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Award Number: W81XWH-04-1-0209

TITLE: Notch Signaling and Schwann Cell Tranformation: Development of a Model System and Application to Human MPNSTs

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REPORT DATE: March 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2005	Annual (1 Mar 2004 - 18 Feb 2005)

4. TITLE AND SUBTITLE
Notch Signaling and Schwann Cell Tranformation: Development of a Model System and Application to Human MPNSTs

5. FUNDING NUMBERS
W81XWH-04-1-0209

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
This is an annual report that presents data obtained during the grant's initial year of funding. The grant addresses the potential role of Notch signaling in the malignant transformation of neurofibromas to MPNSTs in patients with NF1. Our previous work has shown that constitutive expression of Notch can transform rat Schwann cells and that at least on MPNST-derived human Schwann cell line (of three examined) signals via Notch. This report includes novel results pertaining to two Tasks of the Statement of Work, including our observations that 1) Notch transformation of rat Schwann cells is accompanied by a loss of contact inhibition, 2) Notch signaling transiently down-regulates CyclinD1 expression in rat Schwann cells although full transformation is associated with abundant levels of CyclinD1, and 3) human MPNST cell lines down-regulate CyclinD1 in response to Notch, suggesting that these cells are not as fully transformed as NICDtransduced rat Schwann cells. The significance of these data is discussed in the context of additional Tasks described by the Statement of Work.

14. SUBJECT TERMS
NF1, neurofibroma, MPNST, Notch, Schwann, transformation

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The goals of the project are to gain insights into the mechanism by which Notch (in the form of its constitutive form, NICD) transforms rat Schwann cells, and to establish the relationship, if any, between Notch signaling and human MPNSTs.

Notch comprises a family of transmembrane receptors whose interaction with ligand leads to proteolytic cleavages that liberate the Notch intracellular domain, NICD, from the plasma membrane. NICD then enters the nucleus where it activates transcription. Notch's role in several cancers is well established, most notably in T-ALL where a rare chromosomal translocation interrupts the Notch1 gene, resulting in the constitutive expression of NICD. Recent work has shown that nearly 50% of T-ALLs carry more subtle mutations in the Notch1 gene(*1*). We have shown that forced expression of NICD can transform rat Schwann cells and that one of our three MPNST cell lines expresses detectable NICD(2). We therefore proposed that Notch signaling may contribute to the malignant transformation of a subset of neurofibromas in NF1 patients.

Note: During the past year there have been no published studies concerning the mechanism of Schwann cell transformation nor other studies that would influence the Statement of Work.

BODY

Results generated over the past year have addressed Tasks 1a and 2c of the Statement of Work.

Task 1. Determine the cause of NICD expression in MPNST cells.

a. Determine genomic organization of the Notch genes and expression of Notch ligands in sNF96.2 cells.

We reasoned that the expression of NICD in sNF96.2 cells may be due either to chromosomal rearrangements involving any one of the four the Notch genes (leading to ligand-independent activation) or to over-expression of any one of the five different Notch ligands. Data thus have far ruled out rearrangements of the Notch1 gene (Southern blots; data not shown) and most likely the other Notch genes as well. Although tentative, the latter conclusion stems from a) the relatively low level of NICD expression in sNF96.2 cells (compared to a T cell line carrying a translocation of the Notch1 gene; data not shown) and b) the dramatic increase in NICD that results when sNF96.2 cells are depleted of calcium (data not shown). Removing calcium activates endogenous Notch genes by shedding their extracellular domains and thus the observation indicates that the endogenous Notch proteins in sNF96.2 cells are not constitutively active. As for ligands, we have determined thus far that Jagged1 is not highly expressed in sNF96.2 cells (data not shown). Expression of the other four ligands has yet to be evaluated.

Conclusions. We are relatively confident that the NICD expression in sNF96.2 MPNST cells is not due to a grossly rearranged Notch gene. However, recent work from

Jon Aster and Thomas Look has identified subtle Notch mutations that can activate signaling and play a participatory role in hematopoietic malignancies. Such mutations have not yet been looked for in sNF96.2 cells. We have yet to fully evaluate the potential role of ligand-induced signaling.

Task 2. Identify the pathways and proteins that collaborate with Notch to induce transformation of rat Schwann cells.

c. Determine effect of Notch ligands on transformation-associated phenotypes.

Growth properties of NICD-transduced rat Schwann cells. One of the questions we addressed concerns *why* NICD-transduced Schwann cells are transformed. We considered two possibilities: 1) they grow faster and/or 2) they do not undergo contact inhibition. Growth curves comparing parental and NICD-transduced cells (Figure 1) showed conclusively that NICD is not mitogenic (indeed, NICD-transduced cells grow somewhat slower), but does allow cells to grow well past confluence, to densities much higher than those observed with parental cells. As with the parental cells, NICD-transduced cells remain forskolin dependent (data not shown), arguing further that NICD does not act as a mitogen.

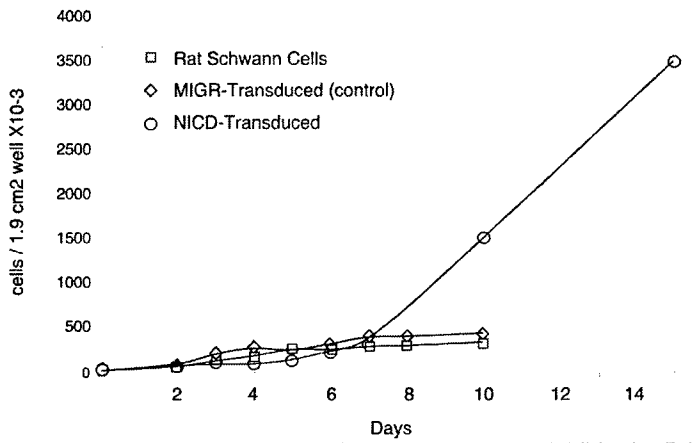

Figure 1. NICD-transduced rat Schwann cells are not contact inhibited. Primary rat Schwann cells, MIGR (control vector) transduced and NICD transduced Schwann cells were plated at day zero and cell counts were determined at the indicated times.

The effect of Notch ligands on rat Schwann cells: the CyclinD1 paradox. It is generally believed that forced expression of NICD leads to much higher levels of Notch signaling than does ligand-mediated activation of endogenous Notch receptors. It was therefore of interest to determine if Notch ligands were able to mimic any of the effects of NICD. We have established techniques in the lab to activate endogenous Notch receptors through ligands (Jagged1 and Delta4) immobilized on tissue culture plates(*3*). When rat Schwann cells were grown on such plates, they did NOT become morphologically transformed (by appearance), nor did they escape contact inhibition (data not shown). This suggests that the ability of NICD to transform rat Schwann cells a consequence of high level signaling.

We have reported that NICD-transduced Schwann cells express abundant CyclinD1(*2*). By contrast, we have observed that Notch signaling leads to the *down*-regulation of CyclinD1 in many other cell types, including epithelial cells, fibroblasts and myoblasts (data not shown). Since these latter cell types are not transformed by NICD, *we hypothesized that the response of CyclinD1 to Notch may be symptomatic of a particular cell type's ability to be transformed, those cells that down-regulate CyclinD1 being refractory.* Accordingly, we examined the response of CyclinD1 to ligand-induced Notch signaling in rat Schwann cells. As shown below (Figure 2), rat Schwann cells grown on control plates (Fc) expressed a readily detectable level of CyclinD1 while those grown in the presence of either Delta4 (D) or Jagged1 (J) expressed much less protein. By contrast, rat Schwann cells transduced with NICD expressed an elevated level of CyclinD1 that was not affected by either Delta4 or Jagged1. These data contradict our hypothesis and show that while the inhibitory effect of Notch signaling on CyclinD1 is not symptomatic of a cell type's resistance to transformation by NICD, they do show that once transformed by NICD, CyclinD1 expression is refractory.

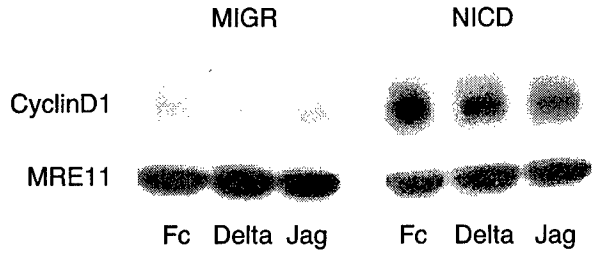

Figure 2. Notch signaling down-regulates CyclinD1 in Rat Schwann cells. MIGR (control) or NICD transduced rat Schwann cells were grown on control plates (Fc) or plates coated with either Delta4 (Delta) or Jagged1 (Jag) and harvested after 72 hours. Levels of endogenous CyclinD1 and MRE11 (control) were assessed by Western blot.

Our experiments have shown that the negative effect of Notch signaling on CyclinD1 is post-transcriptional; that is, Notch does not significantly affect the level of CyclinD1 mRNA (data not shown). The stability of CyclinD1 protein is affected by at least two pathways. One involves GSK3-mediated protein destabilization through phosphorylation of Thr286(*4*). The other involves an APC-related destabilization through a Destruction Box in CyclinD1's N-terminus(*5*). To better understand the mechanism by which Notch signaling lowers the level of CyclinD1 protein we introduced into NIH 3T3 cells retroviruses that express various Flag-tagged CyclinD1s, either wild type or mutant. The L32A mutant lacks the Destruction Box while the T286A mutant destroys the primary GSK3 phosphorylation site. As shown below (Figure 3), only the T286A mutant was refractory to the effect of Notch signaling. Although this result implicates GSK3 in mediating the effect of Notch on CyclinD1 expression, we have not observed a difference in either GSK3 levels or GSK3 phosphorylation (phospho-GSK3 has reduced activity) in cells exposed to Notch ligands (data not shown).

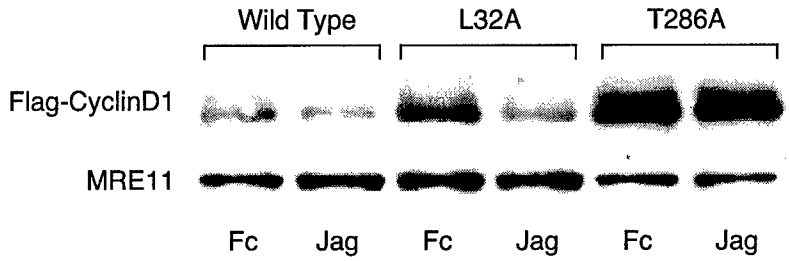

Figure 3. The CyclinD1 T286A mutant is resistant to the inhibitory effects of Notch signaling. Retroviruses expressing Flag-tagged Wild Type, L32A or T286A CyclinD1 were introduced into NIH 3T3 cells and then the cells were grown on plates coated with Fc (control) or Jagged1 (Jag) for 24 hours. CyclinD1 levels were assessed by Western blot using an anti-Flag antibody and an MRE1 antibody as control.

We next examined the response of CyclinD1 to Notch signaling in our three MPNST cell lines. We reasoned that if Notch signaling is largely responsible for transformation, particularly in the sNF96.2 cells which express NICD(*2*), then CyclinD1 in those cells should not be responsive to increased Notch signaling, similar to what we observed for NICD-transformed rat Schwann cells (Figure 2). As shown below (Figure 4), all three MPNST cell lines in addition to our NF cell line down-regulated CyclinD1 when grown on Notch ligand for either 48 hours (upper panel) or 72 hours (lower panel).

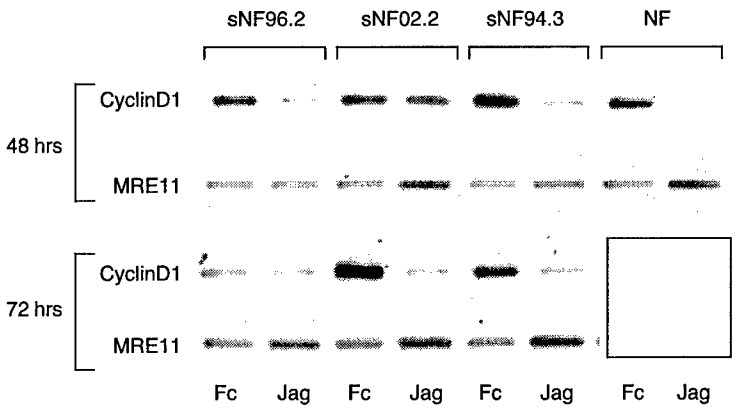

Figure 4. MPNST cells down-regulate cyclinD1 in response to Notch signaling. MPNST cell lines (sNF96.2, sNF02.2, sNF94.3) and an NF cell lines were grown on control plates (Fc) or plates coated with Jagged1 (Jag) for 48 or 72 hours as indicated. Endogenous CyclinD1 was assessed by Western using an anti-MRE11 as loading control. (Note that the NF cell line was not evaluated at 72 hours.)

Conclusions. If the resistance of CyclinD1 to Notch signaling is a hallmark of Notch-mediated transformation, then we conclude that none of our MPNST cell lines, including sNF96.2, is transformed as a consequence of Notch signaling alone. Furthermore, since NICD-mediated transformation of rat Schwann cells is due to a loss of contact inhibition, our future experiments with MPNST cell lines will have to address specifically the role of Notch, if any, in reducing contact inhibition (i.e. their growth in soft agar).

KEY RESEARCH ACCOMPLISHMENTS

1. Transformation of rat Schwann cells is due to a loss of contact inhibition.
2. Notch signaling down-regulates CyclinD1 expression in most cell types, including rat Schwann cells. Transformation, however, is accompanied by abundant CyclinD1 expression, suggesting a requirement for additional events in transformation.
3. Human MPNSTs, including those that express NICD, display reductions in CyclinD1 as a consequence of increased Notch signaling. This suggests that these cells are not as fully transformed as NICD-transduced rat Schwann cells.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Perhaps the most important conclusion derived from our work thus far is that human MPNST cells lines are not as fully transformed as NICD-transduced rat Schwann cells. Mouse modeling has shown that although Schwann cells are the primary targets of transformation, the micro-environment is also important, as genetic lesions in the Schwann cells alone is not sufficient to generate tumors. Accordingly, absolute proof for a role for Notch signaling in the development of MPNSTs may require mouse modeling. Although not addressed in the Statement of Work, such experiments are not planned at this time.

During the coming year we will continue to study NICD-mediated transformation in rat Schwann cells, focusing on Task 2 and Task 4 (Identify primary Notch target genes in rat Schwann cells). We have obtained a NICD-ER fusion construct from Dr. Tony Capobianco (Wistar Institute, Philadelphia) that may allow us to ascertain direct Notch targets (using micro-arrays) in the absence of new protein synthesis. Such targets, as indicated in the initial Statement of Work, may aid in the eventual diagnosis of human MPNSTs.

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