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Wnt genes encode a large family of secreted signaling molecules related to the *Drosophila* pattern regulating gene *wingless*. Several members of the *Wnt* family are implicated in mammary tumorigenesis suggesting that Wnt-activated pathways are a normal component of mammary gland development. Consistent with this hypothesis, we have demonstrated that six *Wnt* genes are expressed in the developing mammary gland. Two of these, *Wnt-6* and *Wnt-10*, are expressed from the earliest stages suggesting roles in induction and growth of the mammary gland. To address their functions, we are analyzing their expression in detail and have initiated approaches to generate mutants. Further, we are developing strategies which will eventually allow tissue specific activation and mutation of genes in the mammary epithelium. Finally, recent work suggests that *frizzleds* encode wingless receptors. We have initiated studies examining *frizzleds* in the mammary gland. *Frizzled-6* is expressed quite specifically in the early mammary gland. It seems reasonable to expect that an understanding of Wnt/frizzled interactions will afford new insights into normal development of the mammary gland, and the pathways that lead to tumor formation.

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5. INTRODUCTION

Nature of problem/Background of previous work.

The mammary gland develops from an epithelial outpocketing of the ventral ectoderm at 11dpc in the mouse embryo [1-4], in response to an initial inductive signal from the underlying mesenchyme [5]. In the female mouse embryo, there is little change in the primary bud over the next four days. However, in the male, mesenchyme surrounding the epithelium condenses from day 14 and this is followed by a rapid necrotic degeneration of the epithelial rudiment. Tissue recombination experiments have convincingly demonstrated that this process is dependent upon testosterone [6], and correlates with the acquisition of testosterone receptors by the mammary mesenchyme [7], which occurs in response to epithelial derived signals [8], and the initial production of testosterone by the embryonic testes. In addition to testosterone responsiveness, the epithelium also induces estrogen responsiveness but, at this stage of development, there appears to be no in vivo role for estrogen [1]. Thus, by the end of this resting period, which is characterized by the appearance of the mammary bud (16.0 dpc), the female mammary gland is poised for further development whereas the male gland is destroyed.

From 16.0dpc to 2dpp, the mammary epithelium extends as the primary mammary sprout into the mesenchyme reaching the fat pad precursor where epithelial branching is initiated. The trigger for elongation of the mammary epithelium is not known, however outgrowth follows the resumption of proliferative activity in the epithelium. The trigger for epithelial branching clearly resides in the mesenchyme of the fat pad precursor. Epithelial morphogenesis, which forms slim epithelial ducts with secondary lateral buds, is specific to the fat pad mesenchyme [9]. Growth into other sources of mesenchymal tissue *in vitro* , e.g. salivary mesenchyme, produces epithelial outgrowths typical of the organ from which the donor mesenchyme was removed. [10]. Thus, shortly after birth in the mouse, and in other mammals [2], the female mammary gland consists of a primary duct connecting with a rudimentary branched epithelium within the presumptive fat pad.

From the period after birth to approximately 4 weeks pp there is very limited growth of the branching epithelium of the mouse mammary gland. However, at 4 to 6 weeks, coupled with the acquisition of sexual maturity, there is a period of extensive cell growth in which the epithelial ducts elongate and branch, extending throughout the fat pad. [11,12] Renewed growth correlates with the reappearance of end buds, a monolayer of unspecialized epithelium at the ends of the ducts. The end buds are thought to contain stem cells which generate differentiated ductal epithelium and myoepithelial cells of the gland. Thus, post natal branching morphogenesis is regulated largely at the termini of the ducts, by controlling the proliferative activity of the end buds. End bud activity is in turn dependent upon ovarian hormones, as ovariectomy results in a rapid loss of end buds and cessation of growth [11,12].

After reaching sexual maturity (6 to 8 weeks pp), further ductal development stops until pregnancy is established. At this time, a second extensive period of ductal growth and branching occurs to fill all the remaining interductal space in the fat pad. During the later phase of pregnancy there is an accompanying development of lobuloalveolar epithelium. Along with the morphogenetic changes in the gland during pregnancy, there is a progressive development of secretory epithelium, such that by birth, a fully functional lactogenic epithelium is established. Interestingly, cytodifferentiation of secretory epithelium will occur in vitro in the absence of morphogenesis, indicating that the two processes are not mutually dependent [13]. Finally, on cessation of suckling, there is a massive involution of the mammary gland due to a widespread destruction of epithelial tissue and the cycle of branching morphogenesis is repeated at the next round of pregnancy.

The mammary gland is unusual, with respect to most organs, in that most of its growth occurs in the adult, and that there are cyclical periods of growth and regression. The control of these processes has been extensively studied and compelling evidence exists for complex regulation

mediated by systemic hormonal signals, and locally acting peptide growth factors (for review see [11,12]).

The initial observation that ovariectomy leads to a cessation of end bud growth implicated hormones in the control of mammary development. There is an absolute requirement for estrogen for proper development of epithelial branching. Maximal growth also appears to require growth hormone or prolactin [11,12]. However, whether these hormones act directly, or sensitize the epithelium to the action of other factors, is not clear. Lobuloalveolar growth requires, in addition to the above, progesterone which accumulates later in pregnancy. Finally, the onset of lactation correlates with the increase in prolactin and glucocorticoids and a decrease in progesterone [11,12].

Evidence for involvement of peptide growth factors in the regulation of mammary development has come from the direct observation of growth factor expression, and implant and transgenic studies which have manipulated growth factors in the mammary gland. Slow release implants of EGF stimulates local growth of end buds in quiescent mammary epithelium [14], whereas implantation into growing mammary glands causes local inhibition of ductal growth, and a down regulation of EGF receptors [15]. Thus, EGF may have a dual specificity depending upon the particular stage of development. TGF-β1 implants also suppress ductal growth [16] acting specifically on the end buds to inhibit DNA synthesis [17] whereas TGFα stimulates alveolar and ductal growth [18-21].

Additional evidence for peptide factors in growth regulation has come from the analysis of mammary tumors in which growth controls have been uncoupled following expression of genes not normally active in the mammary gland. A number of loci, have been shown to undergo MMTV mediated insertional activation in mouse mammary tumors (for review see [22]). Four of these encode secreted peptide factors, *Wnt-1* [23] and *Wnt-3* [24] members of the *Wnt*-gene family, and *FGF-3* [25] and *FGF-4* [26], members of the fibroblast growth factor family. Additional evidence suggests that *Wnt* and *FGF* genes may cooperate in tumor formation as frequently *Wnt-1* and *FGF-3* are co-activated in the same mammary carcinomas [27].

The oncogenic role of *Wnt-1* has been demonstrated by *in vitro* and *in vivo* studies. Transfection of the *Wnt-1* gene into C57MG cells, a primary mammary epithelial cell line, leads to morphological transformation [28,29]. However, these cells do not grow in soft agar or form tumors in syngeneic hosts. In contrast RAC311C cells are rendered morphologically transformed and tumorigenic when transfected with *Wnt-1* [30]. Formal proof of the transforming roles of Wnt-1 has come from transgenic studies which lead initially to hyperplasia, in both the male and female mammary gland, and progress to the formation of adenocarcinomas [31]. As was observed in spontaneously occurring tumors, there is also a synergistic affect of *FGF-3* on *Wnt-1* transformation in the transgenic model [32].

In addition to *Wnt-1* and *Wnt-3*, ten additional members of the mouse *Wnt*-gene family have been identified. Human Wnt-2 was isolated serendipitously in a search for the cystic fibrosis gene [33,34]. Like *Wnt-1* and *Wnt-3*, Wnt-2 is implicated in tumorigenesis as it appears to be amplified and highly expressed in some MMTV induced tumors [35]. Amplification appears not to be related to MMTV, but is a novel mechanism which presumably acts in conjunction with MMTV activated genes to transform epithelial cells [35]. *Wnt-3a* was identified on the basis of its close relationship to *Wnt-3* [36], and *Wnt's-4, 5a, 5b, 6, 7a, 7b*, on the basis of a PCR cloning approach [37] which has been successful in identifying *Wnt*-genes in many species, as well as two new mouse members (*Wnt-10* and *11*; A. McMahon, unpublished data).

All Wnt-proteins have several features in common including a putative signal peptide sequence, one conserved glycosylation site, and 20 absolutely conserved cysteine residues. Typically Wnt proteins are 38 to 45kd. Although only *Wnt-1* and *Wnt-2* have been studied, and these analyses have been restricted to cell culture systems, both genes appear to encode poorly secreted glycoproteins with strong affinity for cell surface and/or extracellular matrix [29,38-44]. Thus, it is likely that they are involved in short-range signaling. Functional analyses of several members

indicates these important regulatory roles in invertebrate and vertebrate development [reviewed in 45,46].

The observation that *Wnt* expression leads to morphological transformation of mammary epithelial cells *in vitro* and hyperplastic growth *in vivo* indicates that mammary epithelium is responsive to *Wnt* gene products. If, Wnt-proteins act as signals (a conclusion greatly strengthened by studies on the Drosophila Wnt-1 orthologue *wingless*, [46]), then by analogy with other families of peptide signals, it would seem likely that the responsiveness of mammary epithelium reflects the expression of functional Wnt-receptors.

Recent evidence demonstrates that unlike *Wnt-1* and *Wnt-3*, six family members are expressed, and developmentally regulated, during normal adult mammary gland development [47]. Thus, the responsiveness to ectopic expression of *Wnt-1* or *Wnt-3* presumably reflects some modulation of *Wnt*-signaling pathways which normally respond to endogenously expressed Wnt-factors. For example, if Wnts normally stimulate cell growth, ectopic expression of *Wnt-1* or *Wnt-3* may lead to hyperstimulation of a proliferative Wnt-signaling pathway. Conversely, if endogenously expressed *Wnts* suppress proliferative activity, ectopic *Wnt-1* or *Wnt-3* expression may block Wnt-mediated growth suppression, possibly by interfering with receptor function.

The situation is likely to be complex on the basis of our studies of *Wnt*-transcription in the adult mammary gland [47b] . *Wnt-2* expression is very weak and confined to virgin or nonpregnant mice [47b]. Thus although *Wnt-2* causes C57MG cell transformation, its expression does not correlate with proliferative activity. Quite the opposite, it is limited to the quiescent state. *Wnt-5a* and *Wnt-7b* are also expressed at low levels in virgin mice [47]. However, expression extends into mid but not late pregnancy showing decreasing levels of expression despite the large increase in mammary epithelium. In contrast, *Wnt-5b* and *Wnt-6* are expressed at low levels prior to pregnancy and increase considerably to midpregnancy, declining by parturition [47]. Thus, these two members show a better correlation with epithelial expansion. Finally *Wnt-4* expression is uniform from in the virgin gland until late in pregnancy when it rapidly declines [47].

Transformation assays on C57MG cells indicate that several Wnt-members which are normally expressed in the mammary gland are transforming in this assay [48]. Wnt-2, -5b and -7b are moderately transforming, weaker than Wnt-1, 3a and 7a, whereas Wnt-4, 5a and 6 are non transforming. *Wnt-4* and *Wnt-5a* are normally expressed by C57MG cells, thus elevation of endogenous expression several fold does not lead to transformation. These results suggest that hyperplasia *in vivo* may result from inappropriate activation of Wnt-2, Wnt-5b and/or Wnt-7b signaling pathways.

In summary, the data clearly support a model in which normal mammary, epithelial growth is regulated by one or more *Wnt*-genes. They demonstrate that uncoupling of these regulatory pathways leads to hyperplasia [31,49] and adenocarcinomas *in vivo* [31]. However, without a better understanding of the normal spatial expression of Wnt-proteins and their putative receptors, and the transforming activity of the family as a whole *in vivo*, we are not in a position to grasp the full significance of their functions in the normal and transformed mammary tissue, nor the relevance that this family may have to human breast cancer.

Purpose of present work/Methods of approach

As discussed above it is now over ten years since Nusse and Varmus identified a locus in the mouse associated with the generation of mammary tumors. It is now clear that the associated gene, *Wnt-1*, is one member of a large family of putative signaling molecules which normally regulate embryonic development. Several members have now been implicated in epithelial cell transformation in the mammary gland from the analysis of spontaneously occurring mouse tumors (*Wnt-1, Wnt-3, Wnt-3a*), transgenic experiments (*Wnt-1*) and *in vitro* studies (*Wnt-1, 2, 3, 3a,*

5b, 7a, 7b). Thus, it would appear that hyperplasia, and eventual adenocarcinoma formation, in the mouse mammary gland result when normal growth regulatory pathways which are presumably controlled by Wnt-proteins, are perturbed by deregulated expression of certain Wnt-family members.

Understanding growth control in the mammary gland is essential for designing strategies which will treat mammary tumors. Further, potential growth regulators are likely mediators of mammary transformation, as exemplified by studies on *Wnt*-genes in the mouse, and should thus be examined for contributory roles in human mammary cancer. This proposal set out to examine the normal and oncogenic roles of Wnt protein in the mammary gland of the mouse and human, and to dissect the Wnt-regulatory pathways at the receptor level. Specifically we proposed to address the issue of whether *Wnt*-genes may be involved in human cancers by directly examining expression in mammary tumors using Northern blot analysis. We propose to use transgenic mice to examine the relationship between normal *Wnt*-gene expression and mammary transformation. As Wnt-signaling is most likely a conventional receptor-mediated process, ectopic expression of specific Wnt-signals presumably exerts its effects through one or more receptor pathways coupled to endogenously expressed Wnt-proteins. If so, we should be able to identify a likely candidate pathway by assaying the transforming potential of endogenously expressed Wnt-proteins when their normal regulation is uncoupled, either by ecoptic expression or gene ablation. Moreover, characterizing the normal expression of *Wnt*-genes and their products in relation to the developing mammary gland may provide strong suggestive evidence as to what growth regulatory pathways may be responsive to Wnt-signals. Finally we propose several approaches toward identifying Wnt-receptors which will be an essential step in fully defining Wnt-signaling pathways, and their regulatory function in the mammary gland. Thus, the proposed studies are directly relevant to the issue of the genetic alterations involved in the origin and progression of cancer and the changes in cellular and molecular function which may account for the development and progression of breast cancer.

In summary we proposed five specific goals

1) To determine, using transgenic mice, which if any of the *Wnt*-members normally expressed in the mammary gland are oncogenic when ectopically expressed using an MMTV enhancer construct.

2) To determine the relevance, if any, of *Wnt-5b* in normal gland development by studying mice homozygous for a likely null mutation in the *Wnt-5b* gene.

3) To determine the normal temporal and spatial expression of *Wnt* genes, and their protein products, during embryonic and adult mammary gland development.

4) To use various schemes to attempt to identify other proteins, particularly candidate receptors, which interact with Wnt-proteins.

5) To isolate sequences encoding all of the yet-unidentified human *Wnt*-genes, providing clinicians with a broad array of Wnt-probes which may be important in the analysis of human mammary carcinomas.

6. BODY

Over the past two years we have made considerable progress in certain areas and these have suggested some exciting new avenues to follow up. Thus, the research goals as they appeared in the original proposal were substantially modified in the last period and this is also the case in the current period, to take into account our new findings. These substantial changes are discussed in the next section and as documented in the previous reporting period we have dropped some of the original goals.

1) **Transgenic analysis of Wnt-mediated oncogenesis**
Wnt-1 and *Wnt-3* were both identified on the basis of their role in MMTV derived mammary tumors. However, it seems likely that these two genes stimulate hyperplastic growth, a first step towards the generation of adenocarcinomas, by deregulated Wnt-mediated growth regulation in the mammary gland. One strong possibility is that one or more of the *Wnt*-members normally expressed the mammary gland are growth stimulatory, and it is through this pathway that *Wnt-1* and *Wnt-3* act. If this is the case, deregulated expression of these members would be predicted to act like *Wnt-1* and *Wnt-3* , causing hyperplasia initially, and tumors with time. Thus, a transgenic approach, utilizing endogenously expressed *Wnts*, may provide an insight into the regulatory pathway through which *Wnt-1* and *Wnt-3* act.

We proposed to test for the transforming potential of *Wnts-2, 4, 5b, 6, 7b*, the six members which we had previously shown to be expressed in the mouse mammary gland, by ectopic expression utilizing an MMTV-LTR, as previously reported for *Wnt-1* [31].

We have not pursued these studies in the current funding period, preferring to change the focus towards an understanding of earlier development and the relevance of *Wnt-6* and the newly identified *Wnt-10* in embryonic mammary gland development (see later).

2) ***Wnt-5b* mutant analysis**

One approach towards examining the role of Wnt members during mammary development is to generate specific mutants by gene targeting in ES cells. To date we have mutated three of the six mammary expressed genes (*Wnt-4, Wnt-5b, Wnt-7b*), but due to embryonic requirements, only *Wnt-5b* homozygous mice are viable. Wnt-*5b* was mutated by insertion of a PGK-neo cassette into the fourth exon. Insertion produces a short deletion and places PGK-neo into the normal *Wnt-5b* open reading frame. Thus the mutated allele is predicted to produce a carboxyl truncated protein. Our experience with similar truncations suggested that this would generate a null allele *Wnt*-5b is moderately trasnforming in the C57MG assay, suggesting that *Wnt*-5b may play a role in epithelial growth stimulation. Moreover, expression of *Wnt-5b* increased dramatically through the first half of pregnancy, a period characterized by the reemergence of mitotic activity in the end buds and reinitiation of branching morphogenesis.

At the time of writing the proposal we had weanlings (3-4 weeks pp) which were homozygous for *Wnt-5b* , the disrupted allele. We proposed to determine whether loss of *Wnt-5b* activity leads to a disruption of growth regulation in the mammary gland, by examining the histology and function of the mammary gland in mutant mice. It is now clear that both males and females are fully viable. Moreover, homozygous females are perfectly able to suckle their offspring and therefore it is likely that mammary development is quite normal.

There are several possible explanations for this result. The simplest would clearly be that *Wnt-5b* by itself does not play an essential role in mouse mammary gland development. This may be true; however, this result presupposes that the targeted allele is indeed a null allele. Our Southern blot analysis of the targeting event indicates that the expected recombination event occurred. However, there is now some question as to whether the allele we have generated is indeed a null allele. Unpublished work from the laboratory of Dr Eric Wieschaus indicates that a similar allele of *wingless,* one in which the carboxyl one-quarter of the protein is missing, is in fact a

hypomorph, a surprising result given that the mutation removes ten absolutely conserved cysteine residues. Thus, to be absolutely certain that *Wnt-5b* plays no role will require that we retarget the Wnt-5b allele. This approach is being pursued by a former postdoctoral fellow, Dr. Shinji Takada, and will not form an important component of the revised plan. Instead our studies will focus on Wnt-6 and Wnt-10.

3) **Wnt expression in the mammary gland**

Although we have demonstrated that several *Wnt* genes are expressed during mammary development, we do not know in which cell types, nor the spatial details. This is of critical importance. Growth and branching morphogenesis is primarily regulated at the end buds. Thus, any growth stimulatory or growth repressive action of a Wnt member is likely to act on this aspect of the epithelial network. As the available evidence suggests that Wnts are short range factors, we would therefore anticipate that some Wnt members will be locally distributed either in the stroma surrounding the end buds, or perhaps in the end bud themselves, and their expression would be predicted to change dramatically with development. Clearly, it is essential for our understanding of their normal regulatory roles that we determine their expression patterns. We proposed to examine this problem by both in situ hybridization and the use of antibodies raised in the laboratory against many of the Wnt-proteins. Further, no one has yet addressed the possibility that Wnt proteins may play a significant role in the early interactions which induce and elaborate the embryonic mammary bud. We proposed to use in situ hybridization to determine whether Wnt-signaling may be involved.

Our initial approach was to use antibody reagents to attempt to immunostain sections and wholemounts of mouse embryos. We chose to examine embryos in the first instance because we have documented sites of Wnt-expression in different regions of the mouse embryo in some detail in our published work. Moreover, we know that several of these sites produce active Wnt-protein as Wnt-mutants are defective in the development of these tissues. We have tested antisera directed against Wnt-1, 3a, 4a, 5a, 5b, 7b but none of these give a reliable, signal despite a number of different fixation conditions (paraformaldehyde, methanol/DMSO, TCA). Thus, we discontinued this approach. It is worth pointing out that this is a considerable problem in the field in general. To date NO ONE has visualized the normal expression of a Wnt-protein, in situ, in any vertebrate embryo. At this time we have not attempted the alternative strategy on adult tissues, that is the RNA in situ hybridization approach, but we have used this approach on whole mounts of mouse embryos, and obtained some interesting results. We have identified two Wnt-genes that are expressed in association with the earliest stages of mouse mammary gland development. These are *Wnt-6* and *Wnt-10*.

Wnt-6 was identified in my laboratory a number of years ago [37]. At the time when the mammary epithelium first thickens, at the initiation of mammary gland development, *Wnt-6* is strongly expressed in the epithelium. Expression of *Wnt-6* is not restricted to the mammary bud epithelium but is present in all ectoderm, suggesting that it may play a widespread role in epithelial signaling. In contrast *Wnt-10* is expressed specifically at two sites of mesenchymal-epithelial interactions, the tooth and mammary buds (Figure 1 in Appendix). Thus, *Wnt-10*, which is a novel mouse family member identified in our PCR-based screens, is a prime candidate for a *Wnt* which might actually induce mammary gland development. In view of the interesting early expression of these members, we will focus our subsequent analysis on determining in detail the expression and properties of *Wnt-6* and *Wnt-10* (see Conclusions).

We have demonstrated that Wnt-10 is expressed in the mammary gland from the time of its initiation until birth. Its expression appears to be epithelial. Currently we are completing this analysis addressing its expression in the postnatal gland. Wnt-6 has a similar pattern, though it is more broadly ectodermal. To address the function of Wnt-6 and Wnt-10 we have generated DNA constructs for gene targeting. We are screening for successfully targeted ES clones in the case of Wnt-6. We will shortly be initiating the targeting of Wnt-10. A considerable amount of a future effort will focus on these mutants. In addition, the expression of Wnt-10 from the earliest stages of mammary development makes the Wnt-10 promoter an excellent tool for designing approaches aimed at tissue specific manipulation of gene expression in the mammary gland which may be of

general utility to the community. To this end we propose to generate two "knock-in" lines [50]. In the first we will generate a line in which the P1 cre recombinase is expressed under Wnt-10 regulation. This will allow the investigator to analyze loss-of-function mutants in genes suspected of a role in mammary epithelial development, but which have an essential requirement at some earlier stage of development. In the second line we will introduce the yeast transcriptional activator GAL4 into the mammary epithelium which will allow mammary specific activation of genes of interest. The GAL4 approach has been widely utilized in Drosophila, and has been shown both in our laboratory and elsewhere to work in mice. These lines will be exploited in our laboratory to address issues related to the Wnt-receptors in the mammary gland (see below).

4) **Wnt receptors**

A major roadblock to our understanding of the regulatory function of Wnt-proteins in embryonic development, as well as in the mammary gland, is our limited knowledge of the signaling pathways. In the absence of compelling data to the contrary the simplest hypothesis to explain all studies on Wnt-factors is that they encode signals which interact through some receptor mediated pathway. The identification of the *Drosophila* segment polarity gene *wingless* as the Wnt-1 orthologue raised the possibility that Drosophila genetics would identify a receptor. This year the work of Nusse, Nathans and their colleagues has demonstrated that a family of transmembrane proteins related to the Drosophila polarity gene frizzled are most likely Wingless/Wnt receptors [51]. As a consequence of this observation we have stopped our search for a general Wnt-receptor and initiated new studies on the role of Frizzled homologues in the mammary gland. As a first step we have isolated or obtained clones encoding ten murine Frizzled genes and have started to compare their expression with our previous studies of Wnt-family members. These studies are still in progress but we have made an interesting observation, that is Frizzled-6 is expressed in a mammary gland specific pattern in the early mammary gland (see Appendix 1). Thus, Frizzled-6 is a candidate for a Wnt-6 or Wnt-10 receptor. We envisage a number of experiments to investigate the relationship of Frizzled-6 to Wnt-signaling in the mammary gland. In conjunction with Wnt-10 and Wnt-6 we will determine the normal distribution of Frizzled-6 at different periods in mammary gland development. We will attempt to determine whether either of these Wnts can interact with Frizzled-6, and whether such an interaction is specific to this Frizzled. Interestingly, Frizzled-6 does not appear to bind to wingless, in contrast to other mammalian Frizzleds [51]. Thus, one approach would be to determine whether Wnt-6/10 and Frizzled co-injection into Xenopus embryos can elicit phenotypes that either component alone is incapable of generating, Using this approach Dawid and colleagues (personal communication) have demonstrated that co-injection of Frizzled-5 and Wnt-5 into the Xenopus embryo results in axial duplication, a response normally elicited by the Wnt-1 class of signals, but only elicited by Wnt-5a in conjunction with Frizzled-6. Finally, using the genetically engineered strains of mice discussed above, we may be able to perform functional experiments to address the role of Frizzled-6 in regulating the mammary gland. For example, ectopic expression of Frizzled-2 in Drosophila is sufficient to activate the wingless pathway. Does ectopic activation of Frizzled-6 lead to mammary hyperplasia? Further, Frizzled binding to Wnts maps to a cysteine-rich extracellular domain [51]. Does expression of this domain in the mammary gland act as a dominant negative arresting growth of the gland?

5) **Human *Wnt*-clones**

The demonstration that deregulated expression of Wnt-genes contributes to adenocarcinomas of the mouse mammary gland suggests that Wnt-family members may play a role in human breast cancer. This problem has been only superficially addressed. Using PCR primers we isolated exonic sequence for human *Wnt-3a, 4, 5a, 7a, 7b*, and together with available probes for *Wnt-2* and *-3*, Wnt-transcription was examined in normal, benign and malignant breast tissue [52]. Some evidence for increased expression of *Wnt-2* and *-4* in fibroadenomas and *Wnt-7b* in malignant tumor was obtained. To systematically address this problem will require the identification of probes for all human Wnt clones. As this is easily achieved, and these probes

will be valuable to clinicians, we propose to complete this task by PCR cloning the remaining family members and making these available.

This was a relatively minor goal in our original proposal. Since submission of the grant it has come to our attention that several groups are systematically exploring the human *Wnt*-family. We have entered into a collaboration with one of these, Dr Tom Strachan, in Newcastle, England, and are sharing our mouse expression data with him. Through our initial efforts and Dr Strachan's more recent work, there are human counterparts for almost all the mouse Wnt-genes, including *Wnt-10*, which, given our findings in the mice, may be of considerable interest to follow up in the human. Dr Strachan has been made aware of our results and will initiate his own efforts to study the human side of *Wnt* genes. We will focus at this time on mouse studies.

7. CONCLUSIONS

There are several implications of the studies thus far.

1) **Transgenic analysis of Wnt-mediated oncogenesis**
In the light of our discoveries and those of other groups in the last year, we believe that our resources are best focused on other aspects of the proposal which are likely to lead to more original findings. It is important to know whether Wnt-10 is capable of transforming mammary epithelium. However, the experiment has apparently been performed by nature as a recent report indicates that MMTV activation of Wnt-10 (as originally shown for Wnt-1), leads to mammary tumorigenesis [53].

2) ***Wnt-5b*** **mutant analysis**
There is no obvious phenotype in mice homozygous for an insertion in the *Wnt-5b* locus. It remains possible that this is not a null allele. Alternatively, Wnt-5b may not be essential for mammary gland development. Our studies have identified that two Wnt-members, Wnt-6 and Wnt-10 are excellent candidates for regulating the initial development of the mammary gland. We will explore their roles by gene targeting, generating mutants in both genes. Further, the specificity of Wnt-10 expression will allow us to use a "knock-in" approach to generate strains of mice which will allow mammary epithelial gene expression to be modified from its earliest stages. These reagents should be of great value in the community and will be used in this proposal to look at aspects of Frizzled action. The relevant DNA constructs will be generated for introduction into mouse ES cells.

3) ***Wnt*** **expression in the mammary gland**
We have determined that our antibodies are not likely to be useful for direct immunolocalization of Wnt-proteins and we will not pursue this aspect further. We have identified two Wnt members, *Wnt-6* and *Wnt-10*, which are expressed in the early mammary epithelium at the initiation of mammary gland development. We will concentrate our efforts on addressing the early aspects of mammary gland induction and growth as it occurs during embryonic life. It is likely that at least some of the signals which operate in the embryo are likely to be important in the adult as well, as new cycles of growth and differentiation take place. Moreover, studies on the embryonic regulation of mammary gland development have clearly lagged behind the research effort on the adult structure. Thus, this shift in emphasis, which plays to the strength of this laboratory, seems a timely one. We will complete our analysis of Wnt expression in mammary gland development.

4) **Wnt receptors**
The problem of how Wnt-proteins signal, most specifically their receptors, is important not only to our understanding of the many important roles that Wnt-proteins play in the regulation of invertebrate and vertebrate development, but also to their oncogenic actions in the mammary gland. The identification of Frizzleds as candidate Wnt-receptors, and more specifically our identification of Frizzled-6 as a mammary specific Frizzled, paves the way for examining the role of Frizzleds in mammary development. To this end we will complete our general survey of Frizzled expression and a more detailed analysis of the expression of Frizzled-6 in conjunction with Wnts. In addition, we will determine whether Frizzled -6 interacts with Wnt-6/10, and if so, whether this interaction is specific.

5) **Human** ***Wnt*****-clones**
We have not pursued the cloning of additional human *Wnts* as this would duplicate efforts in other groups that we have become aware of since submission of this grant. However, we will continue to share unpublished data to facilitate a rapid follow up of potentially interesting areas such as the expression of *Wnt-10*, which may have relevance to human breast cancer, in our collaboration with groups in England.

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9. Appendix

Figure 1. Wholemount in situ hybridization of *Wnt-10* , *Wnt-6*, and *Fz-6* in the mammary ridge (arrow) and early mammary bud (arrowhead) at 11.5 dpc of development (top line). Expression of Wnt-10 is restricted to the developing mammary gland by 14.5 dpc (arrowhead, bottom).