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14. ABSTRACT
Notch proteins are activated upon binding to ligands of the Delta/Serrate family. In previous experiments I hadfound that activated allele of Notch1 cooperates with low levels of oncogenic Ras expressing HMLE cells(termed HMLER). Further investigations revealed that Notch-IC confers protection to HMLER cells againstanoikis, a form of apoptosis triggered upon anchorage-independent growth. Eventhough Notch-IC cooperateswith oncogenic Ras HMLER cells to initiate tumors in vivo, they form well encapsulated, non-metastatictumors. Inhibition of Notch signaling, however, reverts the EMT phenotype of Sum1315 breast cancer cell line,as detected by increase in epithelial E-cadherin and loss of fibroblastic marker, vimentin. In order to assesswhether the observed Notch-Ras cooperation in transformation of HMLE cells in vitro also holds true in vivo innaturally arising breast tumors, immunohistochemical analysis was undertaken. While in normal breast tissueactivated Notch, its downstream target Hes5, or phospho forms of Erk1/2 were not detected, abundant amountsof all these proteins were detected in the same areas within breast cancer tissue. This suggests that the Notch-Rascooperation might indeed be involved in initiating breast carcinogenesis as it occurs in vivo.

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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, cooperation between the Notch and Ras pathway in breast carcinogenesis has been evaluated.

Results:

A) Characterize the functional contribution of Notch activation to breast cell transformation

Since HMLE-ErbB2 ^{CA} expressing Notch1^{IC} could not be derived, this part of the experiment was done using HMLER cells, as proposed in the Statement of Work.

As reported earlier, HMLER cells overexporessing Notch1^{IC} can both give rise to soft agar colonies and initiate tumors in nude mice. The ability to give rise to soft-agar colonies measures the ability to survive in the absence of anchorage, a prime characteristic of cancer cells, while non-transformed cells typically undergo cell death by anoikis, a form of apoptosis. In order to assess whether Notch1^{IC} protects HMLER cells from anoikis, HMLER cells with and without Notch1^{IC} were seeded in methyl cellulose. This prevents attachment of cells to the substratum, thereby providing a way for measuring anchorage-independent survival. Cells were seeded on methylcellulose for 24 hrs, 48 hrs, and 72 hours, at the end of which they were stained with Hoechst 33342 dye to detect apoptotic nuclei. While at 24 and 48 hr time points both HMLER and HMLER- Notch1^{IC} cells showed equal survival, at 72 hours, HMLER cells expressing Notch1^{IC} showed ~25% more survival as compared to HMLER cells (Fig. 1A). This suggests that Notch1^{IC} confers protection to HMLER cells in anchorage independent conditions.

In order to assess whether Notch1^{IC} promotes in vivo transformation of HMLER cells by promoting angiogenesis, analysis of expression levels of pro angiogenic molecule (VEGF) and anti-angiogenic molecule (thrombospondin) was undertaken. Initially, western blot analysis was undertaken using commercially available antibodies against VEGF and thrombospondin. However, since these antibodies failed to work, an RT-PCR analysis was undertaken to estimate the levels of transcripts of VEGF and thrombospondin. Total RNA was extracted from HMLER and HMLER- Notch1^{IC} cells and then converted into cDNA using a commercially available kit. Using primers specific for VEGF and thrombospondin, and using GAPDH as internal control, RT-PCR analysis was undertaken. As shown in Fig 1B, not much difference was seen in the VEGF/GAPDH and thrombospondin/GAPDH ratio between HMLER and HMLER-Notch1^{IC} cells.

Tumors generated by HMLER-Notch1^{IC} cells were analyzed for metastasis to see if Notch1^{IC} enabled the tumors to become metastatic. Gross phenotypic observation revealed that HMLER-Notch1^{IC} cells formed well defined, encapsulated tumors. In order to score for micrometastasis, small chunks of the mouse lung, liver and brain were analyzed for presence of HMLER-Notch1^{IC} cells by DNA PCR for SV40 LT antigen that are present within the HMLER-Notch1^{IC} cells. While the murine tissue showed positive for GAPDH genomic PCR, the PCR for SV 40 LT Ag did not show any amplification, thereby indicating lack of micrometastatis.

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

In my earlier experiment I had observed that in Sum1315 breast cancer cell line, inhibition of Notch signaling by addition of DAPT (an inhibitor of presenilin) resulted in the loss of EMT phenotype. In order to assess whether these morphological appearances corroborated with an alteration at the molecular level, cell extracts were prepared from control DMSO treated and DAPT treated Sum1315 cells, and western blot analysis was undertaken to assess expression of EMT markers. EMT is typically characterized by the appearance of fibroblastic markers (such as vimentin and fibronectin) and loss of epithelial markers (such as E-cadherin). Accordingly, a western blot analysis was performed using antibodies against vimentin and E-cadherin. Beta actin antibody was used as loading control. Western blot analysis revealed a slight increase in

levels of E-cadherin upon addition of DAPT; however, expression of vimentin was completely lost (Fig 2), indicating that inhibition of Notch signaling indeed causes the Sum1315 cells to revert to a more epithelial phenotype compared to their native fibroblastic appearance.

While the above experiment shows a role for notch signaling in maintaining the EMT phenotype, I was also interested in seeing the role of Notch in maintaining the transformed phenotype itself. To address this issue, I had planned to generate siRNA constructs against Jagged1 and/or Notch1 and infecting into different breast cancer cell lines with it, thereby causing suppressiin of Jagged1 and/or Notch1 expression. Eventhough I made several attempts to generate siRNA constructs against both Jagged1 and Notch, however, after screening several constructs, and selecting clones, I failed to procure an siRNA construct that would stably shut down either Jagged1 or Notch1.

C) Assess Notch-Ras cooperation in breast cancers in vivo:
Since the major observation in this project has been the cooperation of Notch and Ras in HMLE cells in transformation, I went ahead to see if this cooperation indeed occurs in vivo in naturally arising breast cancers. To do so, I undertook immunohistochemical analysis of the Notch and Ras pathway in breast cancer tissues. An antibody against the activated, cleaved version of Notch (which will not recognize the full length Notch), and a downstream effector in the Notch pathway, Hes5, was used to detect Notch pathway activation. Similarly, a phospo-ERK1/2 antibody was used to detect activation of the MAPK pathway, as a measure of Ras activation. I find that in normal tissue cleaved Notch1, Hes5, and phospho ERK are not detected, indiciating that these pathways are not active. However, in tumor tissue, same areas expressed high levels of activated Notch, Hes5, and phospho-ERK (Fig 3), thus providing further support for Notch and Ras pathway cooperation in breast carcinogenesis.

Key Research Accomplishments:

1) The cooperation between Notch and Ras in HMLER cells confers protection under anchorage-independent growth conditions.
2) The morphological changes seen in reveral of EMT by blocking Notch signaling is corroborated at the molecular level.
3) The in vitro cooperation between Notch and Ras signaling in HMLE cells in mimicked in breast tumor in vivo.

Reportable outcomes

Experience Gained: Attended a Keystone symposia on Stem Cells, Senescence, and Cancer in Singapore

Conclusion: The results obtained thus far suggest that Notch-Ras interaction may play a critical role in breast cancer development. Inhibition of Notch signaling impairs EMT which plays a critical role in tumor metastasis. The in vitro cooperation between Notch and Ras in HMLE cells is mimicked in naturally arising breast cancers in vivo. Further dissection of the Notch-Ras interaction is likely to identify targets against which new chemotherapeutic drugs can be directed.

References

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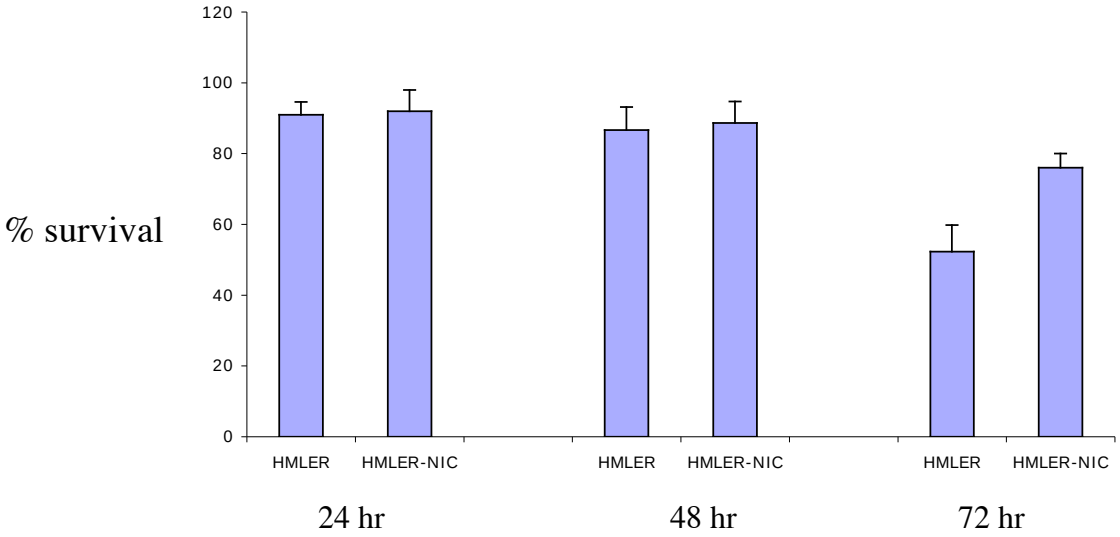
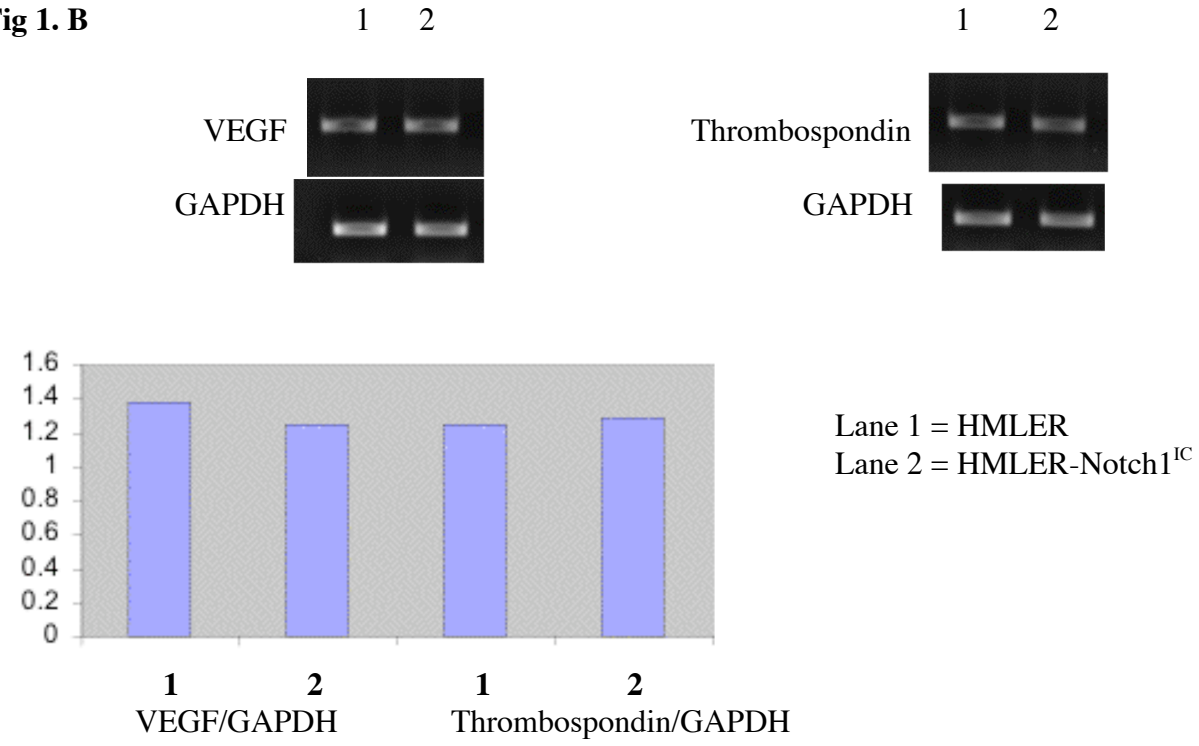
Fig 1. A



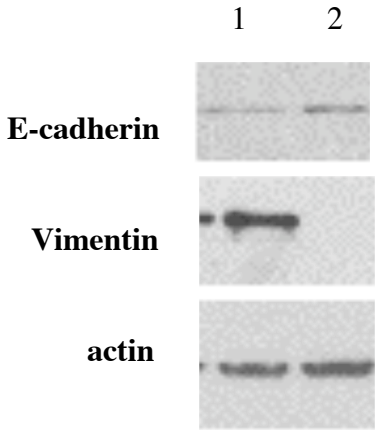
Effect of Notch-IC in anchorage independent growth of HMLER cells. Percentage apoptotic nuclei were counted after 24, 48 and 72 hrs of culturing in methylcellulose coated plates by Hoecsht staining and represented as percentage survival.

Fig 1. B



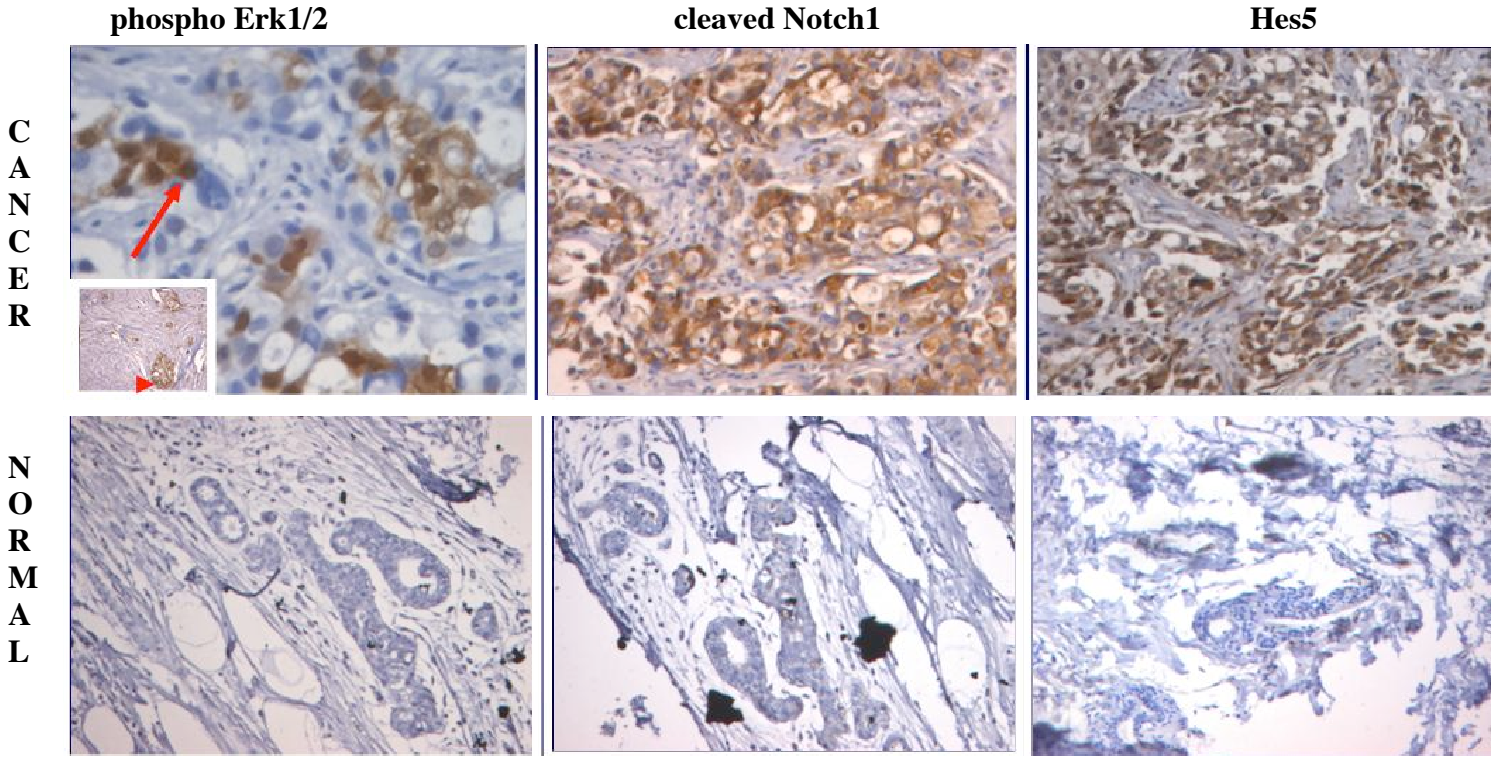
RT-PCR analysis for VEGF and Thrombospondin transcript levels.

Fig 2



Western blot analysis for EMT markers vimentin and E-cadherin.
Lane 1 is Sum1315 DMSO treated cells, and lane 2 is SUM1315 treated with DAPT.

Fig 3



Immunohistochemical analysis of normal and cancer breast tissue. Figure shows staining for phospho-Erk1/2, cleaved form of Notch1, and Hes5 in normal and cancer breast tissue.