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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this work is to assess the contribution of the tyrosine kinase Jak2 in the development and progression of breast cancer. To accomplish this, it is first necessary to define the role of Jak2 in normal mammary gland development. This is being investigated using two distinct approaches a) isolation of embryonic mammary glands from Jak2-null embryos and transplantation into the cleared fat pad of wild type mice and b) generation of a mammary-specific conditional knockout of the Jak2 gene. An analysis of mammary gland transplants has revealed an essential role for Jak2 in normal mammary epithelial development during pregnancy and lactation. Based on histology and marker expression, a secretory phenotype is not attained in the absence of Jak2. Furthermore, the epithelium maintains ductal-like features suggestive of a premature arrest in mammary cell proliferation and differentiation. Knowledge of the activation status of Jak2 in normal mammary gland and mammary tumors will help identify a key target for the development and testing of small molecule inhibitors (e.g. AG490) that specifically target the inhibition of Jak2 kinase activity and slow down tumor development and progression.

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INTRODUCTION

The proposal aims to examine the role of the tyrosine kinase, Jak2, in the development and progression of breast cancer. Numerous publications have demonstrated the constitutive activation of members of the signal transducer and activator of transcription (Stat) in cancer cell lines and primary tumors. These Stat transcription factors are activated by a phosphorylation event mediated by members of the Jak kinase family. The purpose of this research is to elucidate whether Jak2, a central kinase involved in normal mammary gland development and proliferation, contributes to constitutive activation of Stats leading to the promotion of tumorigenesis. To this end, the role of Jak2 in normal mammary gland development is being investigated by a) transplantation of Jak2-deficient embryonic mammary glands into wild-type hosts and b) generation of a conditional Jak2 gene which can be specifically removed from mammary epithelial cells. This will establish whether Jak2 is required or dispensable for mammary gland development. Finally, the activation status of Jak2 and Stat5 in tumors isolated from mice that carry mutations in both the Brca1 and p53 genes will ascertain whether this signaling pathway contributes to tumorigenesis in this mouse model.

BODY

Analysis of Jak2/Stat5 protein in the mammary gland

It has previously been shown (Ref. 1) that Stat5a, which is activated by the prolactin receptor, is essential for the formation of a fully developed mammary gland at lactation. This is coincident with its activation via phosphorylation during mid (day 13) pregnancy (Ref. 2). While the data concerning activation of Stat5a are well established, few reports have appeared in the literature concerning the role of the upstream Jak kinases in the activation of Stats in the mammary gland. In particular, a developmental profile of Jak2 protein and its activation status is lacking. To address this issue, immunoprecipitations with anti-Jak2 antibodies were performed. Examining its phosphorylation state using an anti-phosphotyrosine antibody subsequently assessed the activation status of Jak2. However, despite numerous attempts to immunoprecipitate Jak2 and examine its phosphorylation status it was unsuccessful. This suggests that phosphorylation of Jak2 in the mammary gland is transient and/or unstable. Other methods are being explored, including the use of phosphospecific Jak2 antibodies and immunohistochemsitry and the generation of a more reliable Jak2 antibody.

Once these details have been addressed, the activation status of Jak2 can then be analyzed in tumors arising in Brca1/p53 mice. A breeding colony of these mice have been established and several tumors isolated for this purpose. Interestingly, a recent publication (Ref. 3) demonstrated that Brca1 expression results in constitutive activation of Jak2 and Stat3 in human prostate cancer cells suggesting that Jak2 may lead to cell survival in tumors (~ 90 %) that express normal Brca1. Therefore it would be worthwhile examining the activation status of Jak2 not only in Brca1/p53 mice but also in a broader variety of mammary tumor models.

Jak2 embryonic transplants

Since the deletion of Jak2 results in embryonic lethality at day 12.5 (Ref. 4), it is not possible to study the role of Jak2 in normal mammary gland development in these mice. Therefore, a transplantation approach was used (Ref. 5) in which embryonic mammary glands from day 12.5 Jak2-null embryos (Fig. 1A) were transplanted into the fat pad of a 3-week-old mouse that had had its mammary epithelium removed. Wild type embryonic mammary glands were transplanted into the contralateral cleared fat pad as an internal control. Subsequently, the ability of the transplanted mammary epithelium to develop and proliferate was assessed. Out of a total of twenty-three Jak2-null transplants, five were successful (22 %). The lack of successful outgrowths in these transplants is probably due to the fact that the embryos are not as viable as wild type embryos. Furthermore, there was evidence of lymphocytic infiltration (Fig. 1G, arrowhead) suggesting a partial immune reaction to the transplanted epithelium. To circumvent these problems, serial transplants into athymic nude mice were performed. Initially, whole mount staining assessed the outgrowth of a Jak2-null transplant. Small pieces of this gland were then retransplanted into the cleared fat pad of athymic nude mice and the outgrowth of the mammary epithelium was analyzed.

No significant difference was noticed in mammary epithelial development 8-weeks post transplantation when comparing wild type glands (Fig. 1B) with Jak2-null glands (Fig. 1C). The ducts grew out normally and filled the entire fat pad. At lactation, however, while wild type glands showed evidence of epithelial proliferation and the formation of alveoli (Fig. 1D and F). In contrast, Jak2-null transplants showed a persistence of ductal structures with very few alveolar structures budding off the ducts (Fig. 1E and G). Sectioning of these glands revealed large expanded secretory alveoli containing numerous lipid droplets and signs of active milk secretion in the wild type transplants (Fig. 2C and E). Little evidence of secreting alveoli was apparent in the Jak2-null transplants and there was no evidence of lipid synthesis or expanded lumina (Fig. 2D and F). Gross histological comparisons revealed the Jak2-null transplants to be similar to wild type at day twelve of pregnancy (cf. Fig. 2A and B). This apparent lack of epithelial development at lactation was also apparent in two other mouse models deficient in an upstream receptor (prolactin receptor) and the downstream effectors (Stat5a and Stat5b) of Jak2 signaling (Miyoshi *et al.*, submitted).

To further examine the observed defect in epithelial development, the presence of a Na-K-Cl cotransporter (NKCC1; see Ref. 6Kaplan *et al.*, 1996) and a Na-Pi cotransporter (Npt2b; see Ref. 7Hlifinker *et al.*, 1998) was investigated. It was established that NKCC1 protein was highly expressed on the basolateral membrane of ductal cells in the virgin mammary gland (Fig. 3A, red). In contrast, NKCC1 protein levels were much reduced in developing alveoli at pregnancy (Fig. 3C, red) and lactation (Fig. 3B, red). Npt2b protein is expressed on the apical membrane of alveoli at late pregnancy (day 18) and throughout lactation (Fig. 3I, red). It was questioned whether the structures present at lactation in the Jak2-null transplants were ductal in nature or were developing rudimentary alveoli. To this end, the localization and expression pattern of NKCC1 and Npt2b was investigated. These results demonstrated that high levels of NKCC1 protein were maintained in both the ducts (Fig. 3F, red) and rudimentary alveoli (Fig. 3H, red) present in Jak2-null transplants, somewhat similar to that observed in the virgin gland (Fig. 3A, red). In addition, no staining of Npt2b was observed on the apical membrane of rudimentary alveolar structures in the Jak2-null transplants. This suggests

that there is a functional arrest of mammary gland proliferation and differentiation in the absence of Jak2.

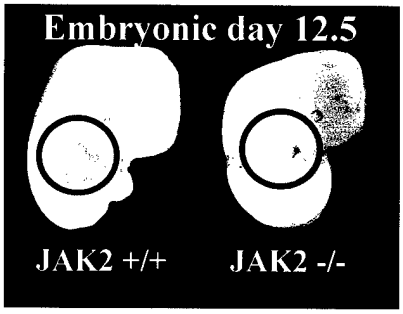
Currently, the proliferative capacity of the Jak2-null cells is being evaluated and electron microscopy will be utilized to examine the apparent undifferentiated nature of these cells on an ultrastructural level. Furthermore, hormone injections will be carried out to determine if Stat5, the downstream transcription factor of Jak2, is able to translocate to the nucleus in the absence of Jak2 activity. This will provide a functional explanation as to why the cells are not able to proliferate and differentiate. It will also provide further evidence that Jak2 is essential for normal mammary gland development.

Conditional deletion of the Jak2 gene

The original targeting construct outlined in the Statement of Work was transfected into SvEv ES cells by electroporation (Fig. 4A). The first round of electroporation generated 3 clones on a total of 6 plates. Typically, at least 300 or so are expected with reasonable transformation efficiency. In order to increase the number of clones, the electroporation was repeated with a higher concentration of linearized plasmid and a lower concentration of FIAU. The second round of electroporation generated a total of 20 clones. These were screened by Southern blot and none of them had the expected targeted event. The lack of clones suggested that the targeting construct had not undergone efficient homologous recombination. The most likely explanation was that the neomycin cassette was not being expressed properly, resulting in few clones due to a lack of drug resistance. To this end, the targeting construct was reengineered so the neomycin cassette was the other side of the floxed exon 2 (Fig. 4B). Repositioning of this cassette altered the detection of the targeting event such that the 3' outside probe would detect a 5.2kb native band and a 6kb targeted band by Southern analysis (Fig. 4B). This targeting construct was electroporated into SvEv ES cells and the cells were plated out. This resulted in greater than 300 clones, 284 of which were subsequently picked and grown up for isolation of genomic DNA. Since the targeting construct was essentially the same except for the repositioning of the neomycin cassette, this indeed confirmed that the position of the neomycin cassette was affecting the function of the cassette. Initial screening of the clones was performed by PCR to identify clones containing the 5' loxP site (Fig. 4C). These clones were then expanded and Southern blots are currently being performed to identify positive clones.

Figure 1: Whole mount analyses of Jak2-null transplants

A: Visual identification of Jak2 -/- embryos



Mammary development in the absence of Jak2

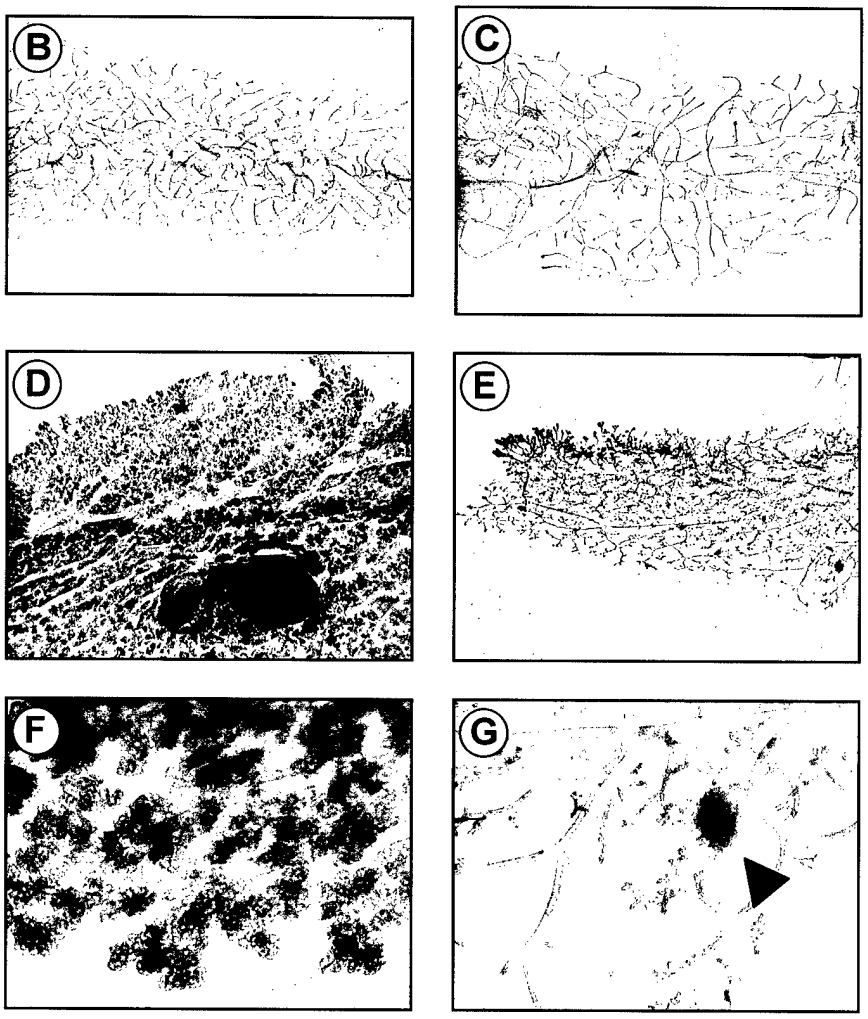


Figure 1: (A) Jak2-null embryos can easily be identified due to the lack of definitive erythropoeisis in the liver. PCR verification was used to confirm the absence of Jak2 in these embryos. (B) Whole mount staining showing ductal development in a representative wild type transplant isolated from a 9-week-old transplanted mouse. (C) Whole mount showing ductal development in a representative Jak2-null serial transplant isolated from a 9-week-old mouse. (D) and (F) Whole mount staining showing alveolar development in a representative wild type transplant isolated from a 12-week-old mouse one day after parturition. (E) and (G) Whole mount staining showing alveolar development in a representative wild type transplant isolated from a 12-week-old mouse one day after parturition. Arrowhead in (G) indicates site of lymphocytic infiltration.

Figure 2: Histological analysis of Jak2-null transplants at parturition

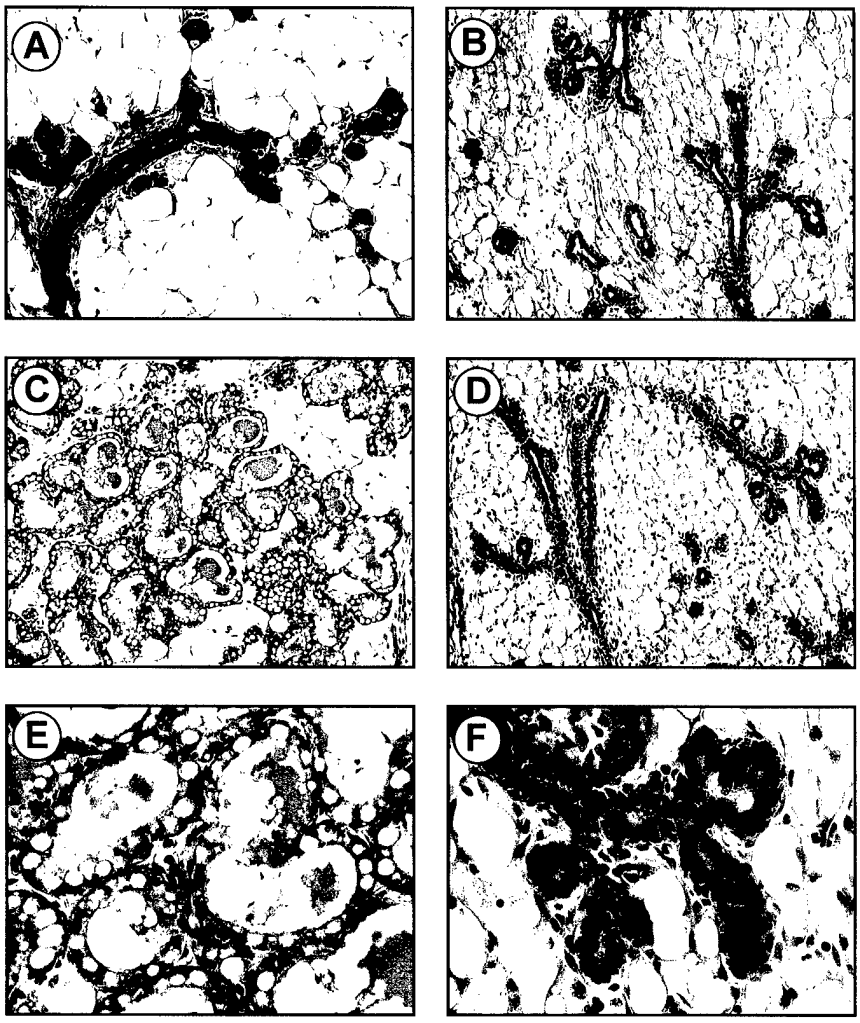


Figure 2: (A) H&E staining of a representative section obtained from a wild type mouse at pregnancy day 12. (B), (D) and (F) H&E staining of representative sections obtained from a Jak2-null transplant one day after parturition. (C) and (E) H&E staining of a representative section obtained from a wild type transplant one day after parturition.

Figure 3: Lack of Npt2b protein and high levels of NKCC1 in the absence of Jak2

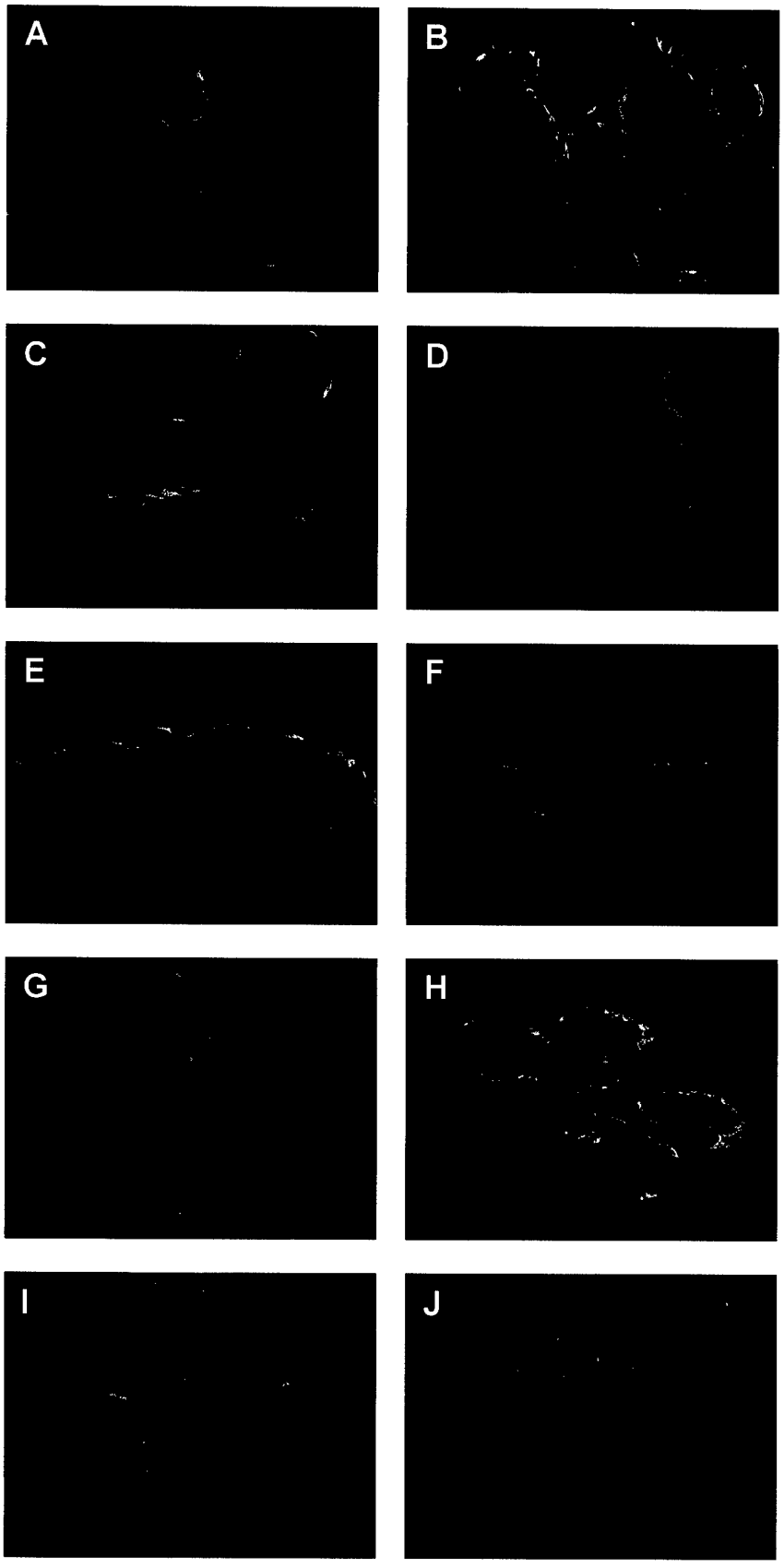
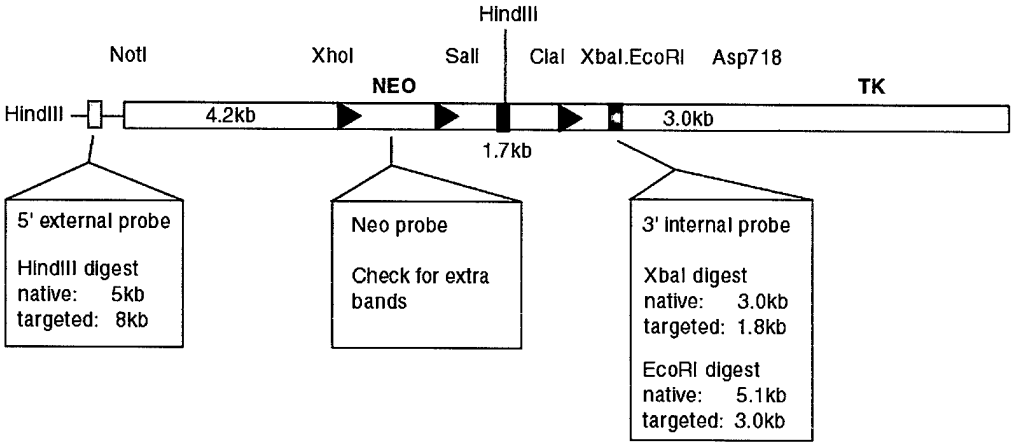


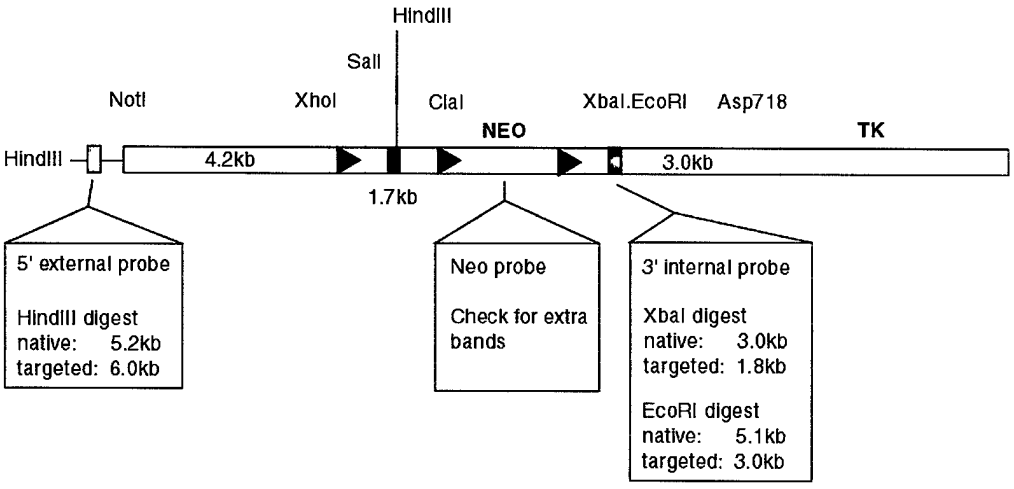
Figure 3: (A) – (H); red represents NKCC1, green represents smooth muscle actin. (I) and (J); red represents Npt2b, green represents E-cadherin. Mammary glands isolated from (A) wild type 11-week-old virgin; (B) wild type one day after parturition; (C), (E), (G) and (I) wild type pregnancy day 13; (D), (F), (H) and (J) Jak2-null one day after parturition. Note the maintenance of NKCC1 protein levels in developing alveolar-like structures in the Jak2-null transplant (D) but not wild type pregnant day 13 mammary glands (C). Further, Npt2b is absent from the apical membrane of Jak2-null transplants (J) but not wild type transplants (I) at parturition.

Figure 4: Jak2 targeting construct and presecreening

A: Targeting construct as outlined in original Statement of Work



B: Revised targeting construct



C: Prescreening for clones containing 5' loxP site

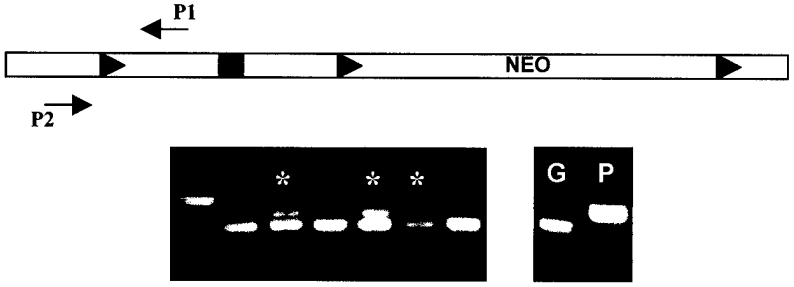


Figure 4: (A) Original targeting construct as detailed in the original Statement of Work. (B) Revised targeting construct indicating repositioning of the neomycin cassette and resultant alterations in the size of the band detected in targeted ES cells with the 5' external probe. (C) The use of PCR to identify clones containing the 5' loxP site. P1, primer 1; P2, primer 2; G, genomic DNA with no loxP site; P, plasmid DNA derived from targeting construct containing 5' loxP site.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of NKCC1 (a Na-K-Cl cotransporter) as a marker of mammary ductal cells and Npt2b (a Na-Pi cotransporter) as a marker of mammary secretory cells.
- Embryonic transplants have demonstrated a requirement for Jak2 in the establishment of functionally differentiated mammary epithelium at lactation but not ductal development in the virgin mouse.
- Absence of Jak2 in the mammary gland leads to continued high synthesis of a NKCC1 and lack of synthesis of Npt2b indicating a deficiency in epithelial differentiation.

REPORTABLE OUTCOMES

Omega 3 Fatty Acids, Breckenridge, CO. June 28th – June 30th. Oral presentation entitled: "Specification of mammary epithelial cells is determined by a pathway including the PrlR, Jak2 and Stat5" (see attached abstract).

Mouse Models of Human Cancer Steering Committee Meeting, Burlingame, CA. July 9th – July 12th. Poster presentation entitled: "Specification of mammary epithelial cells is determined by a pathway including the PrlR, Jak2 and Stat5" (see attached abstract).

Paper submitted for publication: "Signal Transducer and Activator Transcription 5 (Stat5) controls the specification of mammary alveolar epithelium".

CONCLUSIONS

The work completed to date indicates a fundamental role for the tyrosine kinase Jak2 in normal mammary gland development. Thus the absence of Jak2 leads to a failure of mammary epithelial cell development and proliferation during pregnancy and lactation. These observations are accompanied by the maintenance of high NKCC1 protein levels and a lack of Npt2b protein, indicative of a defect in functional differentiation of the mammary epithelium at pregnancy. Work is currently underway to try and address the activation status of Jak2 throughout normal mammary gland development and also in tumors isolated from mice with mutations in the Brca1/p53 genes. Since it has recently been shown that Brca1 can interact and modulate the Jak-Stat pathway in human prostate cancer cells, it is proposed that the status of Jak2 in other mammary tumor models be evaluated. A number of mouse models are already established in the Principal Investigators lab for this purpose. Knowledge of whether Jak2 kinase activity is constitutively active in tumorigenic tissue will allow the development of small molecule inhibitors to specifically target and down regulate its activity. AG490, a relatively specific Jak2 kinase inhibitor, and other analogs may prove very useful in this endeavor.

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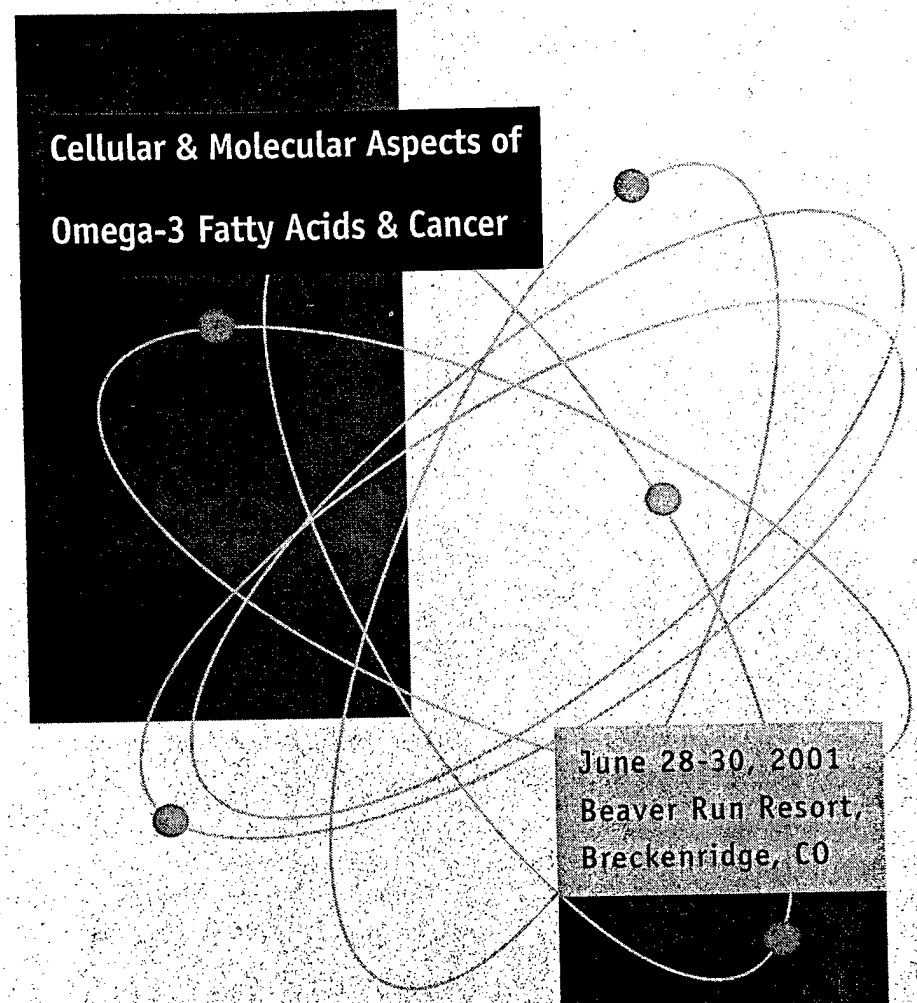
Cellular & Molecular Aspects of Omega-3 Fatty Acids & Cancer



June 28-30, 2001
Beaver Run Resort,
Breckenridge, CO

Marshall University
Omega-3 Research Institute

produces ET-1, causes osteoblastic metastases in a mouse model. Treatment with a specific endothelin A receptor antagonist significantly reduced the osteoblastic metastases. Evidence from other models implicates tumor-produced platelet-derived growth factor (PDGF) as another mediator of osteoblastic metastases.

These data indicate that tumor cells selectively interact with the bone microenvironment. Understanding these interactions at the molecular level has identified novel targets for therapeutic intervention aimed at both osteolytic and osteoblastic metastases.

Using Gene Targeting Approaches To Identify Signaling Pathways Controlling Mammary Gland Development. **Jonathan M. Shillingford**, National Institutes of Health, Laboratory of Genetics and Physiology, Bethesda, MD, USA.

In order to fully appreciate the role that specific genes play in mammary tumorigenesis it is first necessary to establish the role they assume in normal mammary development. Our studies focus on the generation of mouse models to better understand these processes. Using the technique of conditional gene targeting and deletion using the Cre/loxP recombination system, we defined an essential role for the signal transducer and activation of transcription isoform, Stat5a, in pregnancy-mediated alveolargenesis. More recently, using mammary transplantation techniques, we have analyzed the combinatorial effect of Stat5a and Stat5b deletion on mammary gland development. Analyses of these transplants demonstrated that both factors are necessary for the attainment of a secretory phenotype. The observed defects in epithelial development were also apparent in mammary transplants derived from prolactin receptor (PrlR)- and Janus kinase 2 (Jak2)-null mice, highlighting the importance of the PrlR-Jak2-Stat5 pathway in normal mammopoesis. To extend these observations further, we have discovered and utilized membrane markers characteristic of either ductal or secretory epithelial cells. These studies revealed that the epithelial cells present at parturition in the absence of the PrlR, Jak2 or Stat5a/Stat5b retain cellular features suggestive of their arrest at the ductal cell stage, which we have termed 'ductoli'. We propose that an intact PrlR-Jak2-Stat5 pathway is necessary to generate the secretory cell type and the concurrent acquisition of a fully functionally differentiated mammary epithelium.

NATIONAL CANCER INSTITUTE MMHCC Mouse Models of Human Cancers Consortium

Poster Presentation Abstracts

Tuesday, July 10, 2001

PI: Jeff Green

Presenter: Jonathan Shillingford

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Poster: Specification of Mammary Alveolar Epithelium is Controlled by a Pathway Including the Prolactin Receptor, Jak2 and Stat5

Functional development of mammary epithelium during pregnancy is dependent upon intracellular signaling mediated by the actions of prolactin. However, the underlying molecular and cellular events are not clearly understood. We examined the specific contributions of the prolactin receptor (PrlR), Jak2 and Stat5a/b in the formation and differentiation of mammary alveolar epithelium through the transplantation of PrlR-, Jak2- and Stat5a/b-null mammary epithelia. Although virgin PrlR-, Jak2- and Stat5a/b-null mammary epithelia were not morphologically distinct from virgin wild type, they failed to form alveoli and differentiate. There was no evidence of open lumen in either the Jak2- or Stat5a/b-null transplants. In contrast, PrlR-null epithelium underwent partial differentiation and formed alveoli-like structures with small open lumina. Electron microscopy revealed undifferentiated features of organelles and a perturbation of cell-cell contacts in PrlR- and Stat5a/b-null epithelium. High expression of NKCC1, a Na-K-Cl cotransporter characteristic for ductal epithelium, was maintained in the alveoli-like structures of PrlR- and Stat5a/b-null epithelium. In contrast, the Na-Pi cotransporter Npt2b and the gap junction component connexin 32, usually highly expressed in secretory epithelium, were undetectable in the null mice. These data suggest that the absence of intracellular signaling components of the PrlR-Jak2-Stat5 pathway manifest similar phenotypes. Furthermore, these experiments demonstrate that signaling via the PrlR, Jak2 and Stat5 is critical for the determination, proliferation, and differentiation of mammary alveoli during pregnancy.