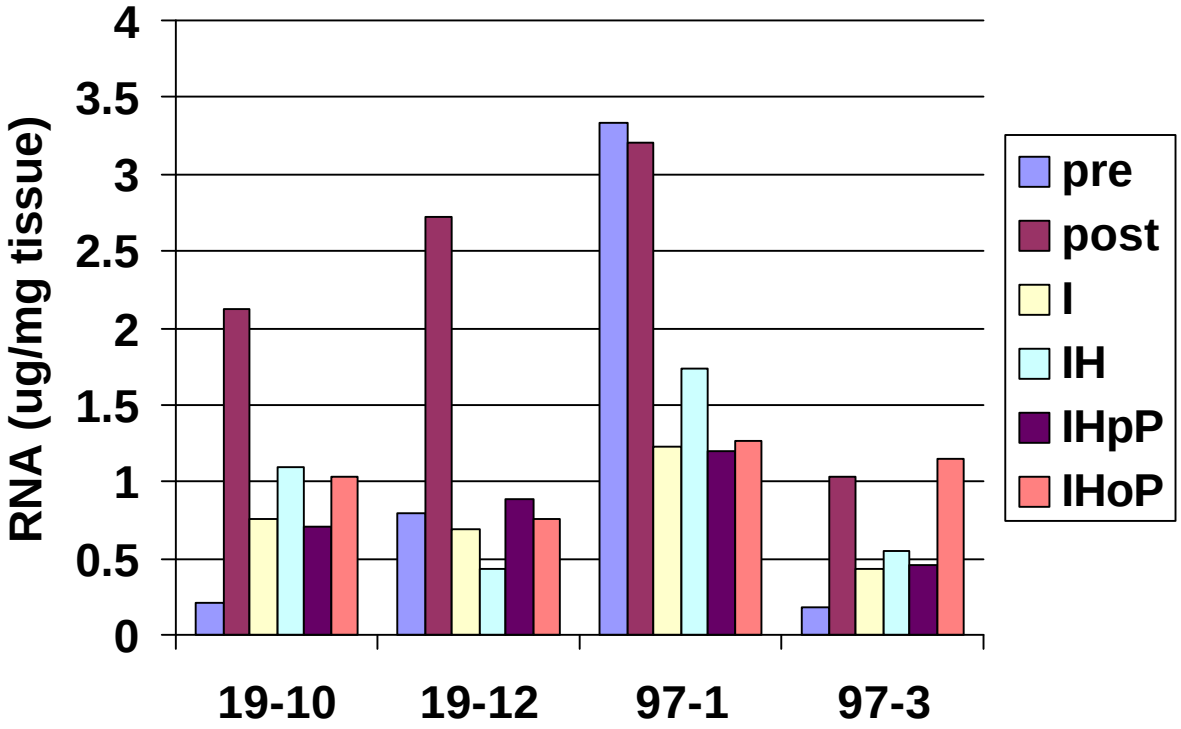


Figure 20. Total RNA - transgenic gilts. Overall, total RNA concentrations of post-implant samples were greater than those of pre-implant samples, indicating gene activation as a result of the induction procedure. Cultured samples demonstrated a large variation in total RNA concentrations, which may be due to inadequate RNA isolation from the samples or inadequate culture conditions to maintain RNA viability or certain cellular functions.

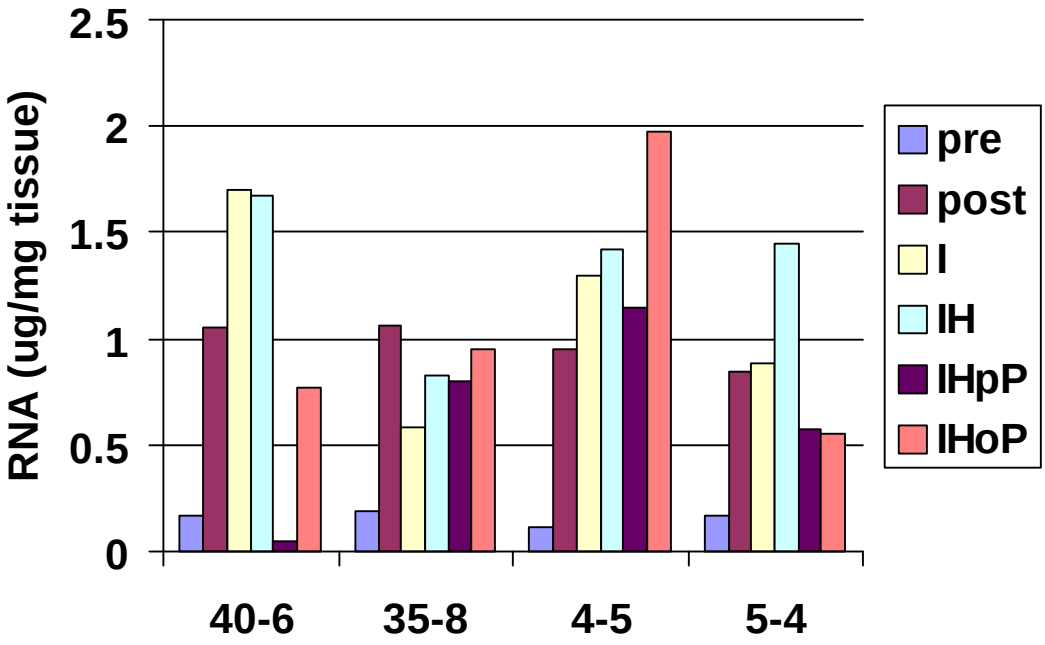


Figure 21. Total RNA - non-transgenic gilts. Overall, total RNA concentrations of post-implant samples were greater than those of pre-implant samples, indicating gene activation as a result of the induction procedure. Cultured samples demonstrated a large variation in total RNA concentrations, which may be due to inadequate RNA isolation from the samples or inadequate culture conditions to maintain RNA viability or certain cellular functions.

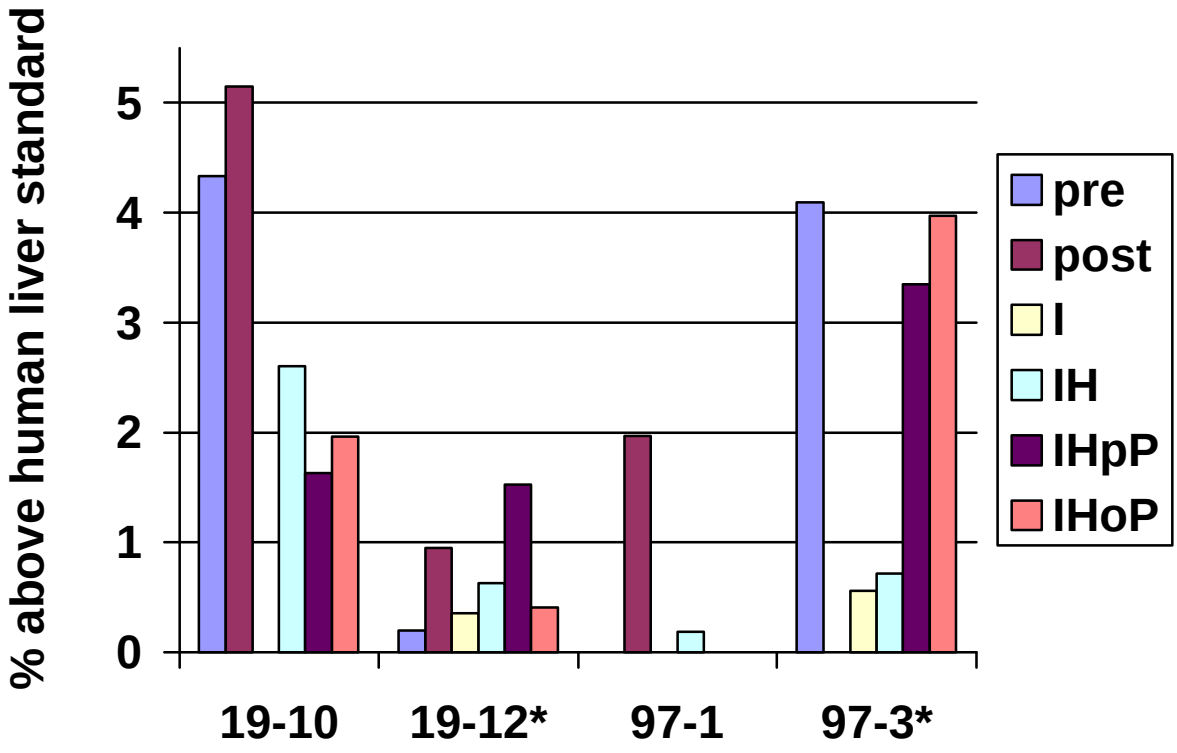


Figure 22. Slot blot analysis for rhFib Aα chain mRNA - transgenic gilts. rhFib Aα chain mRNA was detected in all samples. rhFib Aα chain mRNA responded as expected in culture in two of the four gilts*. (Missing bars indicate degraded sample.)

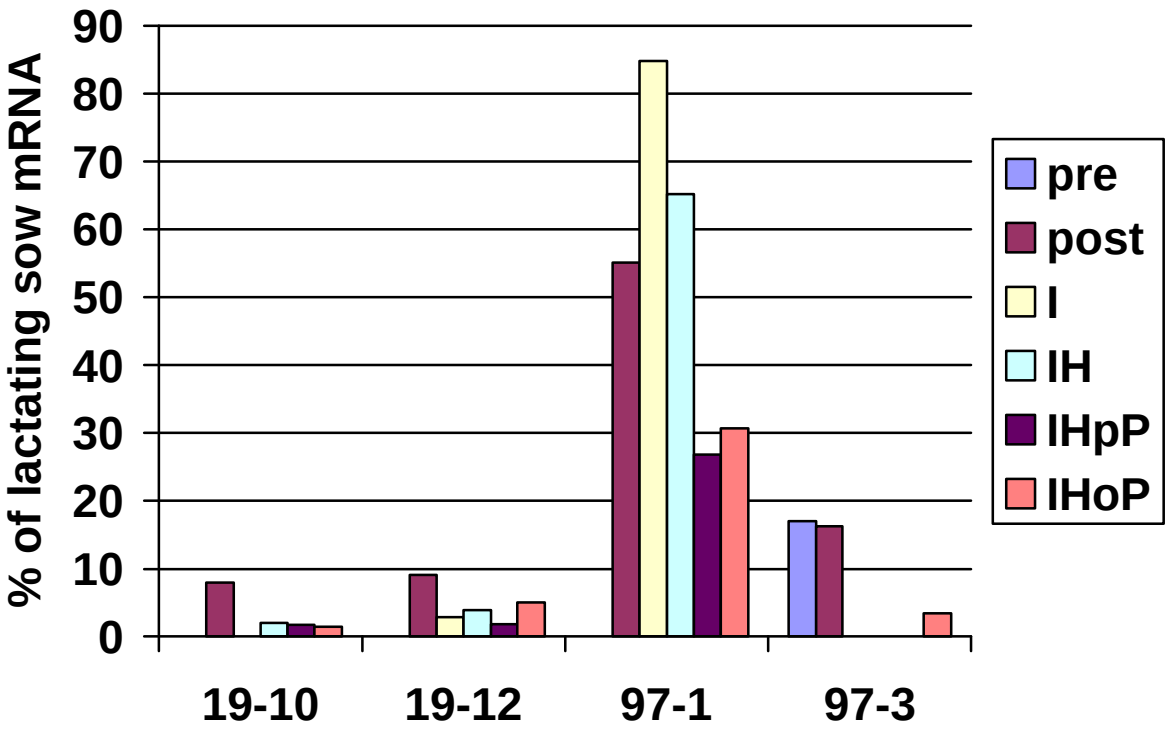


Figure 23. Slot blot analysis for β-lactoglobulin mRNA - transgenic gilts. β-lactoglobulin mRNA was detected in all post-implant samples. Overall, β-lactoglobulin mRNA was not maintained well by the culture system. (Missing bars indicate degraded or missing sample.)

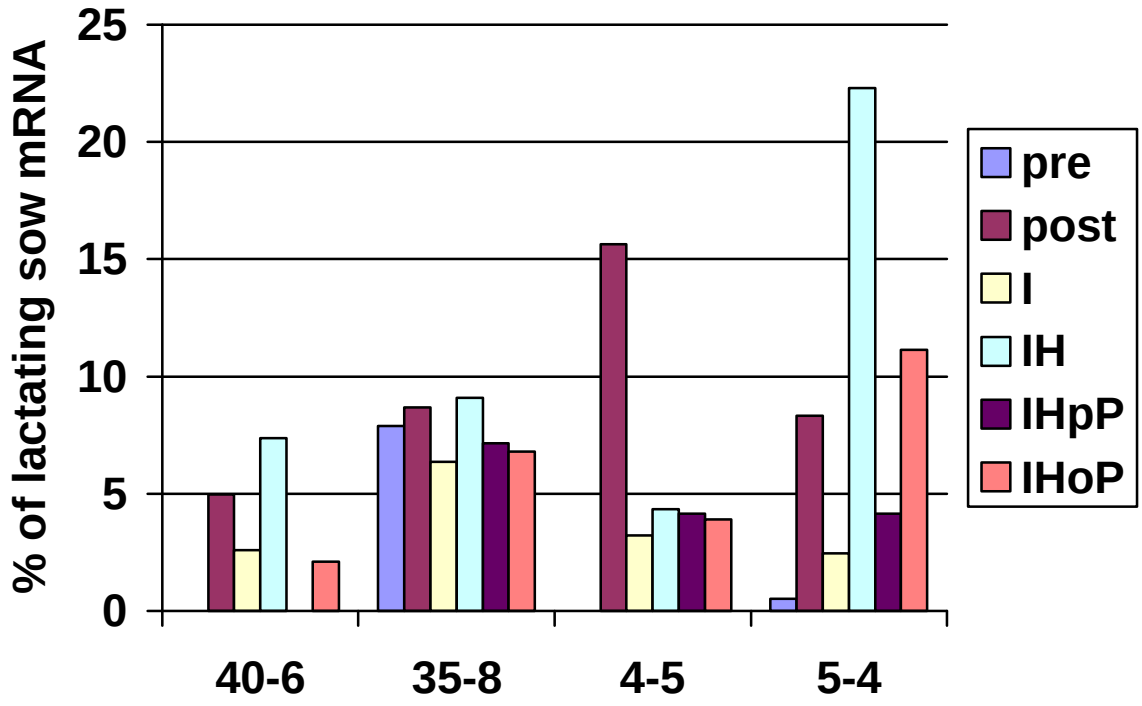


Figure 24. Slot blot analysis for β-lactoglobulin mRNA - non-transgenic gilts. β-lactoglobulin mRNA was detected in all post-implant samples. Overall, β-lactoglobulin mRNA was not maintained well by the culture system. (Missing bars indicate degraded or missing sample.)

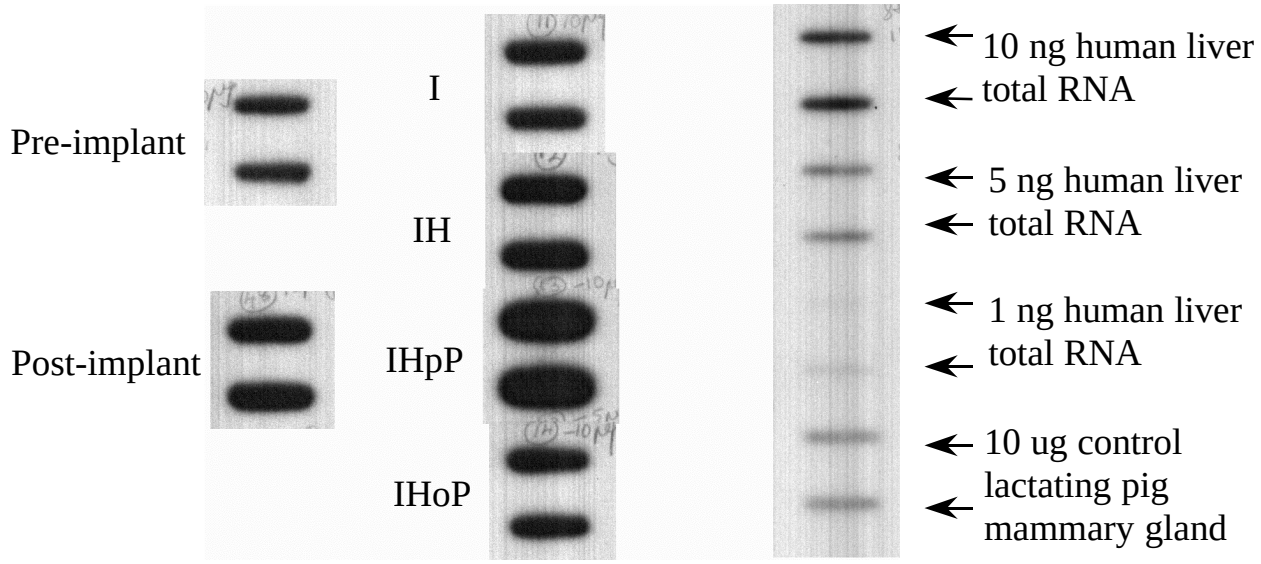


Figure 25. Gilt 19-12: rhFib Aα slot blot analysis. 10 ug of total RNA was loaded per slot. Blot was probed with rhFib Aα chain cDNA. The in vivo induction resulted in increased levels of mRNA. In vitro culture samples responded as expected, with the highest levels of mRNA found in the triple hormone combination using porcine PRL.

Table 3. Dot blot analysis - Least squares means ± SE rhFib (ng) secreted into culture

	Non-Transgenic	Transgenic
I alone	17.8 ± 4.5	31.1 ± 4.5
IH	21.0 ± 4.5	28.9 ± 4.5
IHpP	29.3 ± 4.5	29.0 ± 4.5
IHoP	23.5 ± 4.5	35.3 ± 4.5

Blots were incubated with an antibody that recognizes the three chains of Fib as well as fully assembled rhFib. Values are the averages of four gilts for each treatment. There were no significant differences between transgenic and non-transgenic gilts (P=.326). This is most likely due to the crossreactivity of rhFib and endogenous porcine fibrinogen.

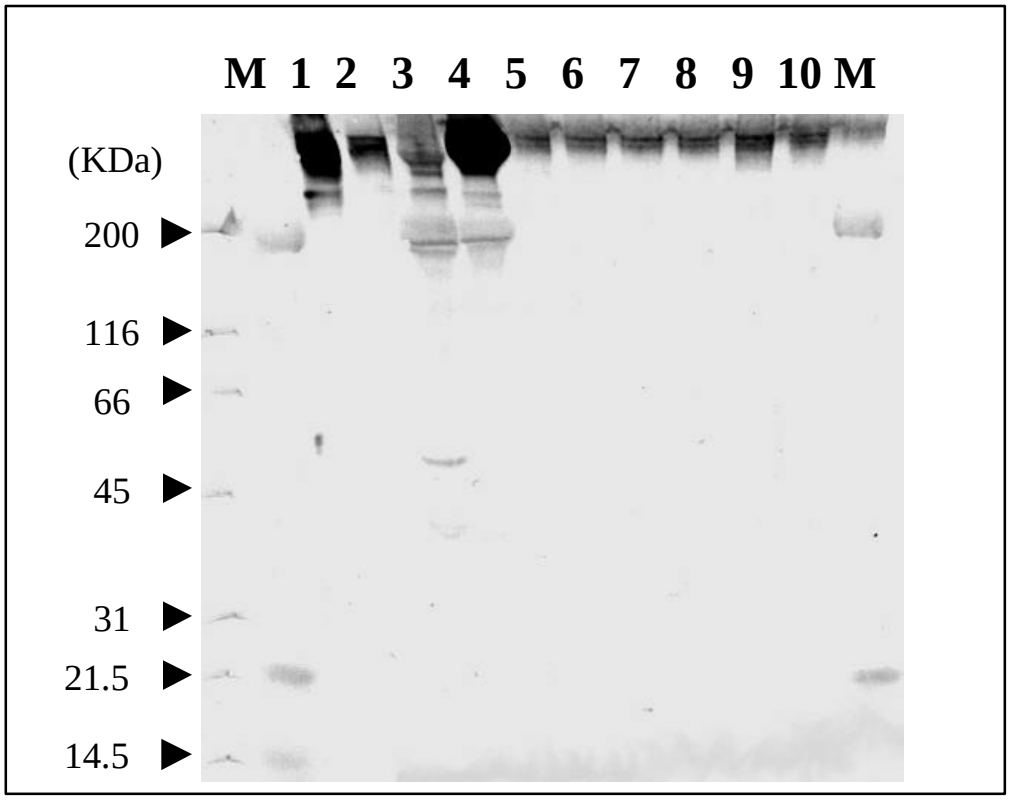


Figure 26. Non-reduced Western blot for Fibrinogen: non-transgenic gilt.

Protein fraction of homogenized tissue was separated by SDS-PAGE, blotted, and probed with a polyclonal anti-hFib antibody. NTG = non-transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, NTG pig serum; 4, NTG pig plasma; 5, Gilt 35-8 Insulin alone; 6, Gilt 35-8 IH; 7, Gilt 35-8 IHpP; 8, Gilt 35-8 IHoP; 9, Gilt 35-8 pre-implant; 10, 35-8 post-implant.

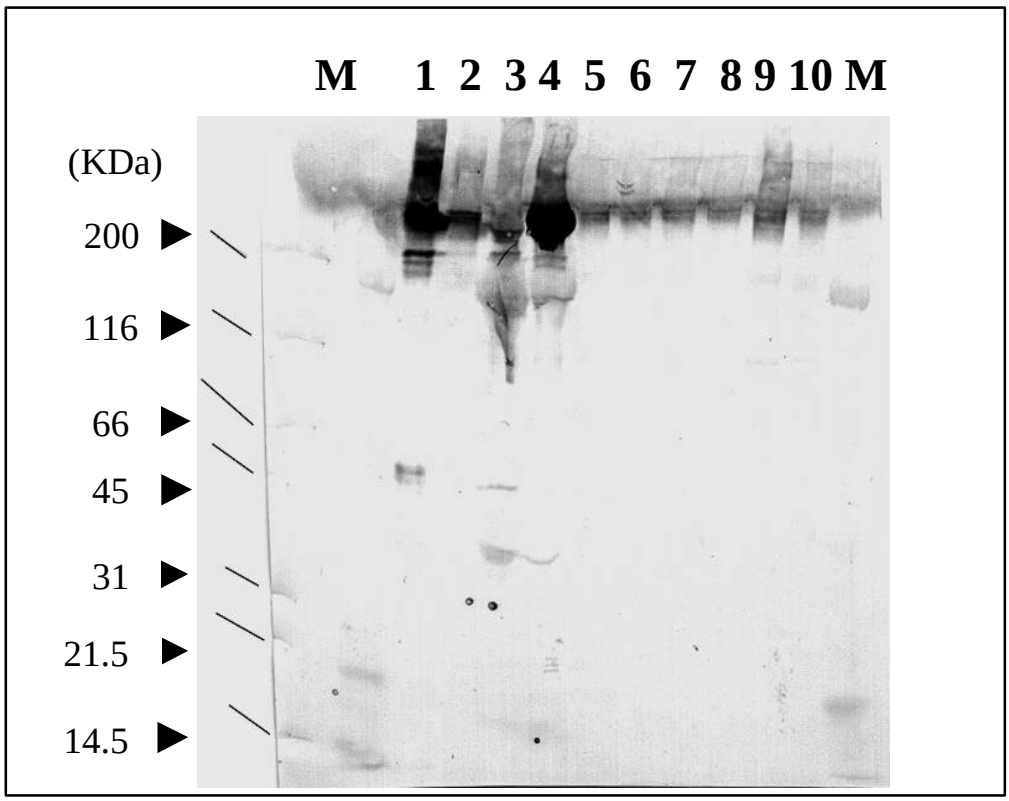


Figure 27. Non-reduced Western blot for Fibrinogen: transgenic gilt.

Protein fraction of homogenized tissue was separated by SDS-PAGE, blotted, and probed with a polyclonal anti-hFib antibody. NTG = non-transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, NTG pig serum; 4, NTG pig plasma; 5, Gilt 97-3 Insulin alone; 6, Gilt 97-3 IH; 7, Gilt 97-3 IHpP; 8, Gilt 97-3 IHoP; 9, Gilt 97-3 pre-implant; 10, 97-3 post-implant.

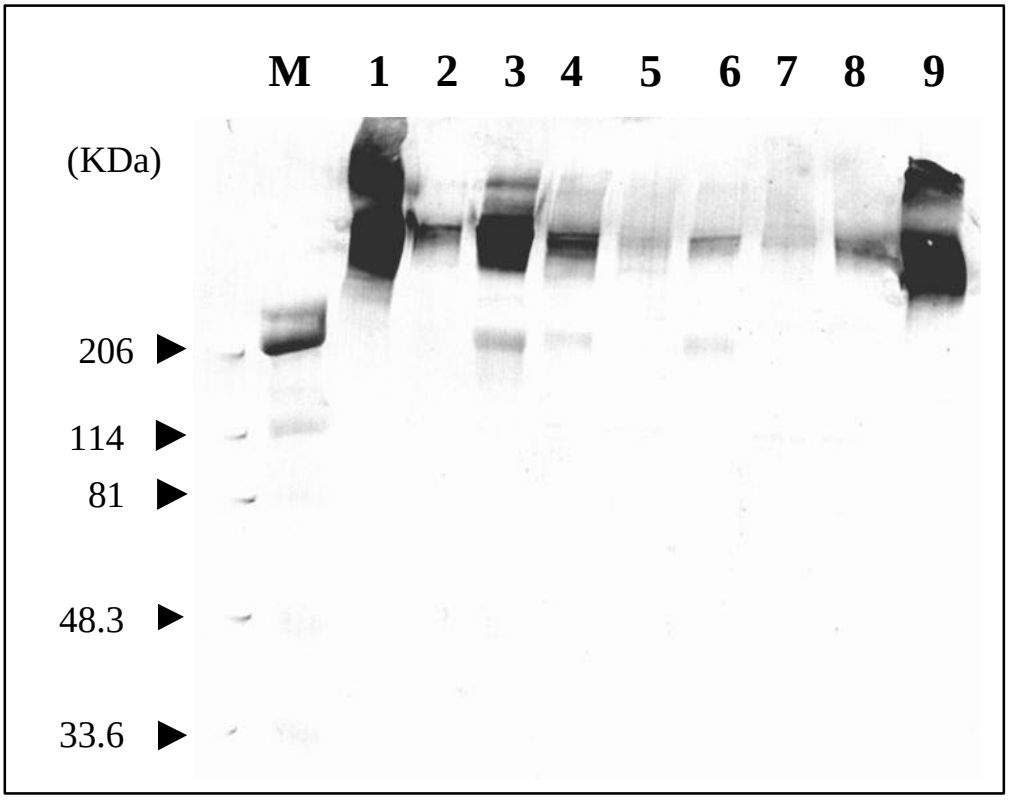


Figure 28. Non-reduced Western blot for Fibrinogen: post-implant and IHpP.

Protein fraction of homogenized tissue was separated by SDS-PAGE, blotted, and probed with a polyclonal anti-hFib antibody. NTG = non-transgenic; TG = transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, NTG pig plasma; 4, Lactating sow; 5, TG Gilt 97-3 IHpP; 6, TG Gilt 97-3 post-implant; 7, NTG Gilt 35-8 IHpP; 8, NTG Gilt 35-8 post-implant; 9, hFib, 75 ng.

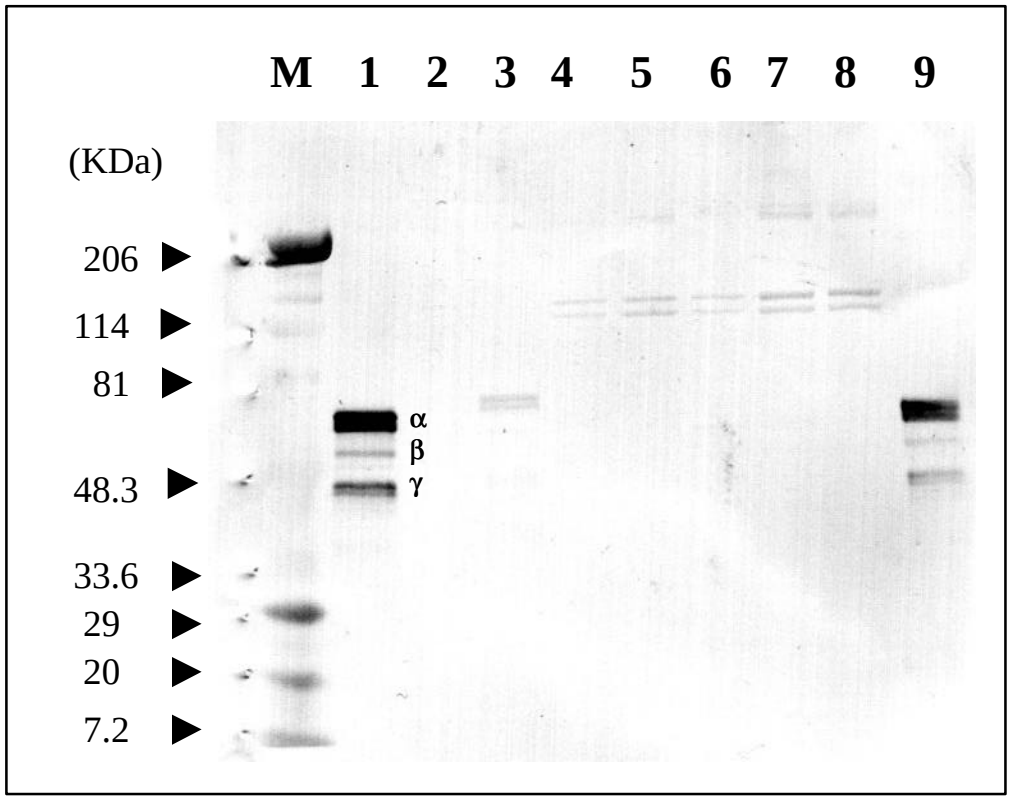


Figure 29. Reduced Western blot 1 for Fibrinogen.

Protein fraction of homogenized tissue was separated by SDS-PAGE under reducing conditions, blotted, and probed with a polyclonal anti-hFib antibody. NTG = non-transgenic; TG = transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, NTG pig plasma; 4, Lactating NTG sow; 5, TG Gilt 97-3 IHpP; 6, TG Gilt 97-3 post-implant; 7, NTG Gilt 35-8 IHpP; 8, NTG Gilt 35-8 post-implant; 9, hFib, 75 ng.

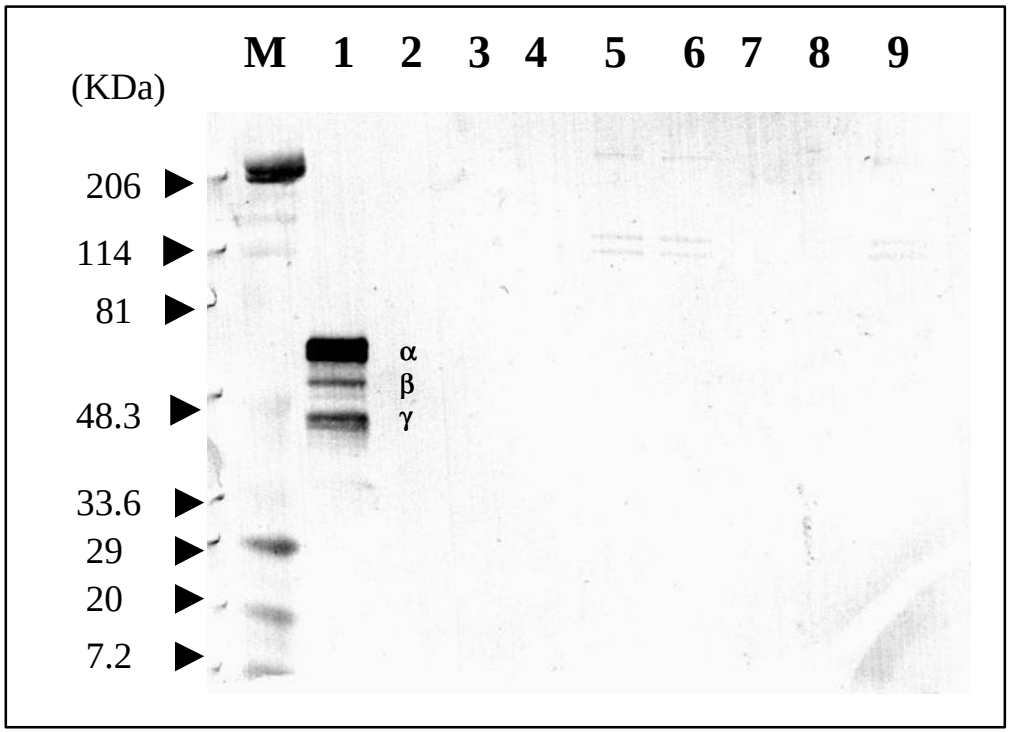


Figure 30. Reduced Western blot 2 for Fibrinogen.

Protein fraction of homogenized tissue was separated by SDS-PAGE under reducing conditions, blotted, and probed with a polyclonal anti-hFib antibody. NTG = non-transgenic; TG = transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, TG Gilt 97-1 IHpP; 4, TG Gilt 97-1 IHoP; 5, TG Gilt 19-12 IHpP; 6, TG Gilt 19-12 IHoP; 7, TG Gilt 97-3 IHoP; 8, TG Gilt 19-10 IHpP; 9, TG Gilt 19-10 IHoP.

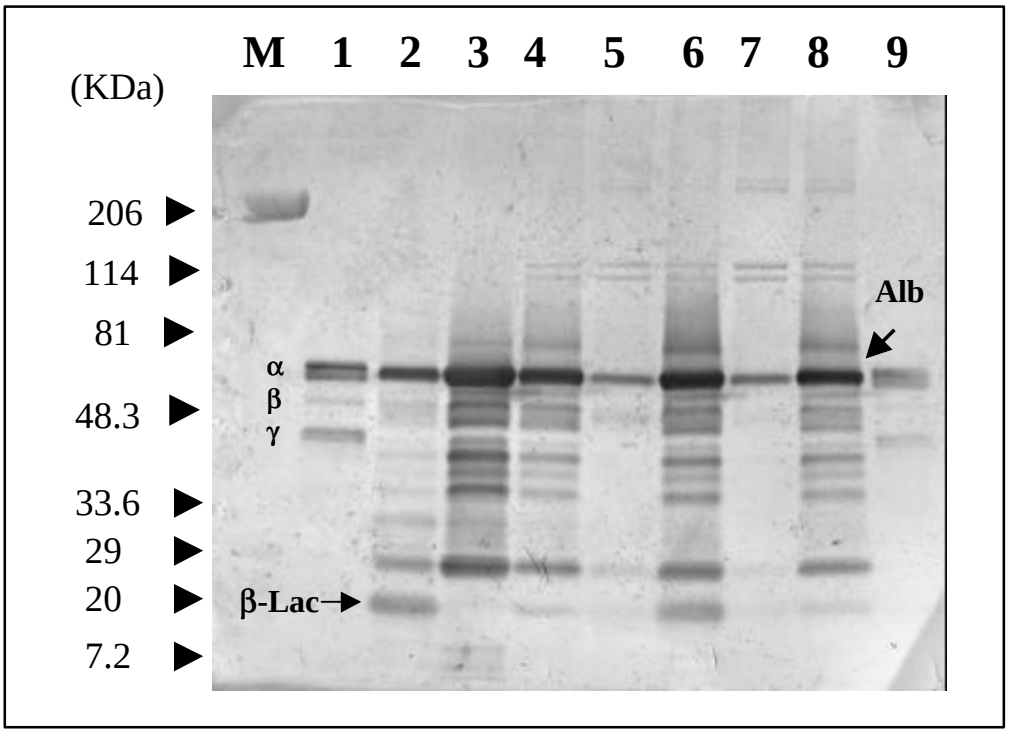


Figure 31. Reduced Western blot for porcine whey proteins.

Protein fraction of homogenized tissue was separated by SDS-PAGE under reducing conditions, blotted, and probed with a polyclonal anti-pig whey antibody. NTG = non-transgenic; TG = transgenic. Lanes: M, molecular weight markers; 1, hFib 150 ng; 2, NTG pig whey; 3, NTG plasma; 4, Lactating NTG sow; 5, TG Gilt 97-3 IHpP; 6, TG Gilt 97-3 post-implant; 7, NTG Gilt 35-8 IHpP; 8, NTG Gilt 35-8 post-implant; 9, hFib, 75 ng.

Discussion

The mammary gland of transgenic swine has been used successfully for the production and secretion of recombinant proteins. In this study, hormonal induction of lactogenesis was examined as a method for identifying those transgenic gilts that are likely to express the mRNA for a transgene and produce the corresponding protein during a natural lactation. Overall, the results of this study indicate that mammary development and a degree of lactogenesis can be induced in gilts by subcutaneous implants designed to release E_2 (7.1 mg/d) and P_4 (19 mg/d) for 21 d. Subsequent tissue analysis detected the presence of mRNA and resultant protein for β-lactoglobulin, an endogenous milk protein, in post-hormone treatment samples. Messenger RNA for rhFib Aα chain was detected at varying concentrations in all samples. However, individual chains or fully assembled rhFib protein were not detected in any of the analyzed samples.

Mammary alveolar development was evaluated histologically in tissue sections taken before and after the 21 d hormone treatment period. Pre-implant sections for all eight gilts were characteristic of prepubertal mammary tissue from gilts (Shamay et al., 1992). Analysis revealed very sparse parenchymal tissue relative to the large amount of adipose and connective tissue stroma present. The parenchyma was poorly developed with few alveoli and ducts. The alveolar epithelium exhibited disorganized layers of cells with irregular shaped nuclei and low secretory capacity.

Following the induction procedure, mammary tissue for all gilts exhibited dramatic parenchymal tissue proliferation and subsequent diminished stromal tissue. This proliferation was accompanied by limited alveolar cell differentiation. These findings support in greater detail those reported by Shamay et al. (1992) who successfully induced mammogenesis in gilts with a similar hormone administration regimen.

Although the post-implant sections demonstrated dramatic tissue and cellular changes when compared to the pre-implant tissue, the post-implant tissue was not as highly developed and differentiated as one would find in a mid-gestation or lactating sow. The ovarian steroids, E_2 and P_4, do exert the majority of the endocrine control of mammary development and lactogenesis. However, there are other key hormones, such as glucocorticoids and PRL, involved that may have not been sufficiently elevated in the gilts used in this study.

Glucocorticoids and PRL have been implicated in the structural differentiation of mammary epithelial cells (Akers, 1994). Glucocorticoids have been shown to synergize with PRL to enhance lactogenesis (Delouis et al., 1980) and PRL is hypothesized to sensitize the mammary gland to the action of E_2 and P_4 (Gardner and White, 1941). Further mammary development and differentiation may have been induced if these hormones had been included in the treatment regimen. However, fully lactating tissue does not respond as well in the explant culture system. Therefore, further investigation of the details of hormone administration would be necessary.

All eight gilts expressed β-lactoglobulin mRNA after hormonal induction in vivo. The tissue analyzed for the corresponding protein showed the presence of the protein in post-implant samples as well. This indicates that lactogenesis was successfully induced by the steroid implants. In a similar study, Shamay et al. (1992) also showed that β-lactoglobulin mRNA levels after in vivo induction were comparable to those found in fully lactating sows.

However, expression of β-lactoglobulin mRNA and resultant protein was not maintained by in vitro culture with any of the four hormone combinations: Insulin alone, IH, IHpP, or IHoP. Lockwood et al. (1966) reported the secretion of β-lactoglobulin by pregnant mouse mammary

explants cultured in media supplemented with the triple hormone combination. It is possible that porcine β-lactoglobulin is under a different hormonal control than in other species.

Shamay et al. (1992) found that gilt mammary explants cultured in similar conditions were unable to maintain β-lactoglobulin mRNA at levels comparable to lactating sows, but PRL did have a stimulatory effect on the expression. They also demonstrated that β-casein expression was maintained in vitro with the triple hormone combination. This indicates a different mechanism of control for these two endogenous milk proteins. Due to this difference, β-casein is probably a better indicator of lactogenesis induction in vitro. Further work should include analysis of β-casein mRNA concentrations.

Our results indicate that the rhFib transgene (Aα chain) was activated by the hormonal induction in vivo and in vitro. However, actual levels of expression were variable across gilts. Results for gilt 19-12 followed the expected pattern of expression (Figure 25). Post-implant rhFib Aα chain mRNA was increased compared to pre-implant mRNA. Concentrations of rhFib Aα chain mRNA in cultured samples responded increasingly better as the hormone supplementation changed from insulin alone to IH, and from IH to IHpP. Ovine PRL did not have as great a stimulatory effect as porcine PRL.

Despite the presence of rhFib Aα chain mRNA, Western analysis revealed no recombinant protein present in the tissue samples, under either non-reduced or reduced conditions. Also, single chains or fully assembled rhFib were not secreted into the culture media. However, there seems to be substantial crossreactivity between hFib and endogenous porcine fibrinogen. In order to eliminate this crossreactivity, the dot blot analysis must be performed under reducing conditions. Further investigation should include a reduced dot blot analysis of the media samples.

Shamay et al. (1992) reported successful expression of a transgene and corresponding protein in two gilts in a study using similar hormone induction procedures. The transgene used in that study was full-length mouse WAP, a milk protein. In our study, the transgene rhFib used the regulatory region of mWAP as a promoter to direct its expression to the mammary gland. The difference in transgene expression between the two studies may be attributed to inadequate hormonal stimulus for activation of the mWAP promoter region when it is coupled to another transgene. Pittius et al. (1988) found differences in the expression patterns of the endogenous WAP gene and WAP-transgene hybrid gene. They speculated that the non-promoter regions of the WAP gene may contain additional regulatory regions, which would account for increased transcription compared to a transgene using the WAP promoter.

In summary, mammary gland development and lactogenesis can be induced in virgin pigs with subcutaneous implants of E_2 and P_4. This procedure can be used to detect the presence of transgene mRNA. However, this study cannot conclusively demonstrate that this procedure can be used to detect the presence of recombinant protein.

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Vita

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