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14. ABSTRACT:
This is an annual report that presents data obtained during the grant's third 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 1) that the Notch targets Hes5 and c-Myc alone are unable to mimic the constitutive form of Notch, NICD, to effect transformation and 2) that NICD is NOT sufficient to transform primary Schwann cells. This latter observation is in stark disagreement with our earlier results. Accordingly, we have added a new Task that will address this discrepancy and elucidate the specific pathways that NICD alters in Schwann cells.

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NF1, neurofibroma, MPNST, Notch, Schwann, transformation

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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. *However, as explained below, the Statement of Work has been significantly altered due to unanticipated results obtained while addressing Task 3.*

BODY

Results generated over the past year were intended initially to address Task 4 and Task 3 of the Statement of Work.

Task 4. Identify primary Notch target genes in rat Schwann cells (Months 24-48).

a. Determine the role of Hes-5 in transformation.
b. Generate, clone and analyze Notch targets using DamID fusion proteins.
c. Compare gene expression in transformed rat Schwann cells and MPNSTs.

A molecular signature of Schwann cell transformation. As indicated in the last annual report, we had begun to use quantitative RT-PCR (Q-RT-PCR) to assess gene expression changes in NICD-transformed Schwann cells. The most notable change involved the dramatic down-regulation of Sox10 mRNA. As shown in Figure 1, we also observed the up-regulation of CyclinD1 and the Notch target genes Hes5, Hey1 and c-Myc. (c-Myc has been identified as a direct target of NICD in T cells, we do not see this in all cell types.)

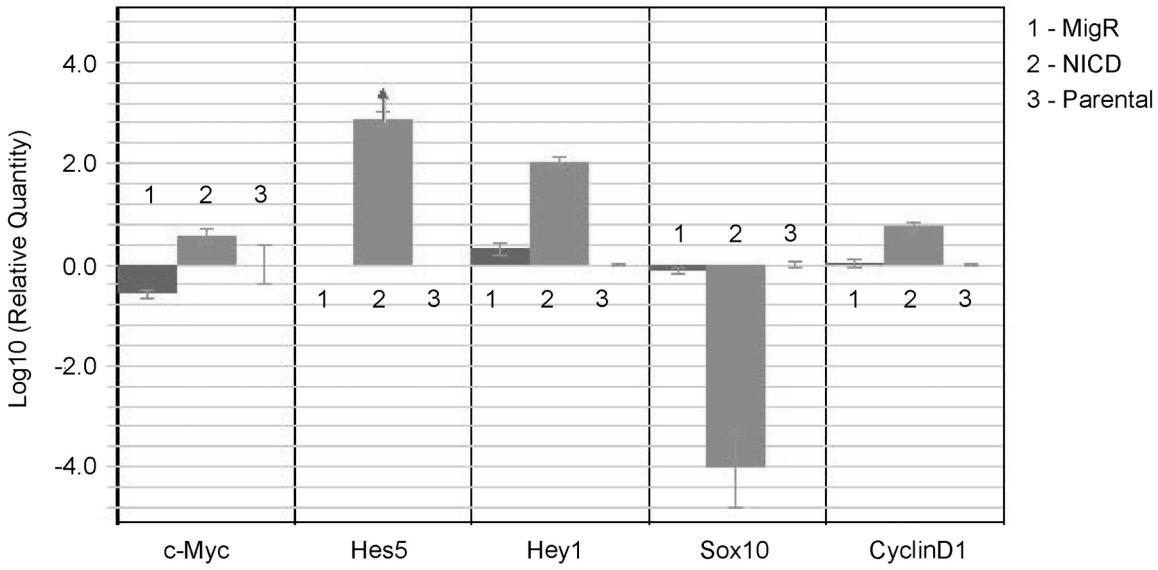

Figure 1. NICD-mediated transformation is accompanied by increased expression of Hes5, Hey1, c-Myc and Cyclin D1 and dramatic inhibition of Sox10 expression. Untransduced cells (3 - Parental), empty virus-tranduced (1 - MigR) or NICD-transduced cells (2 - NICD) were evaluated for the expression of the indicated genes using quantitative RT-PCR (Q-RT-PCR).

Constitutive expression of Hes5 or c-Myc is not sufficient for Schwann cell transformation. We sought to determine if either Hes5 or c-Myc was sufficient to induce a transformed phenotype in Schwann cells. Accordingly, we obtained retrovirues that constitutively express either Hes5 or c-Myc and used them to transform primary rat Schwann cells. As shown in Figure 2, constitutive expression of Hes5 did not affect expression of Sox10, Cyclin D1 or c-Myc and so we conclude that Hes5 is unable to mimic the effect of NICD. While constitutive expression of c-Myc was able to enhance the expression of Cyclin D1, it did not down-regulate Sox10 (it actually up regulated Sox10) and it did not result in the loss of contact inhibition (data not shown). We conclude that constitutive expression of c-Myc is also unable to mimic the effects of NICD.

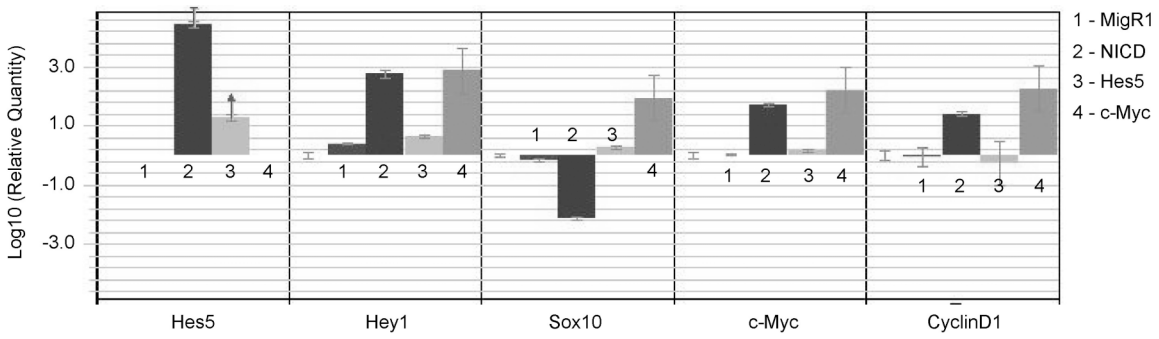

Figure 2. Constitutive expression of Hes5 or c-Myc does not lead to Schwann cell transformation. Schwann cells transduced empty virus (1 – MigR1) or virus expressing NICD (2 – NICD), Hes5 (3 – Hes5) or c-Myc (4 – c-Myc) were evaluated for expression of the indicated genes using Q-RT-PCR.

Task 3. Determine if Notch-mediated transformation is reversible (Months 6-36).

a. Generate and analyze reversibly inducible forms of Notch.
b. Examine effects of Notch inhibitors (γ-Secretase inhibitors, DN-Mastermind).
c. Establish effects of Notch inhibitors on NICD-expressing MPNST cells.

Normal Notch signaling requires three proteolytic events that occur in order within the Notch protein to generate NICD, the active form of the receptor. The first occurs independently of ligand while the second is ligand dependent. The third cleavage is constitutive, but depends on the second having occurred beforehand. The third cleavage is carried out by a multiprotein complex referred to as g-Secretase. g-Secretase inhibitors (GSIs) therefore inhibit signaling by preventing the generation of NICD. An artificial form of Notch that mimics the receptor after the first two cleavages is active since it can be acted on by g-Secretase to generate NICD. This form of Notch, which we refer to as NDE, is inactive in the presence of GSI.

Our approach to determine if Notch signaling is reversible employed the use of NDE. We anticipated first transforming Schwann cells with a retrovirus that expresses NDE and then treating the cells with GSI to inactivate NDE. The transformed phenotype would then be assessed over time.

However, to our surprise (and dismay) NDE did not transform Schwann cells. As shown in Figure 3, while both NICD and NDE induced expression of Hes5 and Hey1, only NICD-transduced cells displayed elevated levels of c-Myc and Cyclin D1. NDE expression did not repress expression of Sox10 (data not shown). To ensure that this was not due simply to low expression from the retrovirus, we flow sorted NDE cells into high expressers (Hi) and low expressers (Lo) based on GFP levels. The high expressers also did not show evidence of transformation (Fig. 3).

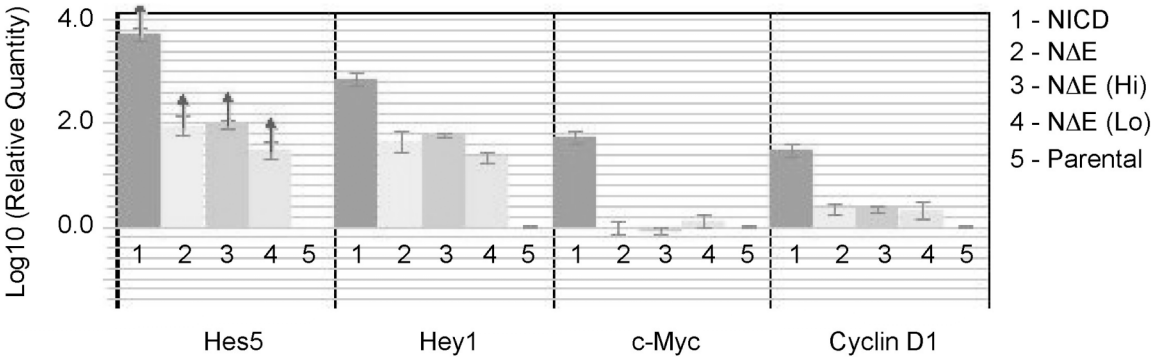

Figure 3. NDE does not transform Schwann cells. Cells expressing NDE (or sorted into high (Hi) or low (Lo) expressors were evaluated for expression of the indicated genes by Q-RT-PCR and compared to parental Schwann cells and NICD-transformed Schwann cells.

NICD expression is NOT sufficient to transform rat Schwann cells. The results of Figure 3 forced us to re-examine our original observation that NICD is sufficient to transform Schwann cells. It should be stressed that our early experiences were very consistent and were reproduced many times: each time we transduced Schwann cells with a retrovirus expressing NICD we obtained transformed cells at high frequency. However, this has NOT been the case over the past year. Using exactly the same retrovirus, we do not consistently observe transformed cells. The question is why?

It is known that simply growing primary cells in serum can lead to chromosomal rearrangements and transformation, particularly when the cells are "primed" with a known oncogene(*3*). Accordingly, we considered two possibilities. First, we explored the idea that the Schwann cells become transformed only after harboring NICD and grown in serum for extended periods of time. Second, we asked if the age of the cells at the time of transduction plays a role, with older cells being more susceptible. Our original experiments were carried out with cells that had been passed 30 times (which is fairly old). Our experiments did not support either of these ideas. As shown in Figure 4, using cells that had been transduced and immediately frozen three years ago, evidence of transformation (Sox10 down regulation) was apparent as soon as there was sufficient material to evaluate (passage 5). As shown in Figure 5, cells passed 30 times were not susceptible to transformation by NICD, unless the transformation had been carried out in 2004.

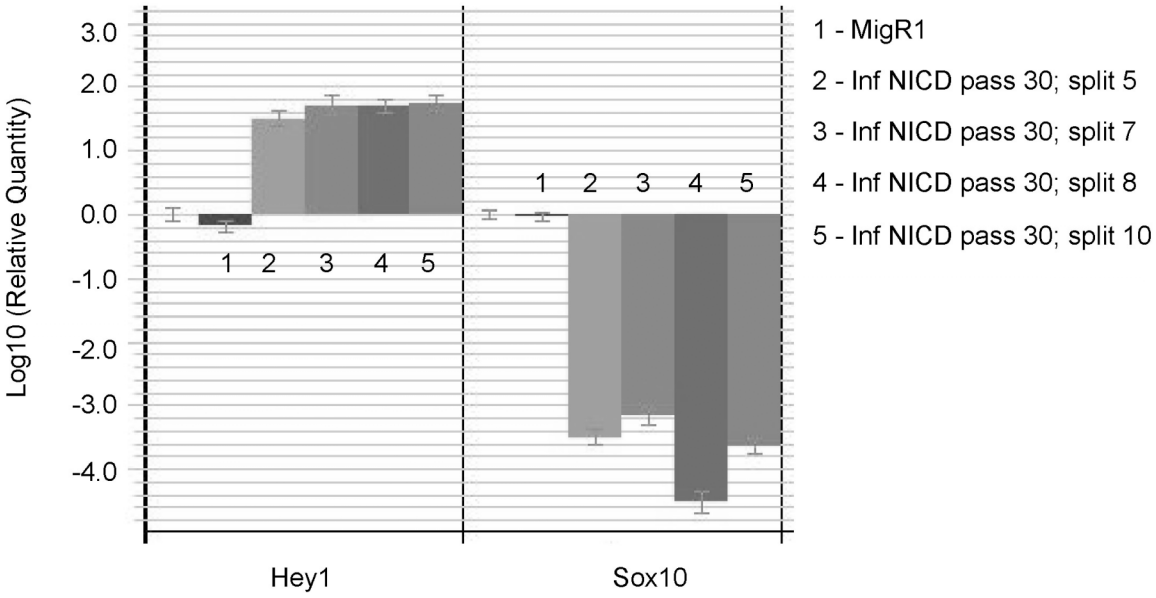

Figure 4. Cells transduced with NICD in 2004 show early evidence of transformation. Passage 30 Schwann cells transduced with NICD and immediately frozen in 2004 were thawed and passed (split) the indicated number of times and assessed for Hey1 expression and Sox 10 expression as indicated by Q-RT-PC.

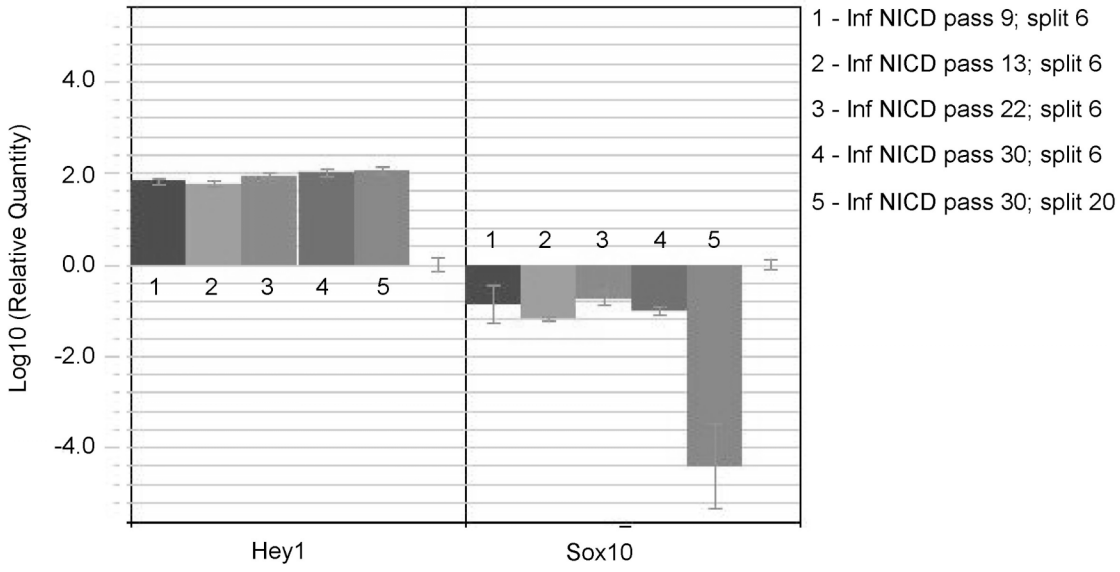

Figure 5. Old age does not guarantee transformation by NICD. Primary Schwann cells were passed for various times prior to transduction by an NICD-expressing virus. Populations 1 (passage 9), 2 (passage 13), 3

(passage 22) and 4 (passage 30) were transduced in 2006 and analyzed after 6 splits, while population 5 (passage 30) was transduced in 2004 and analyzed after 20 splits.

We conclude that under the conditions used in 2003-2004, NICD was capable of transforming Schwann cells, but those conditions do not exist today and remain elusive. Our current theory is that a particular batch of serum used in 2003-2004, which is no longer available, cooperated with NICD to transform the cells. As mentioned above, there is precedent for this possibility(*3*).

New Task: Determine which class of oncogenes cooperates with NICD to transform Schwann cells.

Dr. Allison Lloyd and colleagues have described a general model for the transformation of rat Schwann cells(*4*). Experiments employing activated Ras and various versions of SV40 Large T antigen showed that mitogen-independent growth can be distinguished from mitogen-independent *plus* anchorage-independent growth. Both are required for Schwann cells to be tumorigenic: the former required both activation of Ras and inactivation of p53, while the latter required, in addition, the inactivation of Rb family members, most likely p107 and/or p130. Interestingly, the ability of T antigen to repress Rb family proteins could be phenocopied by mutations in the cyclin-dependent kinase inhibitor $p16^{Ink4a}$. This suggests that $p16^{Ink4a}$ mutations, while broadly stimulating CDK4 and CDK6, affect primarily the activities of p107 and p130, and not Rb. The reason for this is unknown.

We will test the hypothesis that NICD requires additional events to transform Schwann cells and that these will reflect one or a combination of those events described by Lloyd. Accordingly, we will generate Schwann cells that have been transduced with NICD plus either a) activated Ras, b) SV40 Large T antigen or c) a dominant-negative p53. We anticipate that Ras plus T antigen will be sufficient to transform Schwann cells in the absence of NICD(*5*). Thus, we expect NICD to fulfill the role of either activated Ras or T antigen. Given that activate Ras is sufficient to cause Schwann cells to dedifferentiate(*6*), it is more likely that NICD will be able to substitute for SV40 T antigen.

KEY RESEARCH ACCOMPLISHMENTS

1. Neither Hes5 nor c-Myc, each a target of Notch, is able to mimic the ability of NICD to transform Schwann cells.

2. NICD expression is NOT sufficient to transform Schwann cells as previously thought.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

While our data describing NICD-transformed Schwann cells remain valid (see earlier reports), we can no longer conclude that NICD is sufficient for oncogenic transformation. It is now an *overriding priority* to determine the type of additional event that occurs which cooperated with NICD in our earlier experiments. Given what is already known about Schwann cell transformation (requiring activated Ras, inactivated p53 and inactivated Rb family members), we should now be able to fit NICD into one or more of these pathways. This will be critical for our understanding of NICD-mediated transformation. Although our inability to transform Schwann cells with NICD alone was viewed initially as a step in the wrong direction, the new Task should allow us to gain better insight into the precise mechanism of NICD action.

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