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To test if Neu differentiation factor (NDF), an activator of the c-neu gene, is proliferative or differentiative in the mammary gland, we have generated a transgenic mouse line overexpressing NDF in the mammary epithelium. These mice develop mammary adenocarcinomas in a stochastic fashion, develop Harderian gland hyperplasia and have subtle developmental abnormalities of the mammary gland. Together these data point to a proliferative effect of NDF in the mammary gland.

The role of the c-neu proto-oncogene in normal development is unclear, though its expression is seen in a variety of fetal tissues. We have used homologous recombination and gene targeting strategies to disrupt the c-neu gene to understand its potential role in development. An ES cell clone carrying the altered neu gene was used to generate chimeric animals able to pass that gene through the germ line. Analysis of several generations of these animals has revealed that the c-neu gene is vital to development. No animals carrying two copies of the mutated gene develop to term. Homozygous mutants do not live past day 10 of gestation. Studies are ongoing to determine the mechanism by which the absence of Neu can lead to premature death.

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Introduction

Recently, a family of stimulatory ligands for the EGF/*erb*B family of receptor tyrosine kinases has been described. This family includes Neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor inducing activity (ARIA), and the glial growth factors (GGFs). This family of ligands, known collectively as the neuregulins, is comprised of alternatively spliced isoforms of a common gene. Interaction of the products of this gene with heterodimers and/or homodimers of the *erb*B receptors leads to their auto and trans-phosphorylation and subsequent interaction with SH-2 proteins including phospholipase C-γ (PLC-γ), the p85 subunit of posphotidylinositol 3'-kinase (PI-3 K), and the GTPase activating protein of *ras* (*ras*-GAP).

The EGF receptor (EGFR) has been associated with cancers of the breast, bladder, lung and stomach. Another member of the EGFR family, HER2/*erb*B2/c-*neu*, has been implicated in a large proportion of human malignancies, including breast and colon carcinomas, and its amplification and/or overexpression has been associated with a poor prognosis. Our laboratory has demonstrated that the overexpression of an activated form of this receptor, when targeted to the murine mammary gland using the mouse mammary tumor virus promoter (MMTV), is transforming. Overexpression of the cellular form of this receptor in the mammary gland is also transforming, though in a more stochastic fashion.

Binding of NDF to the cell surface through one or more of the *erb*B receptors is thought to stabilize receptor dimers, similarly to the effect produced by the point mutation found in the transmembrane domain of the activated *neu* oncogene, a mutation not found in human tumors. Although the role of NDF in breast cancer is still unclear, its biological effects on mammary epithelial cells *in vitro* are quite profound. Some mammary epithelial cell lines can be stimulated to proliferate more rapidly, while others enter G_0 arrest in response to NDF, and begin to exhibit markers of mammary cell differentiation such as casein and lipid production. Elevated expression of NDF in a number of human mammary tumor cell lines has been shown to correlate with the expression of vimentin, a marker of metastatic disease; and it has also been demonstrated that the human breast epithelial cell line, MCF-7, which is unable to form tumors in nude mice, will form tumors when stably transfected with NDF, the growth of which can be inhibited by anti-NDF antibodies (R.Lupu, personal communication).

We have used a transgenic mouse model to test if the overexpression of NDF in the mouse mammary gland can lead to the transformation of the mammary epithelium, or lead to other, differentiative, effects on mammary gland development. We have also targeted the disruption of the c-*neu* proto-oncogene to examine its potential role in development of the mouse as a whole as well as its mammary gland development.

Body

NDFβ2 transgene construct and tissue-specific expression

In order to study the biological effects of high levels of NDF in the breast, we have created transgenic mice overexpressing NDF mRNA in the mammary epithelium. A recombinant plasmid containing the MMTV LTR fused to the murine β2c NDF isoform cDNA was microinjected into the male pronucleus of a one-cell mouse embryo. Ligated to the MMTV promoter/enhancer is a 1.2 kb cDNA containing the entire coding sequence of murine β2c NDF isolated from a v-Ha-*ras* -induced murine mammary gland tumor using the polymerase chain reaction (PCR). This *ras*-induced tumor was chosen as a tissue source of NDF as the original NDF clone descibed was isolated from a *ras*-transformed RAT-1 fibroblast cell line. To ensure proper expression of this cDNA, simian virus 40 (SV40) splicing and polyadenylation signals were added to the 3' end of the transgene construct.

Three transgenic founder animals carrying the fusion gene were generated (TG.IJ, TG.IK, and TG.IM), and each animal passed the transgene to its progeny in a Mendelian fashion. The best characterized line, TG.IJ, will be described here.

The tissue-specific expression of the transgene was determined by Northern blot analysis of 10 μg of total RNA isolated from a variety of organs. The antisense probe used in this analysis was an SV40-fragment specific to the transgene construct. Interestingly, highest levels of transgene expression are seen in the salivary gland of virgin animals, a gland rarely transformed by overexpression of other oncogenes using the MMTV LTR. Otherwise, only the mammary gland expresses the transgene, though expression of the transgene in two different mammary glands from the same animal may vary. These results are consistent with the expression of other transgenes driven by the MMTV LTR.

NDFβ2 transgene expression leads to Harderian gland hyperplasia

Approximately 50% of all TG.IJ animals, male and female, exhibit a unilateral or bilateral exophthalmous resulting from a progressive enlargment of the Harderian gland, a tubuloalveolar gland located within the orbit of many terrestrial species, though absent in primates. This phenotype is observed as early as the time of weaning (3 weeks of age), and is coincident with transgene expression, as animals exhibiting unilateral exophthalmous show no detectable transgene expression in the unaffected contralateral gland, but demonstrate considerable levels of transgene messenger RNA in the affected gland, higher than those seen in the mammary gland. After histologic evaluation, these enlarged Harderian glands are characterized as hyperplastic adenomas. While growing *in situ*, they do not invade the bone of the surrounding orbit, and they fail to grow when transplanted into a syngeneic host indicating they are not transformed.

Persistence of terminal end bud structures

We studied possible effects of transgene expression in the developing mammary gland by examining ductal morphogenesis in these animals. Although pregnancy and lactation stimulate the highest levels of transgene expression from the MMTV LTR in the mammary gland, we have demonstrated that the MMTV-NDF transgene is clearly expressed in virgin glands. To determine if there are any developmental abnormalities associated with transgene expression in these virgin glands, we prepared mammary gland whole mounts stained with carmine red alum to examine the growth of the mammary gland ductal tree. In the normal developing mouse mammary gland, as the animal passes through sexual maturity, ductal epithelial structures gradually fill the mammary fat pat in response to mesenchymal signals. The terminal end bud in the developing gland functions as a growth point, driving ductal morphogenesis by providing differentiated ductal and myoepithelial cells for the formation and elongation of secondary ducts. In a wildtype virgin female, these multi-layered terminal end bud structures have virtually disappeared by ten weeks of age, as the multiple layers of end bud cells undergo regression. We sacrificed a number of virgin transgenic female mice at various developmental stages and found that, although the mammary glands of younger animals appeared normal, glands from older animals (>10 weeks) had increased numbers of TEBs as compared with age-matched control animals.

Stochastic appearance of breast tumors NDF transgenic mice

To examine the potential ability of the MMTV-NDF transgene product to elicit a tumorigenic phenotype in the mice, female TG.IJ transgenic mice were set up to breed continuously, upon reaching sexual maturity, in order to maximize expression of the transgene from the MMTV LTR. After the first round of pregnancy, animals were monitored weekly for the appearance of tumors. By 14 months of age, each animal in the study had developed at least one mammary gland tumor, with the average age at tumor onset being approximately 10 months after birth. Examined histologically, all tumors were similar in nature and distinct from other MMTV-oncogene-derived mammary gland tumors described previously in our laboratory. Each tumor was characterized as an adenocarcinoma with a squamous cell component, highlighted by abundant keratinaceous debris. Northern blot analysis of total RNA from these tumors confirms high levels of transgene expression, though the mRNA source is naturally more homogeneous than the "unaffected" contralateral mammary glands typically used for comparison. These tumors continue to grow well when transplanted into the mammary fat pad of syngeneic hosts and can be adapted to cell culture conditions.

The c-neu proto-oncogene is vital to murine development

In addition to the study of the biological effects of NDF in the mammary gland through stimulation of the *erb*B receptor complex, we were interested in the role of one of these receptors, *erb*B2, in development. We created a targeting construct in which the exon encoding the transmembrane domain

of the receptor was removed and replaced with a neomycin resistance cassette (including in-frame stop codons), making what we predicted would be a null mutation of the *neu* gene. An embryonic stem cell clone carrying the disrupted gene was injected into blastocysts and several chimeric animals were isolated. These animals carried the disrupted allele and were able to pass it through the germ line. No homozygous mutant animals have been born so far and we believe that they die in utero at approximately day 10 of gestation.

Conclusions

The *erb*B2 gene has been shown to be transforming *in vitro* by overexpression, truncation, or point mutation. Our laboratory has demonstrated that the activating point mutation is transforming *in vivo* using a transgenic mouse model. The same mouse model has been used to show that the cellular form of *erb*B2 (c-*neu*) is transforming as well, though with a slower onset of tumor formation. We initiated this study to determine if the same transgenic model could be used to assay the proliferative potential of Neu differentiation factor, a molecule known to stimulate the *erb*B2 receptor through a heterodimeric receptor complex formed with other members of the EGF receptor family. In vitro experiments with NDF on different target mammary epithelial cell lines have yielded somewhat ambiguous results, though different responses to NDF may be a function of the complexity of the receptor types expressed on the surface of these cells. We hypothesized that if a normal receptor such as Neu, when overexpressed in the mammary gland, can provide an initiating event for tumorigenesis, then perhaps the overexpression of a gene product known to activate this receptor would have a similar function. The results of our experiments support this hypothesis in three ways. First, each animal in the study devloped a mammary tumor; in some cases two or three independent tumors were found upon necropsy. Second, there is an incompletely penetrant phenotype in which there is a massive hyperplasia of the Harderian gland in approximately 50% of these mice. These hyperplastic and hypertrophic adenomas are benign in that they are non-invasive and they fail to grow when transplanted into syngeneic host animals. Clearly, elevated levels of NDF expression is not sufficient, as high levels of transgene expression are seen in the salivary gland, yet no histolopathological consequences of this expression in that organ has ever been observed. The benign attributes of these growths imply that there are factors missing from the Harderian gland necessary for this hyperplasia to progress to a malignant neoplasm. Among these putative factors could be the right combination *erb*B receptor subtypes (*erbB*1-4). Interestingly, this Harderian gland phenotype has been seen in another transgenic mouse line in our laboratory, the MMTV-*ras* line, *ras* being a gene found to lie on the Neu signalling pathway. In that line, the glands are also hyperplastic and not neoplastic. Taken together with the NDF transgenic animals, these data suggest that there may be factors downstream of Neu and *ras* which influence the susceptibility of a cell type to oncogenic transformation. A second, parallel, pathway may also provide the "second hit" necessary for oncogenesis and this pathway may be active in the mammary epithelium but inactive in the Harderian gland. It is also possible that there are factors inhibitory to the *neu-ras* signalling pathway which are present in the Harderian gland but absent in the mammary gland.

Lastly, a more subtle phenotype observed in the MMTV-NDF animals is that of the persistence of terminal end bud structures in the mammary gland of virgin females. The terminal end buds normally provide a source of

differentiated myoepithelial cells at the growth points of the mammary ductal tree as it responds to mesenchymal signals to fill the mammary fat pad in the developing gland. These signals provide the TEBs with spatial and temporal growth cues, to avoid overgrowth of ductal structures as well as providing information to halt growth when the outer limits of the fat pad have been reached. Concomitant with the cessation of ductal growth, comes regression of the TEBs in a muture mouse. In our transgenic animals, however, the communication between the developing ductal structures and stromal signalling cells appears to be perturbed in that upon reaching the limits of the fat pad, TEBs do not consistently undergo the apoptotic regression seen in wild type animals. Moreover, in mature transgenic females, TEBs are evident in regions of the mammary gland in which they are not normally seen, in the proximal regions of the gland (close to the lymph node in the #4 gland). There also appears to be a disruption in the signalling involving the direction of growth of the ducts, in that some ducts are found to have reversed direction and, in some cases, they have overlapped other ducts. Although subtle, theses results raise two possibilities about NDF action in the developing mammary gland: NDF expression overcomes inhibitory signals provided by the mammary stroma, not necessarily those signals which inhibit the growth of the ducts, but those which signal the direction of growth and those which influence the active regression of the terminal end buds. These potential inhibitory signals are overcome by a normal pregnancy, lactation and regression, as persistent TEBs are not observed in the mammary glands of such animals.

The second part of our work, discussed here, describes the embryonic lethality of generating a null mutation in the c-*neu* gene. It is obvious that this gene is necessary for the proper development of the mouse, and we are currently searching for the mechanism by which the absence of Neu is lethal to the developing animal.

The third part of our proposal concerns our desire to express NDF recombinantly to test its potential growth arresting effects on mammary carcinoma cell lines. We have been unable to generate sufficiently bioactive recombinant product, but we feel that we may be able to use the tumor cell line derived from our MMTV-NDF transgenic mouse line to purify such a product.

References

No papers have been presented or published as a result of the work described here, though two manuscripts are in preparation.