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Development of a Model System and Application to Human MPNSTs

PRINCIPAL INVESTIGATOR: Tom R. Kadesch, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-6270

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6. AUTHOR(S)
Tom R. Kadesch, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-6270

E-Mail: kadesch@mail.med.upenn.edu

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13. ABSTRACT (Maximum 200 Words)

This is an annual report that presents data obtained during the grant's second 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 one Task of the Statement of Work, including our observations that 1) Notch transformation of rat Schwann cells is accompanied alterations in the cell cycle profile, consistent with an up-regulation in CyclinD1 expression, 2) CyclinD1 expression is upregulated, in part, at the level of transcription, 3) Notch-mediated transformation requires signaling through PI3 Kinase and PKA, but not Jak-Stat, and 4) the process of Notch-mediated transformation is qualitatively distinct from that initiated by Notch ligands. The significance of these data is discussed in the context of additional Tasks described by the Statement of Work.

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INTRODUCTION

The goals of the project are to gain insights into the mechanism by which Notch (in the form of the intracellular domain, NICD) transforms rat Schwann cells, and to establish the relationship, if any, between Notch signaling and human malignant peripheral nerve sheath tumors, 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 (T-cell acute lymphocytic leukemia) 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 that would influence the Statement of Work.

BODY

Results generated over the past year have addressed Tasks 2 of the Statement of Work.

Task 2. Identify the pathways and proteins that collaborate with Notch to induce transformation of rat Schwann cells (Months 6-18).

a. Examine involvement of Ras-MAPK, PI-3 Kinase, AKT, Jak/Stat and NF-□B.

b. Evaluate status of p16Ink4a, E2Fs, and Sox10.

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

Although Task 3, "Determine if Notch-mediated transformation is reversible," was initially to be addressed in months 6-36, this task was delayed and will be addressed in the coming year.

As stated in last year's annual report, Cyclin D1 levels were found to be abnormally high in NICD-transformed Schwann cells. This suggests that proteins that promote cell cycle progression may be directly responsible for the transformed phenotype. However, we also presented seemingly paradoxical evidence that growth rates in parental and NICD-transformed cells were

comparable and that the distinguishing feature of transformed cells was that they failed to undergo contact inhibition. We were forced to spend a fair amount of effort during the past year trying to resolve this paradox. One possible explanation was that the amount of Cyclin D1 in the NICD-transformed cells was not actually higher, but that it was abnormally low in the parental cells because those cells were undergoing contact inhibition when cell extracts were made. (When normal cells undergo contact inhibition, Cyclin D1 levels typically drop.) We carried out several experiments to rule in or rule out this possibility.

CyclinD1 levels as a function of cell density. Our first experiment was to confirm that CyclinD1 levels were higher in NICD-transformed cells irrespective of cell density. This would rule out artifacts due to the lack of contact inhibition in NICD-transformed cells. As shown in the figure below, CyclinD1 levels were, in fact, higher in NICD-transformed cells irrespective of cell density.

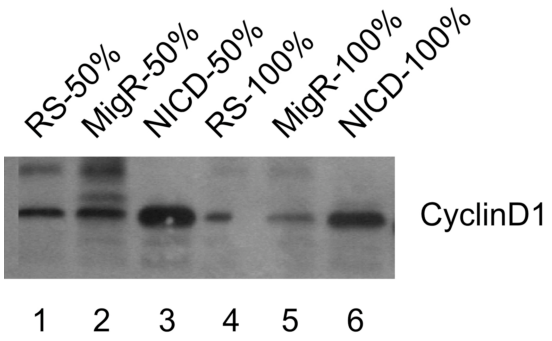

Figure 1. CyclinD1 expression is high in NICD-transformed Schwann cells. Primary rat Schwann cells (RS), and their MigR-transduced and NICD-transduced counterparts were grown to different densities (approximately 50% or 100% confluent as indicated) and CyclinD1 levels were assessed by Western immunoblot.

(Editorial note: Last year's annual report review suggested that Western blots, like that provided above, be presented as densitometric scans. First, this is not standard in the field. Second, only I^{125}-labeled secondary antibodies allow for true quantitation of such data. Consequently, we will continue to present the data in their qualitative form as above.)

Cell cycle analyses. Since Cyclin D1 levels are indeed higher in NICD-transformed cells, then these cells should exhibit an altered cell cycle profile, also independent of cell density. We harvested parental (MigR-transformed) and NICD-transformed cells at different densities (roughly 50% confluent and 100% confluent) and carried out cell cycle analyses using propidium iodide (PI) staining and fluorescence activated cell sorting (FACS). The accompanying table shows that NICD-transformed cells had a higher percentage of cells in S-phase at both low density and high density. This argues that the G1-S transition is faster in NICD-transformed cells, a result expected if CyclinD1 levels are higher.

Although we would generally expect cell populations with a higher percentage of cells in S-phase to be growing faster — in this case they do not — it appears from the above table that cells transformed with NICD are also more delayed in the G2-phase. Thus, these cells may actually not have an overall shorter cell cycle duration and faster rate of division.

	Percentage cells in:		
	G1	G2	S
MigR 50%	77	9	14
MigR 100%	81	8	11
NICD 50%	52	15	33
NICD 100%	69	11	20

Signaling pathways required for growth in soft agar. Our previous data indicated that NICD-transformed cells, but not parental Schwann cells, could grow in soft agar(*2*). These results were purely qualitative. We have now repeated these assays, using Ras-transformed NIH 3T3 cells as a control and have included inhibitors of various signaling pathways. It should be pointed out first that we have been able to repeat the soft-agar results (they were performed initially in the lab of our collaborator Dr. Gihan Tennekoon), but that the inherent efficiency is extremely low, being roughly 1 percent that obtained with Ras-transformed NIH 3T3 cells. With regards to the inhibitors, we saw no growth in the presence of Ly, an inhibitor of PI3 Kinase, and very few colonies in the presence of H7, an inhibitor of PKA. Small or no effects were seen in the presence Bis, an inhibitor of PKC, or PD18059, an inhibitor of ERK 1 and 2. We conclude that Notch is either activating or collaborating with the PKA and PI3 Kinase pathways to transform rat Schwann cells.

Effects of Notch ligands and NICD on CyclinD1 transcription. The CyclinD1 promoter has been proposed to be a direct target of NICD(*3*). Although this is not consistent with our data concerning the down-regulation of CyclinD1 by ligand-induced signaling (see 2005 report), we considered the possibility that *high* concentrations of NICD might be able to transcriptionally activate CyclinD1. Accordingly, we have used quantitative RT-PCR (Q-RT-PCR) to evaluate the effects both of Notch ligands and of NICD on CyclinD1 transcript levels. As shown in the figure below (far right panel), CyclinD1 levels were unchanged when rat Schwann cells were cultured on Fc-Jagged1 and induced slightly (~2.5-fold) in cells transformed with NICD. We conclude 1) that the effect of NICD on the CyclinD1 promoter is dose dependent and 2) that if the CyclinD1 promoter is a direct target of NICD, it is not particularly responsive. Note for comparison the magnitude of NICD-mediated induction of Hes5 or of Hey1.

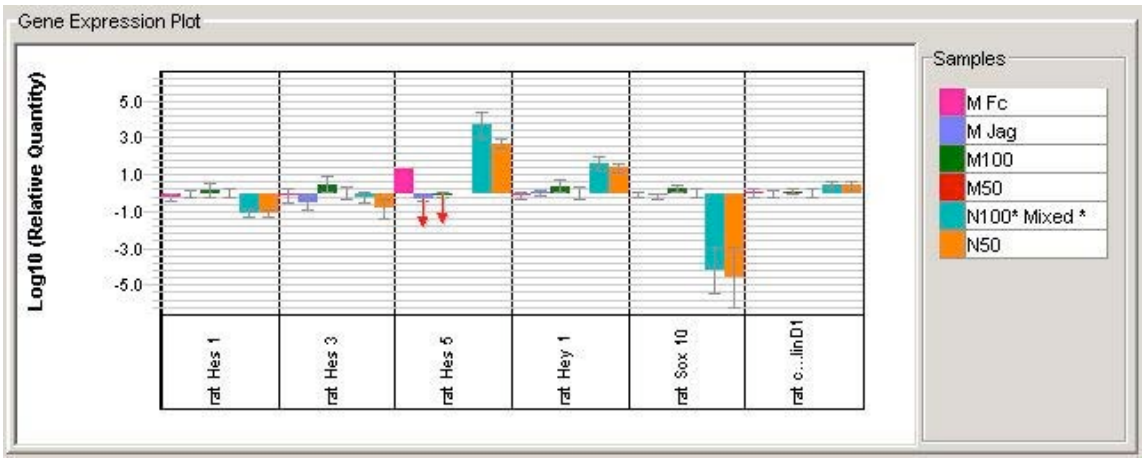

Figure 2. Effects of Notch signaling on the transcript levels of presumptive targets in Schwann cells. Cells were grown under the following conditions, from left to right in each panel: MigR-transduced cells grown a) on Fc-Control (M Fc), b) on Fc-Jagged1 (M Jag), c) to ~50% confluence and d) to ~100% confluence; NICD-transformed cells were grown e) to ~50% confluence and f) to ~100% confluence. RNA samples were analyzed by Q-RT-PCR for expression of the Notch targets Hes1, Hes3, Hes5, and Hey1 and for expression of Sox10. The data represent the averages of three separate experiments.

Growth of NICD-transformed cells in soft agar was completely dependent on signaling through PI3 Kinase (see above). PI3 Kinase is known to activate AKT, which is known to inhibit GSK3, which is known to destabilize Cyclin D1. (In effect, PI3K activity, through AKT, stabilizes CyclinD1 protein). Indeed, some recent reports have shown a correlation between Notch signaling and activation of AKT in T cells(*4*). Thus, the increase of CyclinD1 protein in NICD-transformed Schwann cells could be due to transcriptional effects as well as to effects on protein stability. Our next experiments to address the relationship between NICD and CyclinD1 will be to a) examine the status of AKT[1] and b) determine by ChIP if NICD is actually recruited to the CyclinD1 promoter. (This latter experiment actually falls into the category of Task 4, "Identify primary Notch target genes in rat Schwann cells.")

Expression of additional Notch target genes. The Q-RT-PCR results above also provide data concerning the transcript levels of other Notch target genes. Our initial experiments showed little change in Hes1 protein levels when Schwann cells were transformed with NICD(*2*) and this correlates with an slight decrease in Hes1 transcript levels (panel 1). Hes3 levels were relatively unchanged (panel 2), while levels of Hes5 transcripts were significantly induced by NICD (panel 3), as expected from higher levels of Hes5 proteins in these cells(*2*). Levels of Hey1 transcripts were also increased in the presence of NICD

[1] Preliminary results obtained the day this report was submitted show that the status of AKT is not influenced by NICD. However, these experiments need to be repeated.

(panel 4). Remarkably, the level of Sox10 transcripts were drastically reduced by NICD (panel 5; not that these data are plotted on a log scale). Given the importance of Sox10 in regulating neural crest development and gliogenesis, this result is very exciting and implicates a role for Notch, directly or indirectly through NICD, in regulating Sox10 transcription. Very little is currently known about the Sox10 promoter. It will be a priority for us to ask if we can recapitulate this profound reduction in Sox10 transcription with DNA comprising sequences 5' to the Sox10 coding sequence.

Investigating a possible role for Stat3 in mediating the effects of NICD. It has been shown recently that Notch signaling promotes Jak-Stat signaling in neuronal cells(*5*). This effect is through the direct binding of either Hes1 or Hes5 to both Jak2 and the DNA binding protein Stat3. Presumably, the Hes proteins facilitate the phoshorylation and activation of Stat3 by Jak2. Given that constitutively active Stat3 can be oncogenic(*6*), and that Hes5 is highly induced in NICD-transformed Schwann cells(*2*), we felt that it was imperative to investigate a possible connection between NICD and Stat3 in Schwann cells. However, as shown in the representative experiment below, Notch signaling had no such effect in Schwann cells. First, it is interesting to note that rat Schwann cells have relatively high levels of phosphorylated Stat3 to begin with. Second, NICD-transformed cells did not contain higher levels of phosphorylated Stat3 (in fact, they consistently contained slightly lower levels; compare lane 3 to lane 1). Third, growth of cells on the Notch ligand Delta4 did not influence the total amount of phosphorylated Stat3 (compare lane 2 to lane 1). We conclude that if Hes5 does facilitate an interaction between Jak2 and Stat3 in Schwann cells, it is not sufficient to increase the already-high levels of phosphorylated Stat3 and, thus, does not contribute to the transformed phenotype.

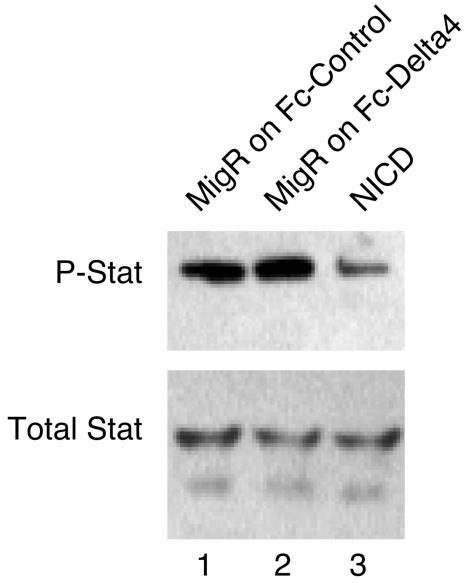

Figure 3. Notch signaling does not influence the level of phosphorylated Stat proteins in rat Schwann cells. Schwann cells were transduced with either a parental retrovirus (MigR) or an NICD-expressing virus. MigR containing cells were cultured on either a control Fc-fusion protein or an Fc-Delta4 fusion protein to initiate Notch signaling. Extracts were made and assessed for expression of total Stat protein or phosphorylated Stat (P-Stat) by Western immunoblot.

KEY RESEARCH ACCOMPLISHMENTS

1. NICD-transformed Schwann cells show an inherent increase in CyclinD1 levels and this correlates with an altered cell cycle profile.
2. Growth of NICD-transformed Schwann cells in soft agar requires signaling pathways emanating from PI3 Kinase and PKA.
3. Transcription of CyclinD1 is not affected by ligand-induced Notch signaling in normal Schwann cells and is increased roughly 2.5-fold in NICD-transformed Schwann cells.
4. The level of Sox10 transcripts is dramatically reduced (>1,000-fold) in NICD-transformed Schwann cells, suggesting a novel and important mode of Sox10 transcriptional regulation.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Our data have refined the relationship between CyclinD1, the cell cycle and the growth rate of NICD-transformed Schwann cells. Solving the apparent paradox presented last year, we have now shown that increased CyclinD1 expression does, indeed, correlate with an increased S-phase population, but that an extended G2 phase may slow the cells growth relative to what we had expected. Our data are also consistent with the increase in CyclinD1 expression being due, in part, to transcriptional effects, possibly mediated by NICD directly.

Our data also show that normal levels of Notch signaling *are not sufficient* to mediate *any* of the observed effects of NICD. Thus, while Notch signaling may promote gliogenesis during development, transformation of Schwann cells appears largely unrelated and, in fact, correlates with de-differentiation.

Relationship between Notch and signaling through PI3 Kinase and PKA appear to be important for transformation, but such a relationship between Notch

and Stat transcription factors, despite supporting evidence in neurons, does not appear to exist in glial cells.

Task 2 is therefore mostly complete. We have only to look at the states of the transcription factors NF-kB and E2F (although E2F proteins should reflect cell cycle status), along with the state of $p16^{Ink4a}$.

In the upcoming year we will begin to explore the possibility that NICD controls CyclinD1 transcription directly, along with the relationship between Hes5 induction and down-regulation of Sox10 (components of Task 4). We will also begin our experiments that address the reversibility of NICD-mediated transformation (Task 3). Although the original Statement of Work indicated that Task 1b, "Examine additional MPNST-derived material," would be carried out throughout the funding period, we will re-assess the importance of this Task as we learn more about NICD transformed cells and how they compare with the three MPNST lines we currently have. As indicated in the last annual report, we concluded that such cells are not as highly transformed as NICD-expressing cells since they down-regulated CyclinD1 when grown in the presence of Notch ligands.

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