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Notch4 expression in the mouse mammary gland is associated with mammary gland tumors. Previously we demonstrated that a member of the nuclear hormone receptor family interacts with Notch1. One functional consequence of this interaction is an anti-apoptotic effect in cells expressing activated Notch1. We hypothesized that Notch4, like Notch1, might interact with other members of the nuclear hormone receptor family in mammary gland epithelium and this may prevent the normal apoptotic cell death that occurs during remodeling in the post-lactating breast. The aims in this proposal are designed to test this hypothesis. Results are discussed suggesting that Notch4 regulates progesterone receptor signaling.

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FOREWORD

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Barbara Osborne 6/27/00

PI - Signature Date

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INTRODUCTION:

This proposal is designed to explore the premise that Notch4 signaling may regulate apoptosis in the mammary gland. The specific aims are 1) to determine if nuclear hormone receptors interact with Notch; 2) to determine the functional role of Notch and nuclear hormone receptors in tumor formation; 3) to elucidate the signaling pathways influenced by potential interaction between Notch and nuclear hormone receptors. During the second year of this grant we have made significant progress toward completing these aims.

BODY:

We have been using the mammalian-2-hybrid assay to look for interactions between Notch and the steroid hormone receptors to address the question in **Aim1**. We had previously found that Notch interacted with Nur77 in a yeast 2-hybrid assay, and can demonstrate a low level of interaction between Notch4 and Nur77. We are making various mutants of Notch and Nur77 to map the domains of interaction between these proteins. We also detect a very low level interaction between PR and Notch4, however we have been unable to co-immunoprecipitate the two proteins suggesting that this interaction may be indirect and involve bridging proteins.

In **Aim 2**, we proposed to look at the role of Notch in tumorigenesis. We are presently gearing up to look at the effects *in vivo*. We have obtained four MMTV-INT3 (Notch4) transgenic mice and are currently breeding them to establish a colony. We will take epithelial cells from these mice and place them into cleared fat pads of wild type mice and look at the effect of estrogen on tumor formation in these mice by administering tamoxifen or by ovarectomizing mice. We will also infect wild type epithelial cells with normal and mutant Notch4 constructs and look at the effect on tumor formation and differentiation.

Transient and stable expression of Notch4 was employed in **Aim 3** to look at the effect of Notch on steroid hormone receptor activity. We have convincingly shown that Notch expression causes a repression of progesterone receptor and glucocorticoid receptor activity in HC11 cells. Notch's effect on the estrogen receptor appears to be more complicated. Low level, constitutive expression of Notch4 represses activity of the estrogen receptor and high level, transient expression appears to enhance estrogen receptor transactivation. We have looked to see if this augmented estrogen receptor activity is responsive to the inhibitory effect of the estrogen antagonist, tamoxifen. We can show that tamoxifen will inhibit the augmented activity, although it appears to take higher amounts of tamoxifen to return estrogen receptor activity back to basal levels.

We have overexpressed wild type and dominant negative members of some of the known proteins down stream of Notch or those which have been demonstrated to effect levels of steroid activity. Using these expression studies in HC11 cells, we have determined that the repression is not likely to be due to increased levels of Grg1 or Hes1, or increased levels of NF-*k*B, Bag-1, or MAP kinase signaling.

We have made mutants of Notch4, which lack the C-terminus after the ankyrin repeats, and have a mutation of a conserved alanine to a phenylalanine in ankyrin domain 4. The ankyrin 4 domain mutant has been shown to inhibit CBF1 activity by disrupting the Notch, SKIP, CBF1 interaction. However, neither of these mutants alleviate Notch's repressive activity on the progesterone receptor. We are presently making N-terminal deletion mutants, which will eliminate the RAM23 domain and portions of the first and second ankyrin repeats to further these observations.

INT3 (Notch4) transgenic expression has been demonstrated to down regulate progesterone receptor expression in ductal epithelial cells. Therefore, we will be infecting INT3 epithelial cells with adenoviral expressed PR and put them back into mice to see if the block in differentiation can be overcome and the tumor formation prevented.

KEY RESEARCH ACCOMPLISHMENTS

-Mutant forms of Notch4 has been generated and cloned into retroviral vectors
-Reporter constructs for ER, PR, and CBF1 are being generated in retroviral and adenoviral constructs
-An INT3 colony of mice has been established
-In vivo techniques for looking at and manipulating mammary gland morphology have been set up

REPORTABLE OUTCOMES

- Abstract presented at the DOD BCRP meeting in Atlanta, Georgia in June 2000 entitled "The Effects of Notch on the Transcriptional Activity of the Steroid/Thyroid Hormone Receptor Superfamily" SW Smith, R Lawlor, DJ Jerry, BA Osborne

CONCLUSIONS

We have extended our results from last year and can now demonstrate that Notch4 modulates not only the progesterone receptor, but can also affect the glucocorticoid receptor and the estrogen receptor. The INT3 transgenic mice show a retardation of ductal growth until pregnancy or hormones mimicking pregnancy are given and a full ductal tree is observed. These hormones would be expected to induce INT3 expression to a higher level because it is under the control of the MMTV promoter. Our *in vitro* data showing low levels of Notch expression repressing estrogen receptor activity and high levels of Notch augmenting the activity of the estrogen receptor, agree with this phenotype. These mice have a block in lobulo-alveolar development and milk production, which is in agreement with Notch repressing the activity of the progesterone and glucocorticoid receptors. Given the ability of estrogen to cooperate with Ras to induce tumors, it is of interest that estrogen receptor activity is upregulated in these cells. We will be investigating the role of the modulation of these hormones in the tumor formation induced by Notch in the next year.

Figure legends:

Figure 1: The progesterone receptor interacts with INT3/Notch4 in a mammalian 2-hybrid assay.

Figure 2: Notch4 represses transactivation of the progesterone receptor. HC11 cells were transfected with a progesterone-luciferase reporter in the presence or absence of Notch4 and luciferase activity was determined.

Figure 3: Notch4 represses transactivation of the glucocorticoid receptor. HC11 cells were transfected with a glucocorticoid-luciferase reporter in the presence or absence of Notch4 and luciferase activity was determined.

Figure 4: Mutations in INT3/Notch4 do not relieve the repression of progesterone receptor activity. HC11 cells were transfected with a progesterone-luciferase reporter in the presence or absence of Notch4 or constructs with targeted mutation of Notch4 and luciferase activity was determined.

Figure 5: Modulation of estrogen receptor activity by expression of INT3/Notch4. INT3/Notch4 was cloned into two vectors, pJ3 or pCDNA. In cells transfected with pCDNA/Int3 where Notch levels are low, ER activity is activated while in cells transfected with pJ3, which drives high level of Notch expression, ER activity is repressed.

Figure 1

Mammalian-2-Hybrid with PR and INT3

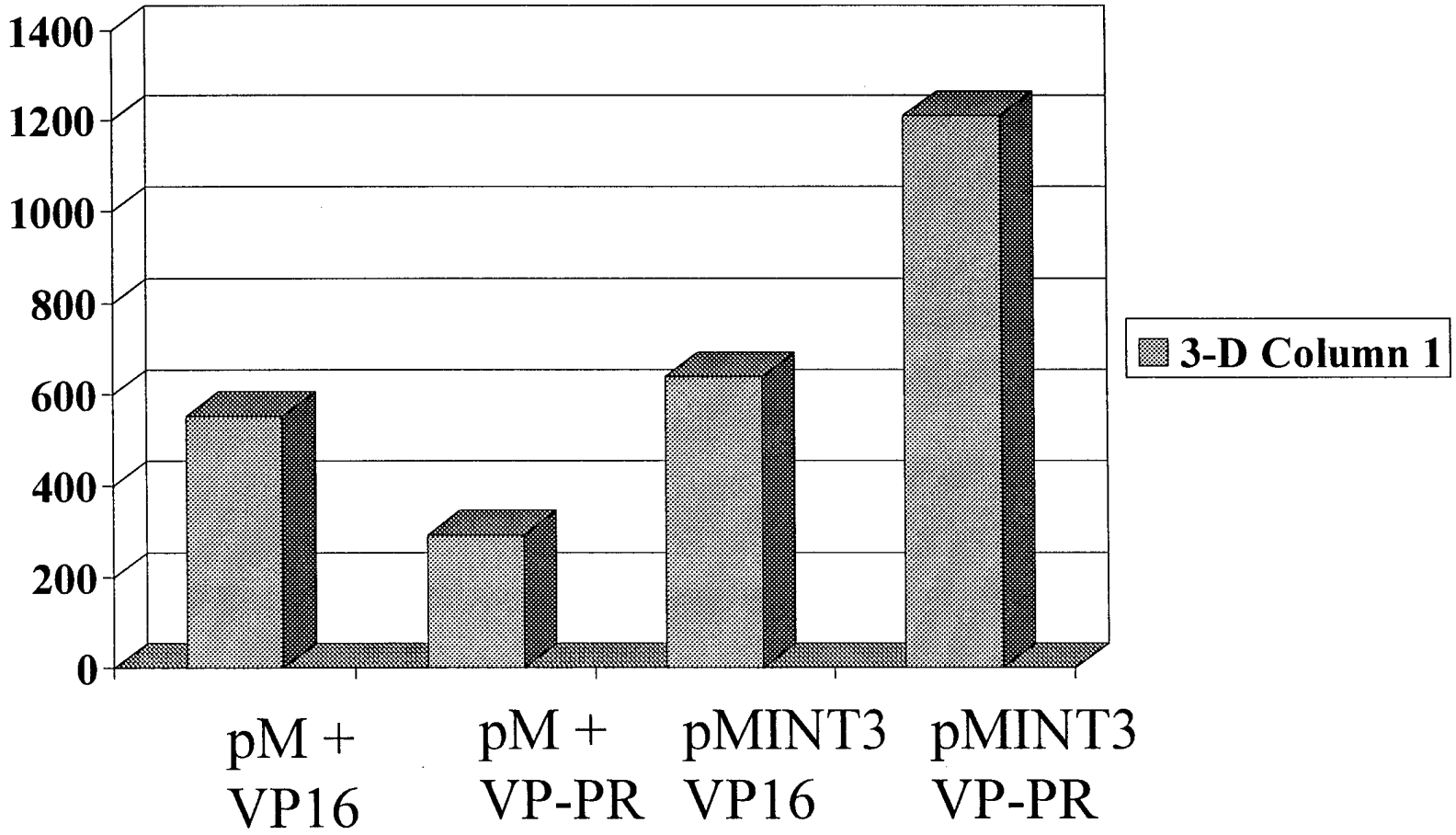

Figure 2

The Effect of Notch4 Expression on Progesterone Receptor Transactivation

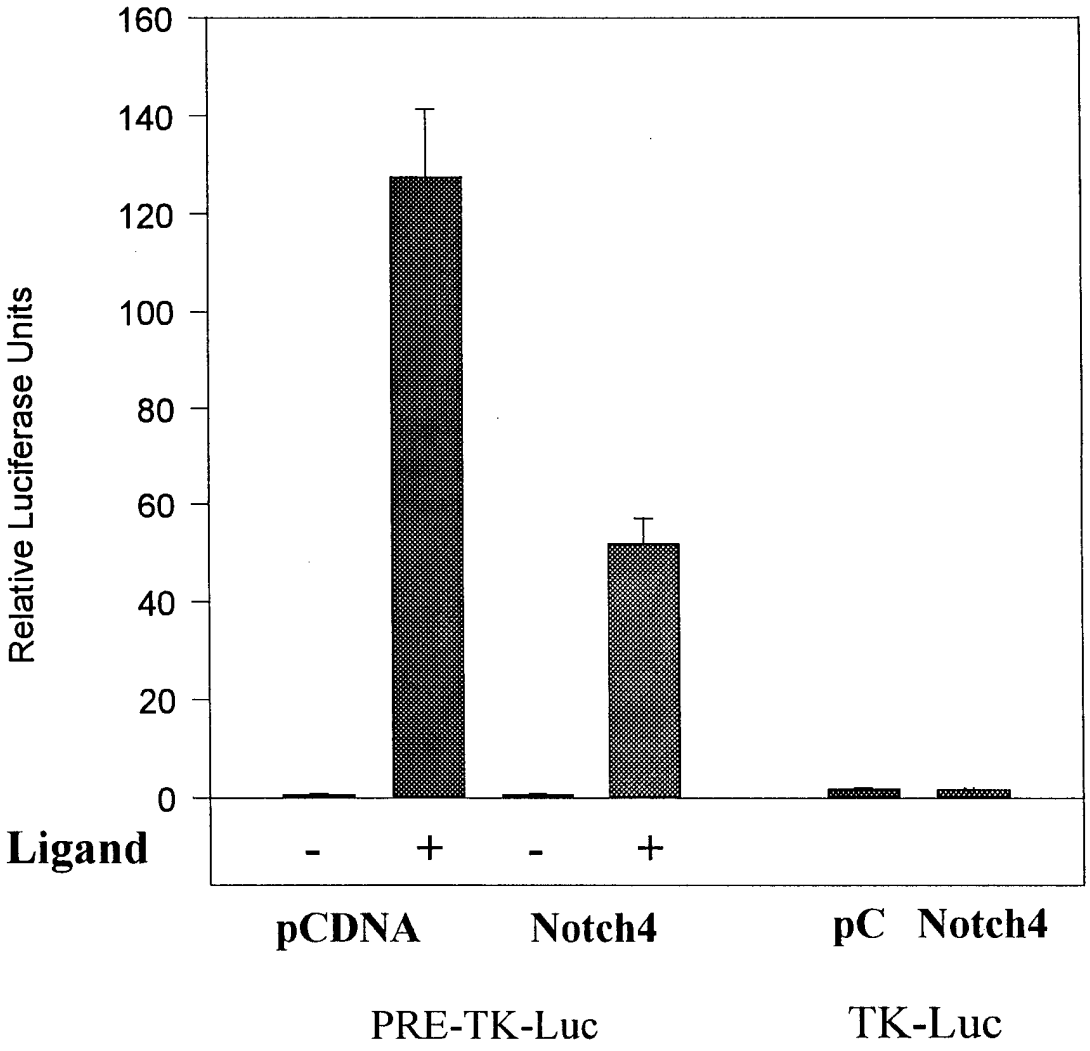

Figure 3

The Effect of Notch4 Expression of Glucocorticoid Receptor Transactivation

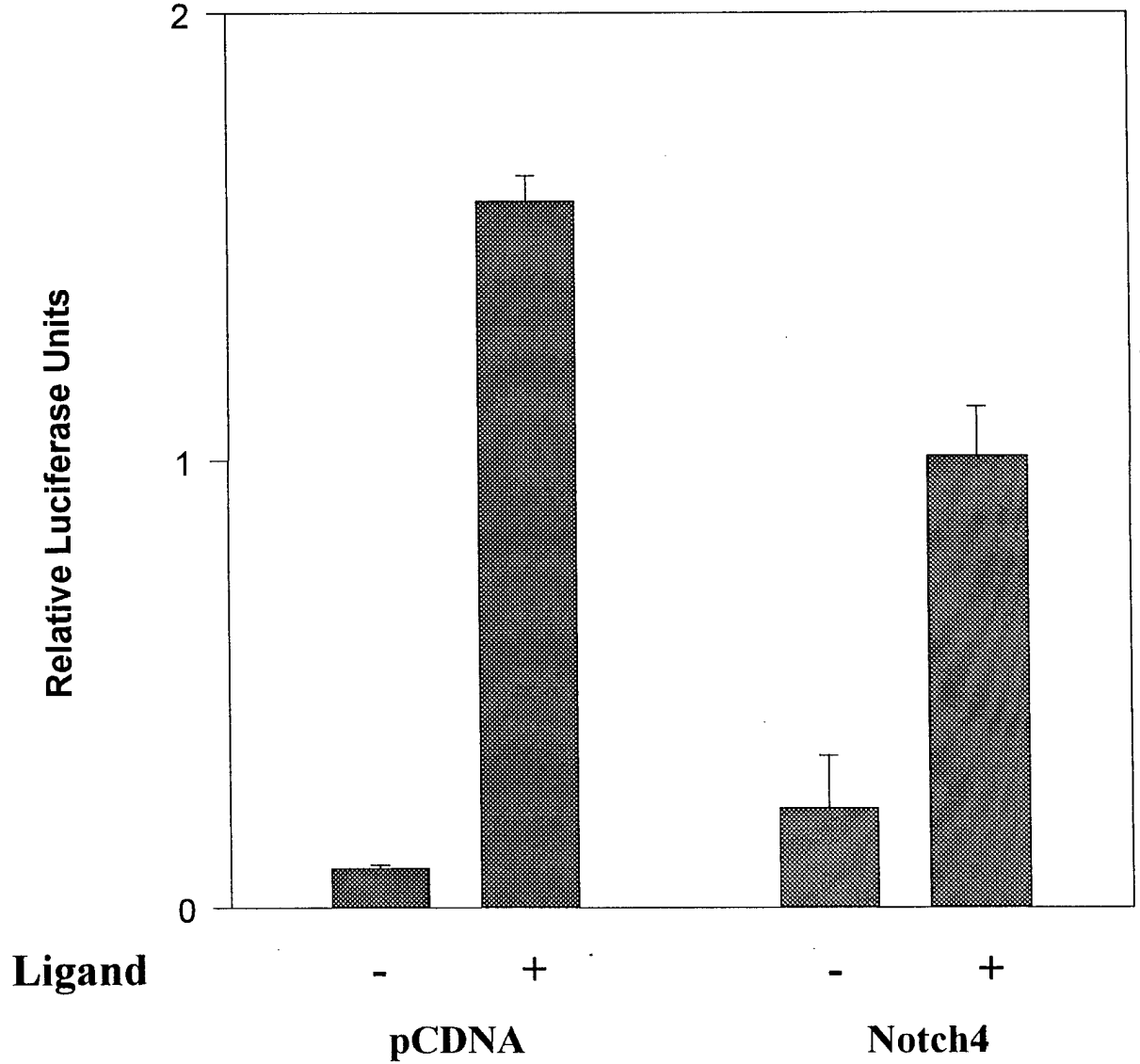

Figure 4

The Effect of Mutant INT3 on PR Transactivation

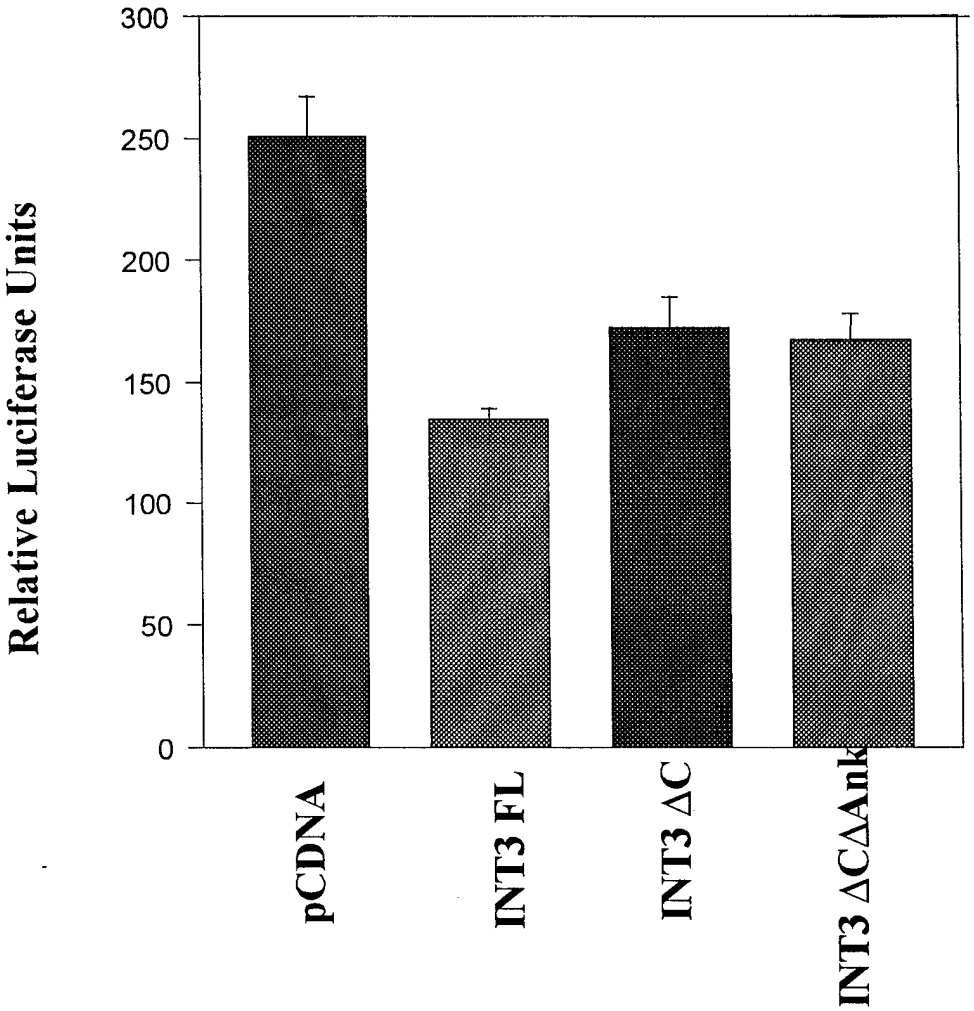

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Figure 5

The Modulation of ER Activity by varying levels of INT3