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PRINCIPAL INVESTIGATOR: Annapoorni Rangarajan, Ph.D.

CONTRACTING ORGANIZATION: Indian Institute of Science
                                              Bangalore 560012 India

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14. **ABSTRACT**
Notch proteins are activated upon binding to ligands of the Delta/Serrate family. In previous experiments I had found that activated allele of Notch1 cooperates with low levels of oncogenic Ras expressing HMLE cells(termed HMLER). Since ErbB2 overexpression is one of the leading causes of Ras activation in breast cancer cells, I tested whether ErbB2 overexpression will cooperate with Notch in HMLE cells. While overexpression of activated Notch1 failed to transform wild-type ErbB2-expressing HMLE cells, it caused cell death inconstitutively active ErbB2-expressing cells. These results indicate that Notch-Ras cooperation in transformation is largely dependant on the extent of signal activation. While low levels of Ras signals fails to cooperate withNotch, too much of Ras activation leads to cell death. My previous experiments based on RT-PCR analysis had suggested that the Notch-ligand Jagged1 may be involved in activating Notch signaling in breast cancers. WhileJagged1 expression in HMLER cells led to soft-agar colony formation, it failed to generate tumors in vivo. In order to further demonstrate a role for Notch signaling in breast cancers, I generated siRNA against Jagged1.Screening several of these siRNA led to the selection of siJagB4 that caused greater than 95% suppression ofJagged1 expression in co-transfection experiments undertaken with siJagB4 construct and a myc-tagged Jagged1expresssion construct. Subsequently, I generated lentiviruses encoding this siRNA and introduced this siRNA into several breast cancer cell lines. Upon further characterization, I found that this siRNA had very moderate effects in downmodulating Jagged 1 expression driven by endogenous promoter. The best effect observed was a 25% reduction in MCF-7 cells. Currently, I am making subclones of the MCF7 cells to see if a single clone showing major suppression of Jagged 1 expression could be isolated for further testing their tumorigenic behavior.

15. SUBJECT TERMS
Notch, Ras, signaling, transformation, tumorigenesis

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The role of Notch Signaling Pathway in Breast Cancer Pathogenesis

Introduction

Notch proteins belong to a family of four receptors, Notch 1-4, which are activated upon binding to ligands of the Jagged/Delta family. Ligand binding induces a series of cleavage in the Notch protein, thereby causing the release of its cytoplasmic region in to the nuclei, where it functions as a transcriptional activator. Truncated and constitutively active forms of all four Notch proteins have been shown to have oncogenic functions in several murine cell-types. In an MMTV insertional mutagenesis model, integration of the integration of MMTV at the *int3* locus caused mammary tumorigenesis through the activation of the *int3/Notch4* gene. Importantly, aberrant expression of Notch pathway components has been detected in human breast cancers (1,2). Taken together, these observations implicate a role for Notch signaling in human breast cancers; however, a clear functional involvement is yet to be demonstrated.

I have been studying the role of Notch signaling using the genetically defined immortalized and transformed human breast cell system developed in the Weinberg lab (3). In this model normal human mammary epithelial cells (HMECs) were immortalized and transformed by introducing a defined set of genetic elements. Thus, introduction of the simian virus 40 early region (SV40-ER) and the catalytic subunit of human telomerase enzyme rendered the HMECs immortalized (these immortalized HMECs are referred to as HMLE cells). Subsequent expression of supra-physiologic levels of oncogenic Ras rendered the HMLE cells fully transformed. Thus, this model provides a unique, genetically well characterized system in which one can study interaction of signaling pathways involved in breast cancer pathogenesis, without the interference of unknown genetic alterations sustained by breast cancer-derived cell lines normally used for such studies.

However, in the model system described above, low or physiologically relevant levels of oncogenic Ras failed to transform HMLE cells. Similarly, overexpression of ErbB2, a receptor tyrosine kinase upstream of Ras normally found overexpressed in many breast cancers, also failed to transform HMLE cells. These observations suggested that in order for physiologically relevant levels of Ras signaling to transform immortalized breast cells, cooperation of additional signaling pathways is essential. I hypothesized that the Notch proteins are a likely candidates for such cooperation.

In this study, I have thus far demonstrated that Notch signals cooperate with low levels of oncogenic Ras in transforming HMLE. In this cooperation, nuclear localization of Notch is essential. Additionally, preliminary evidence suggests that Jagged1 may be the ligand responsible for Notch activation in breast cancers.

Results:
A) Assessing cooperation between Notch1[IC] and ErbB2 signaling in transforming HMLE cells. Breast cancers typically do not harbor oncogenic Ras mutations; nevertheless, Ras signaling is activated in many breast cancers, mainly due to alteration or overexpression of upstream receptor tyrosine kinase. For example, ErbB2 is altered or overexpressed in ~30% of

all breast cancers; however we found that ErbB2 overexpression in HMLE cells failed to transform them. Accordingly, I determined whether Notch1^{IC} could transform wild type ErbB2-overexpressing HMLE cells. However, in previous experiments I found that Notch1^{IC}-expressing HMLE-ErbB2 cells failed to generate colonies in soft agar. One possible reason for this could be that overexpression of ErbB2 only weakly activates Ras. Consequently, I generated HMLE cells expressing a constitutively active ErbB2 (ErbB2^{CA}) (4) via retroviral transduction. This resulted in a greater than 10-fold overespression of ErbB2^{CA} over endogenous levels of ErbB2. In order to determine if activation of Notch signals could cooperate with ErbB2^{CA}, I introduced constitutively activated Notch1 into HMLE cells expressing ErbB2^{CA}. Drug selection was carried out to select polyclonal population of cells stably expressing Notch IC. While the majority of cells died during drug selection, few colonies emerged after two weeks of drug selection. Western blot analysis to detect expression of Notch1 IC however revealed that these cells had somehow managed to shut down expression of Notch1 (Fig.1). Thus, HMLE cells stably expressing both constitutively active ErbB2 and Notch1 IC could not be derived.

Taken together, these results demonstrated that while activated allele of Notch1 could co-operate with low levels of oncogenic Ras to transform HMLE cells, it failed to transform HMLE cells expressing wild-type ErbB2, and caused cell death in constitutively active ErbB2 expressing cells. Previously, I had observed that overexpression of Notch1^{IC} in HMLE cells expressing high levels of oncogenic Ras also caused cell death. Thus, these results suggest that too little Ras signals is insufficient to cooperate with Notch signals for transformation, whereas too much is deleterious, thereby suggesting that cooperation between Notch and Ras is critically dependant on levels of signals.

C) Determine whether Notch-ligand interaction can mimic Notch1 IC in transformation: Notch signaling is activated by interaction with ligands. My earlier experiments had demonstrated that one of the Notch-ligands, Jagged1, is specifically expressed in breast cancer cells, but not in the immortalized HMLE and MCF10A cells. Therefore, I assessed whether Jagged1-mediated activation of Notch signaling could cooperate with Ras for transformation of HMLE cells. Towards this end, I generated HMLER cells expressing Jagged1. Western blot analysis revealed expression of Jagged1 (Fig 2A). I then determined whether Jagged-1 expression results in the activation of Notch signaling. Towards this end, an HES1-luciferase reporter construct, that positively responds to Notch signals, was transiently transfected into HMLER-Jagged1 cells. This caused a 3-fold induction of luciferase activity over control (Fig 2B), indicating that Jagged1 overexpression indeed resulted in Notch activation. The HMLER-Jagged1 cells were subjected to soft-agar analysis. In contrast to HMLER cells expressing a constitutively active allele of Notch1, the Jagged1-expressing cells generated fewer and smaller soft-agar colonies. This suggests that Jagged1-mediated activation of Notch signals is sufficient for soft-agar colonies *in vitro*, albeit at a low efficiency (Fig 2C). When injected subcutaneously into nude mice, however, the Jagged1 expressing HMLER cells failed to generate tumors, suggesting that Jagged1-mediated Notch activation is not sufficient for tumorigenesis *in vivo*. Other ligands of Notch could potentially contribute to Notch signaling in breast cancer cells.

D) Determine whether Notch Signaling is required for the transformed phenotype of breast cancer cells

My previous experiments have suggested a role for Notch signaling in breast cancers. To further investigate the role of Notch signaling in breast cancers, I chose to inactivate Notch signaling in breast cancer cell lines using siRNA approach. My previous experiments indicated that Jagged1 may be the prime ligand which signals to Notch proteins in breast cancer cells. Therefore, I attempted to suppress the expression of Jagged1 in breast cancer cells using an siRNA approach and then determine its effect on their transformation potential. In previous experiments I have reported the screening and identification of an siRNA against Jagged1 called siJagB4 which showed greater than 95% reduction in Jagged1 expression.

As this siRNA was made in a lentiviral-based vector system, I generated lentiviruses containing either siJagB4 or siGFP as control. Four different breast cancer cell lines, MCF7, MDA-MB 435, Sum1315, and the genetically well-characterized HMLER cells, were infected with these viruses. Puromycin drug selection was employed to select those that carried the siRNA. Drug resistant polyclonal population of cells were further expanded and characterized.

A western blot analysis was performed to assess suppression of Jagged1 expression. While MCF7 showed a 25% reduction in Jagged1 expression (Fig 3A), in none of the other three cell lines any change in Jagged1 expression was observed (Fig 3A shows data for MDA-MB 435). Thus, even though the chosen siRNA showed greater than 95% reduction in Jagged1 expression in a cotransfection experiment using a CMV-driven Jagged1 construct, the siRNA seems to have had no effect in suppressing endogenous Jagged1. Currently, I am screening for other siRNA against Jagged1. Notch signaling could be blocked by suppressing Notch expression as well. Towards this end, I tried screening 10 different siRNA constructs against Notch1. however, none of them brought about any reduction in Notch1 expression (Fig 3B; data shown for three constructs). Recently, some papers have reported siRNA against Notch1. I plan to use this sequence to generate lentiviral construct containing siRNA against Notch1. Simultaneously, I am also trying to make clonal population of MCF-7 cells containing siJagB4 to screen for the possibility of singles clones showing a better reduction in Jagged1 expression, compared to the polyclonal population.

Key Research Accomplishments:
1)   Generation and characterization of HMLE cells expressing constitutively active ErbB2 and Notch1[IC]
2)   Generation and characterization of Jagged1-expressing HMLER cells.
3)   Generation and characerization of breast cancer cell lines carrying siRNA against Jagged1.

Reportable outcomes
Abstract presented on "Notch Signaling in Breast Cancer Development" in an international meeting organized by EMBO on the topic "Notch Signaling in Development and Cancer" at Rome, Italy, between April 21-24.

Conclusion: The results obtained thus far suggest that Notch-Ras interaction may play a critical role in breast cancer development. The cooperation between Notch and Ras signal appears to depend on the strength of signal. While too little is insufficient for transformation, too much is toxic. Therefore, regulating the signal strength appears to be critical in breast cell transformation. Further dissection of the Notch-Ras interaction is likely to identify targets against which new chemotherapeutic drugs can be directed.

References
1) Miele L and Osborne B, *J Cell Physiol*, **181**, 393-401 (1999)
2) Weng AP and Aster JC, *Curr Opin Genet Dev*, **14** (1):48-54 (2004)
3) Elenbaas B et al., *Genes Dev*, **15**, 50-65 (2001)
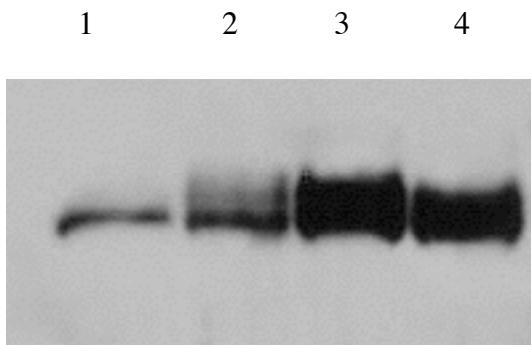4) Seton-Rogers SE et al., *Proc Natl Acad Sci* U S A. **3**;101(5):1257-62 (2004)

Fig. 1

1   2   3
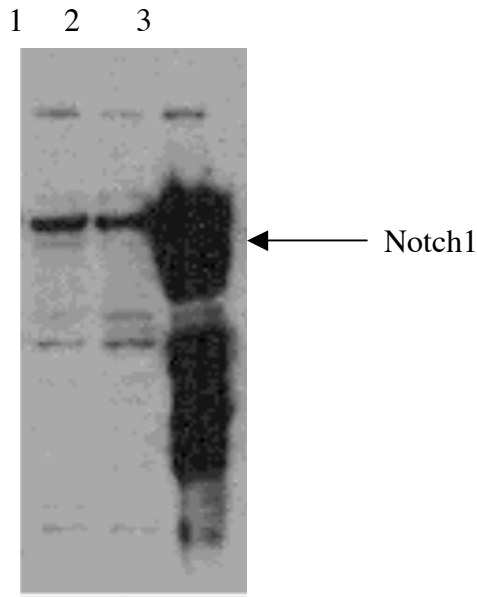
← Notch1

Western blot analysis of Notch 1-IC overexpression in HMLE -ErbB2^{CA}.
Lane 1: un-infected HMLE-ErbB2^{CA} cells
Lane 2: Notch-IC infected and selected HMLE-ErbB2^{CA}
Lane 3: 293T cells transfected with Notch1-IC expression construct (positive control for Notch overexpression).

Fig 2A

1   2   3   4

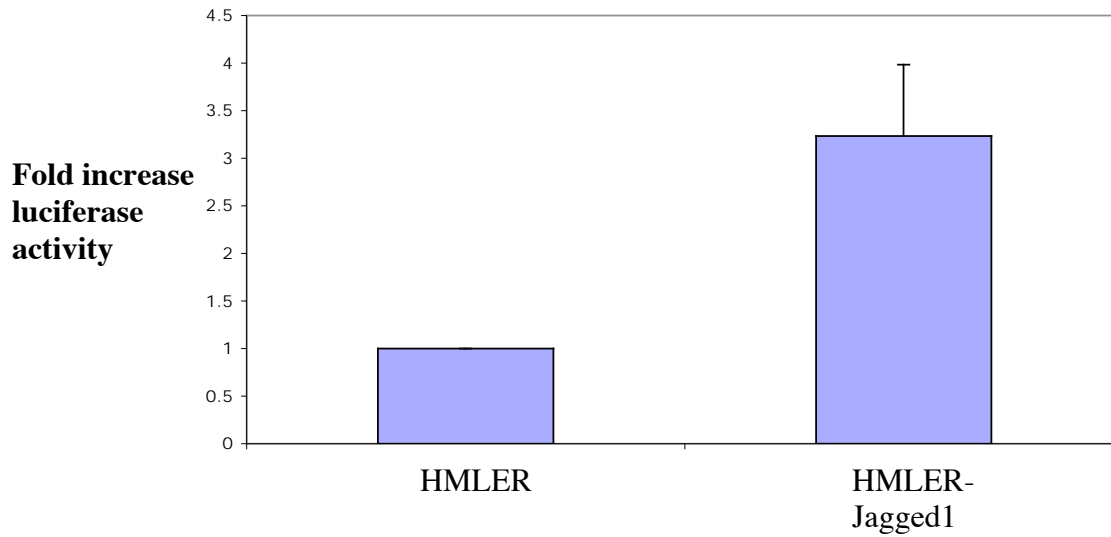**Western blot analysis for Jagged-1 overexpression.**
Lane1: 293T control
Lane2: HMLER cells
Lane3: 293T plus transient transfection of Jagged1 expressing construct (positive control for Jagged1 overexpression)
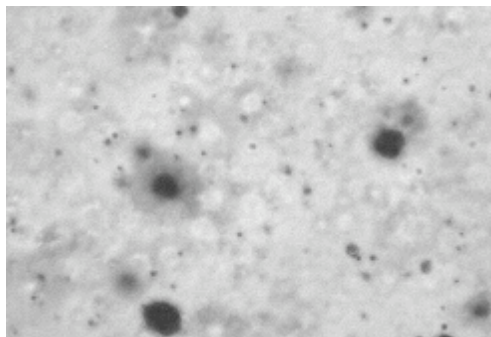Lane4:HMLER cells infected with Jagged1 producing retrovirus

Fig. 2B
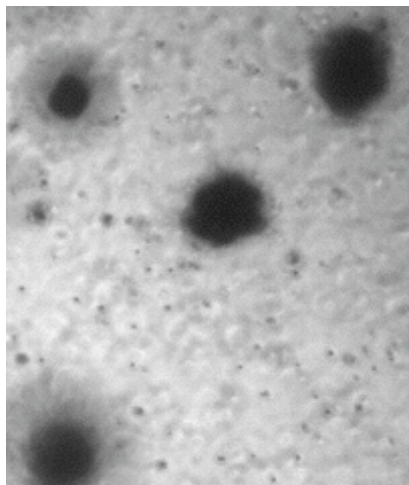

Fold increase luciferase activity

Luciferase activity in HMLER and HMLER-Jagged1 cells transfected with a Notch-responsive HES-luc reporter construct.
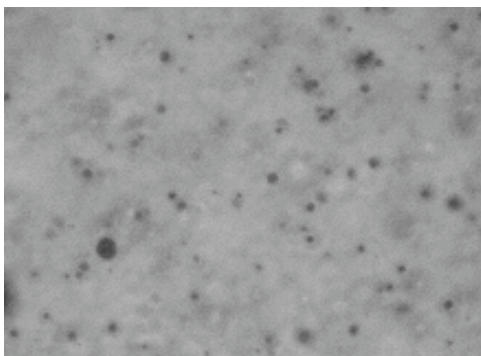
Fig 2C.

HMLER-Jagged1
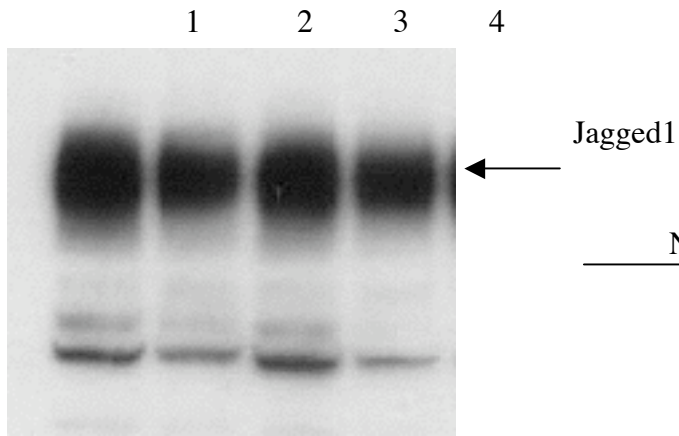

HMLER-Notch1-IC

HMLER

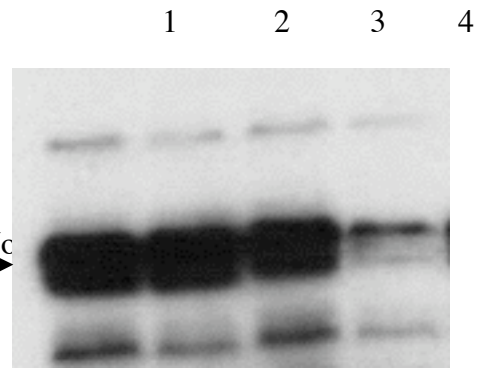**Soft agar colonies generated by HMLER, HMLER-Jagged1, and HMLER-Notch1-IC cells**

Fig. 3 A   **Fig. 3 B**

Western blot analysis for Jagged1.
Lane 1: MCF7 plus siGFP
Lane 2: MCF7 plus siJagB4
Lane 3: MDA MB 435 plus siGFP
Lane 4: MDA MB 435 plus siJagB4

Western blot analysis for Notch1
Lanes 1,2 and 3 are 293T cells
transiently co-transfected with Notch1-
IC construct and different siRNA
constructs against Notch1. Lane 4 is
untransfected 293T cells.