

AD _____

Award Number: DAMD17-99-1-9491

TITLE: Establish an in vitro Model for the Study of NF2 Gene
Function and Gene Therapy

PRINCIPAL INVESTIGATOR: Gene Hung, M.D.

CONTRACTING ORGANIZATION: House Ear Institute
Los Angeles, California 90057-9927

REPORT DATE: October 2000

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010928 059

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 99 - 30 Sep 00)	
4. TITLE AND SUBTITLE Establish an in vitro Model for the Study of NF2 Gene Function and Gene Therapy			5. FUNDING NUMBERS DAMD17-99-1-9491	
6. AUTHOR(S) Gene Hung, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) House Ear Institute Los Angeles, California 90057-9927 E-MAIL: Ghung@hei.org			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 This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Mutation analyses of the NF2 gene from NF2 patients suggest that the inactivation of the NF2 gene, and the consequent lack of gene product (protein known as Merlin/Schwannomin) is the primary cause of this disease. Within the three years period, we plan to develop a laboratory protocol to establish Schwann cell culture using surgical specimens from NF2 patients, and compare the tumor cells from patients with different NF2 gene mutations. In addition, we want to extend the life span of primary culture cells by immortalizing them using a retrovirus which we engineered. This will not only ensure the reproducibility of results within the NF2 community, but also provide scientists greater access to certain materials for the study of biologic function of Schwann cell and also important for testing therapeutic approaches. During the first 12 months, an additional 11 primary cultures were added to the 55 previously banked NF2 tumor cultures. Two primary human vestibular schwannoma cultures from different NF2 patients and one normal Schwann cell culture are in the process of immortalization.				
14. SUBJECT TERMS Neurofibromatosis 2 (NF2), vestibular schwannomas, primary culture, growth rate, cell line			15. NUMBER OF PAGES 12	16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Front Cover.....	1
Standard Form 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	12

Introduction

In the seven years since the NF2 gene was identified, NF2 research has been divided into four main areas: 1) Natural history; 2) NF2 gene function; 3) *In vitro* and *in vivo* models; and 4) Therapeutic intervention. Although there have been some important discoveries, we are still unable to answer key questions about what factors predict the tumor growth rate in patients, the pathogenesis of NF2, whether the current NF2 mouse knock-out model can be used to represent human NF2, and whether gene therapy is the future therapy for NF2. Most researchers feel that the lack of an *in vitro* model system has limited their research progress and development of such a model should be a high priority. The purpose of this study is to develop a NF2 gene deficient *in vitro* model that can be used to further understand NF2 gene function and to facilitate development of new treatments. In this research study, we propose to develop a primary and permanent human Schwannoma cell culture system, and with this *in vitro* model, to test the **hypothesis** that different NF2 gene mutations result in different degrees of loss in NF2 gene function and the loss in NF2 gene function directly controls the tumor growth rate."

To test the hypothesis, we propose the following **Specific Aims**:

- 1) Establish a reproducible protocol for the primary culture of human Schwann and schwannoma cells and characterize NF2 gene function by studying cytoskeleton organization and tumor cell growth rate in culture.
- 2) Immortalize one normal Schwann cell culture and two schwannoma cell cultures with different mutations and phenotypes and characterize the cell lines.

Successful completion of these aims will allow us to: 1) better understand NF2 gene function in Schwann cells at the cell and molecular level and 2) obtain a useful *in vitro* tool for screening of new therapeutic agents for NF2.

Body**STATEMENT OF WORK**

Establish an in vitro model for the study of NF2 gene function.

Specific Aim 1: Establish a reproducible protocol for the primary culture of human Schwann and schwannoma cells and characterize NF2 gene function by studying cytoskeleton organization and tumor cell growth rate in culture.

Task 1: Months 1-20 Establish primary culture protocol by collecting normal vestibular nerve tissues and schwannoma tissues and store all cultures in liquid nitrogen at their passage 3.

Report:

1. The bank of human normal nerve cells and schwannoma and their primary cultures:
During the twelve month period, twenty-five NF2 patients underwent tumor removal surgery at House Ear Clinic. All vestibular schwannoma tissues were collected. Of those 25 tumors, 11 tumors were big enough for primary cultures (See Table 1). In addition, ten normal human sciatic nerve tissues were also collected (See Table 2).

Table 1

Case #	Number of frozen vials	Percentage of S-100 positive cells
#331	5	99%
#337	9	99%
#338	1	99%
#340	5	99%
#341	2	0% (meningioma)
#345	1	98%
#348	3	95%
#351	1	99%
#354	1	99%
#365	3	95%
#367	3	99%

Table 2. Bank of normal sciatic nerves

ID #	Primary culture	Percentage of S-100 positive cells	Number of frozen vials
1	contaminated	N/A	0
2	fail	N/A	0
3	yes	Less 50%	0
4	yes	Less 50%	0
5	yes	>80%	0
6	yes	>99%	7
7	yes	>99%	9
8	yes	>99%	4
9	yes	>99%	4
10	yes	>99%	7

2. Method of improvement for the primary cultures:

a. Growth factors

Previously we have adopted a serum free medium condition (media A) which are more favorable to Schwann cell growth (1-2). In this study, we first addressed whether media A will have any synergetic effect with schwannoma cells in the presence of serum. We have shown that factors contained in media A (insulin 10 ug/ml, progesterone 3×10^{-8} uM, bovine pituitary extract 3 ug/ml, transferrin 10 ug/ml, α -tocopherol 5 ug/ml, forskolin 5 uM) indeed can improve schwannoma cell proliferation in culture ($P < 0.001$, Figure 1).

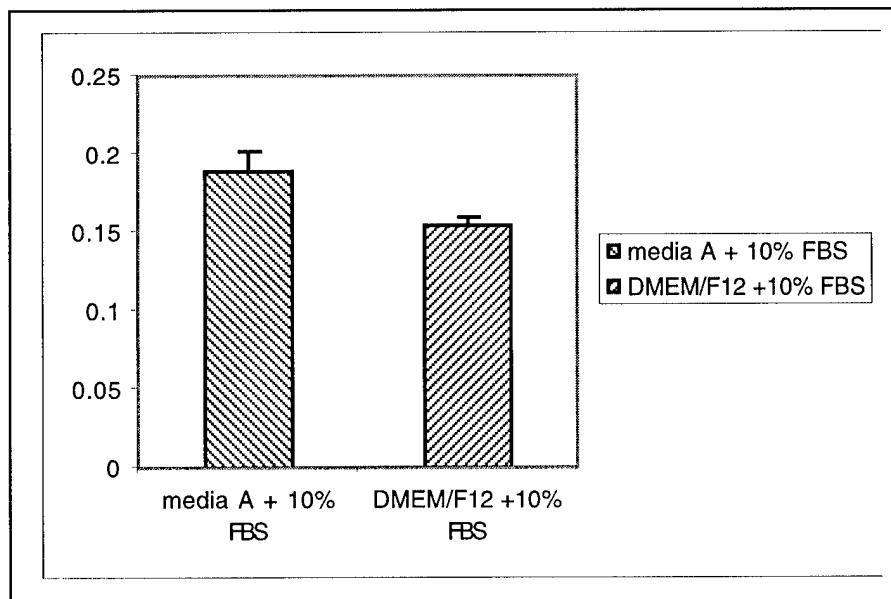


Figure 1: 2×10^4 tumor cells which contains greater than 95% S100 positive schwannoma cells were plated into the well of a 96 well plate. Twenty-four hours later, BrdU labeling media (1umol/BrdU) were added and incubated at 37° C for 20 hours. After washing, the cells were fixed and stained with anti-BrdU (mouse) antibody. The density of staining was measured by a plate

reader.

Next we have compared the growth stimulation effect of two Schwann cell growth factors (Gas-6 and Heregulin) in primary schwannoma cells. No significant effect was seen in cultures that were fed with media A plus Gas-6 (30ng/ml Gas-6 vs media A, P=0.07; Figure 2). However, heregulin was shown, which can promote the growth of schwannoma cells in culture (5nM, P=0.04; 10nM, P=0.02; 20nM, P=0.01, Figure 3). No synergistic effect was observed when both heregulin and Gas-6 were added to the medium (Figure 3).

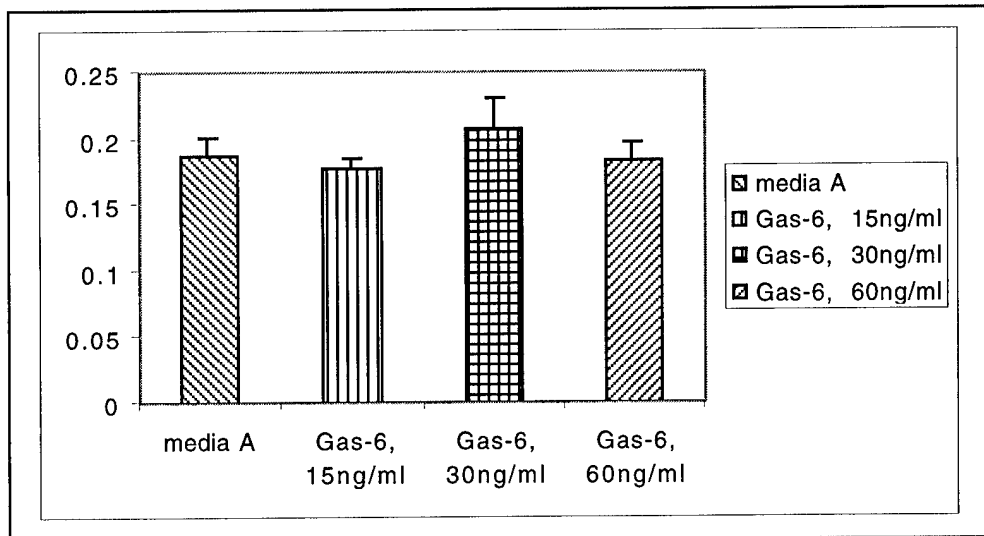


Figure 2: Cell proliferation rate of primary schwannoma cultures treated with different concentrations of Gas-6 added in media A.

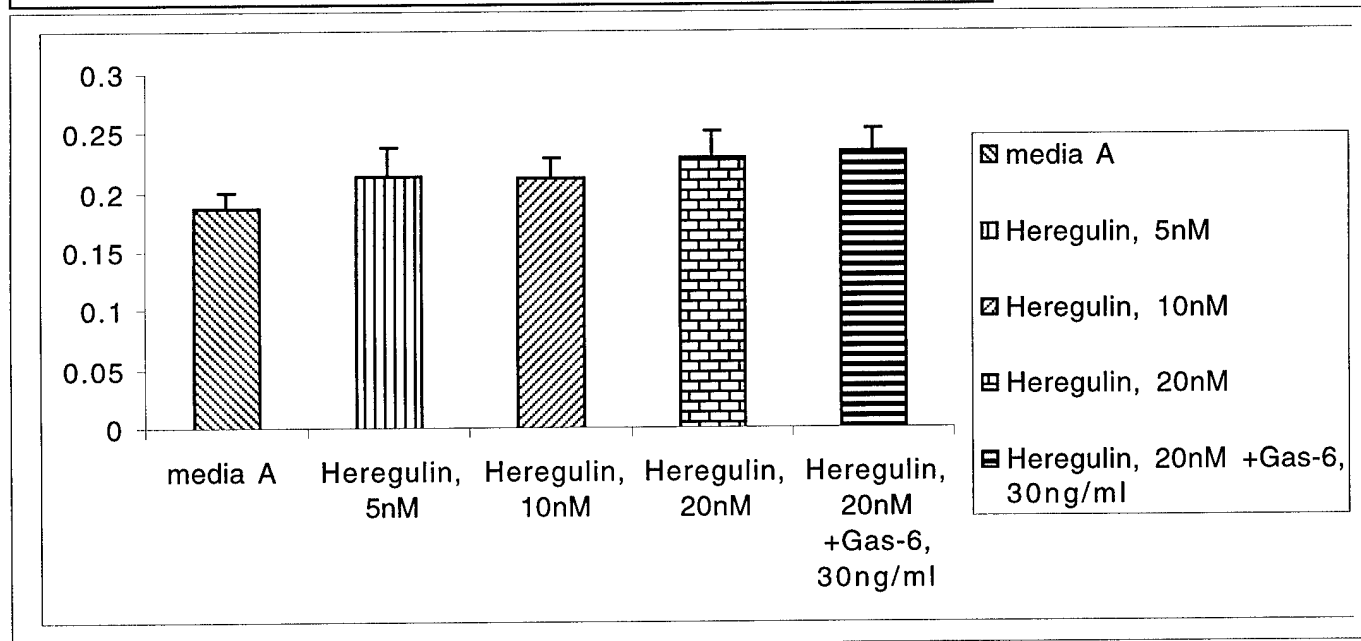


Figure 3: Growth stimulation of heregulin in cultured primary schwannoma cells.

3. Dissection method improvement for culturing primary normal Schwann cells.

We found that prior enzymatic digestion and removal of extra connecting tissues greatly reduces the fibroblast contamination.

Method:

We cut the sciatic nerve into 2 cm long pieces under the dissection microscope and pulled out each fascicle from the encased perinerurium and epineurium (see Figure 4).

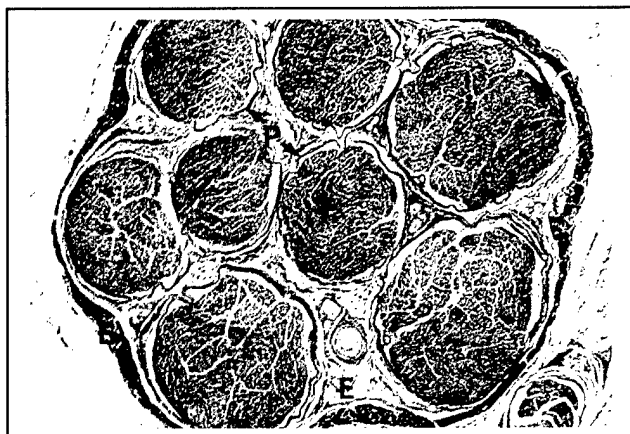


Figure 1: The micrograph illustration (3) of a medium sized peripheral nerve in transverse section. This nerve consists eight fascicles F, each of which contain many nerve fibers. Each fascicle is invested by a condensed connective tissue layer, the perineurium P, and the nerve as a whole is encased in the epineurium E.

Progress on Task 1: In progress.

Specific Aim 2: **Immortalize one normal Schwann cell culture and two schwannoma cell cultures with different mutations and phenotypes and characterize the cell lines**

Task 1: Months 10-14 Primary culture cell immobilization by retrovirus.

Currently, 2 primary human schwannoma cultures and one normal Schwann cell cultures were transduced by retroviral vectors carrying HPV E6-E7 genes and neo^r gene. All cultures are growing under the selection drug G418 (0.4 mg/ml) since 4 days post viral transduction. The drug-resistant cells from Passage 0 and each successive passage were stored and tested for schwannal cell marker S100 expression (Table 3). Culture 286 was established from a normal vestibular nerve tissue which was attached with a unilateral tumor. These cultures have gown beyond 28 passages and carrying a wild type NF2 gene (data not shown). The growth rate of this culture has a significantly slower growth rate by comparing with one previously immortalized schwannoma culture, but is growing significantly faster than the primary non-viral transduced normal Schwann cells (Figure 5).

Table 3:

Primary cultures	Cell type	passage	S100 positive cells at latest passage
286	Normal Schwann	28	>99%
459	schwannoma	3	60%
470	schwannoma	1	N/A

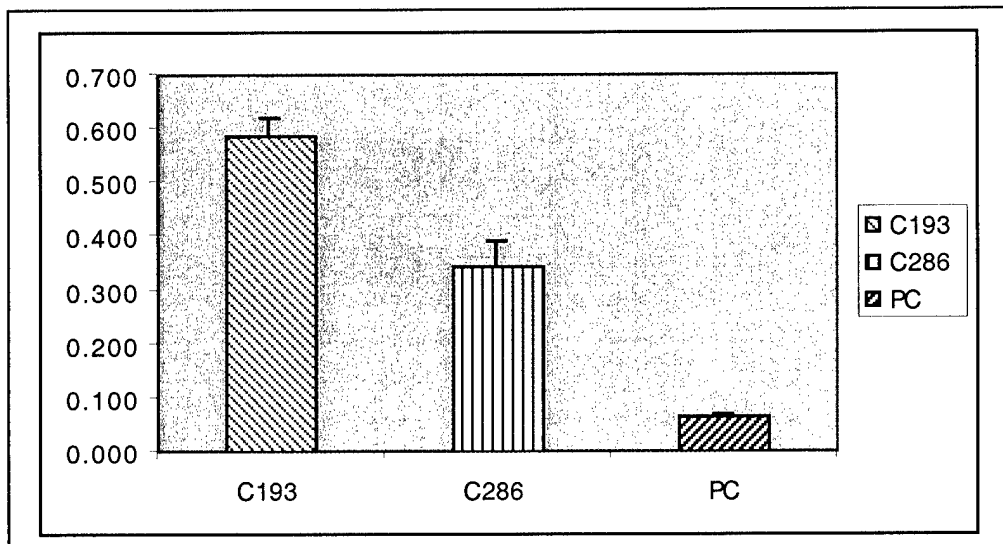


Figure 5: Growth rate comparison between immortalized schwannoma culture (C193, passage 30), immortalized normal Schwann cells (C286, passage 28) and a primary normal Schwann cell culture (PC, passage 2).

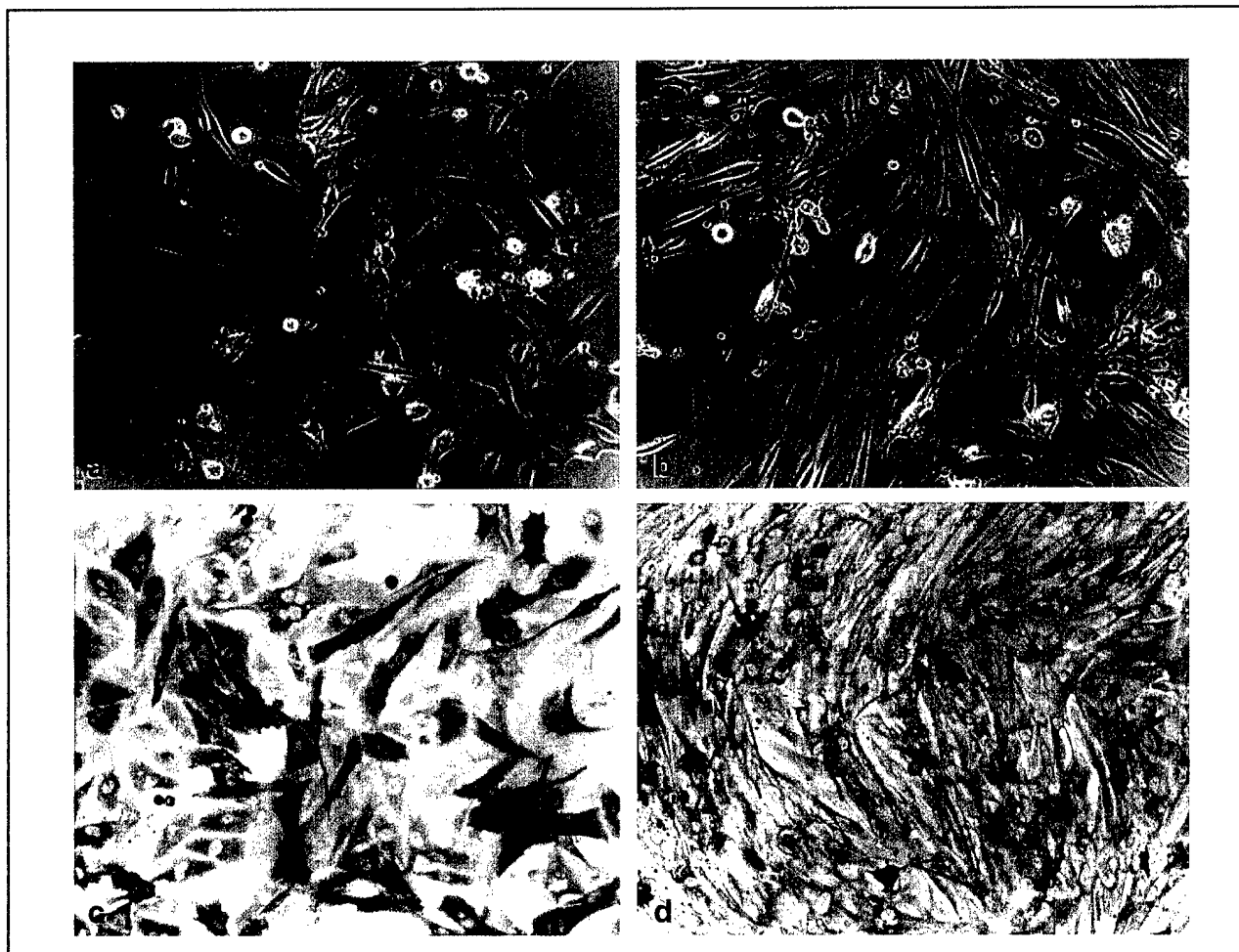


Figure 5: Primary human Schwann cells transduced with retrovirus carrying HPV E6-E7 gene (passage 25). Short spindle shape morphology (a) and with heterogeneous expression of S100 (c) was observed in culture cells plated in a dish for 72 hours. Two weeks later, the cell became more primary normal Schwann cell-like with long processes (b) and homogeneous expression of S100 (d).

Progress on Task 1: In progress.

Key Research Accomplishments

- Establishment of NF2 specific vestibular schwannoma primary culture bank.
- Establishment of human normal Schwann culture bank
- Improvement of protocol for primary culture condition.
- Partially immortalized a normal Schwann cell culture.

Reportable Outcomes

- 9th European Neurofibromatosis Meeting, April 6-8, 2001 Venice, Italy

Abstracts for "Primary culture condition for human vestibular schwannoma" and "Merlin growth inhibitory pathway in human schwannoma cells" will be submitted for presentation

- Human primary vestibular schwannoma tissue and culture bank
- Establish a retroviral vector transduced potential human Schwann cell line.

Conclusions

The goals of this project are to develop a methodology to establish a reliable *in vitro* system and test its credibility for the study of NF2 gene function in Schwann cells. Over the first twelve months of the project, we have collected 25 NF2 related tumors and established 11 primary cultures. Two primary schwannoma cultures and one normal Schwann cell culture were transduced by retrovirus and one has shown signs of immortalization. Both tissue handling techniques and culture conditions were improved.

References:

- 1 Hung, G., R. Faudoa, X. Li, Z. Xeu, D.E. Brackmann, W. Hitzelberger, E. Saleh, F. Lee, D.H. Gutmann, W. Slattery, 3rd, J.S. Rhim, and D. Lim, *Establishment of primary vestibular schwannoma cultures from neurofibromatosis type-2 patients [In Process Citation]*. Int J Oncol, 1999. **14**(3): p. 409-15.
2. Li R *Isolation of human and mouse genes based on homology to REC2, Culture methods for selective growth of normal rat and human Schwann cells..* Methods Cell Biol 1998; 57:167-86 .
3. Wheater P.R., Burkitt H.G., DanielsV.G. *Functional Histology* 1979; p96