行政院國家科學委員會專題研究計畫　期中進度報告

慢性 C 型肝炎之藥物基因體學研究（2/3）

計畫類別：個別型計畫
計畫編號：NSC92-3112-B-002-013-
執行期間：92 年 05 月 01 日至 93 年 04 月 30 日
執行單位：國立臺灣大學醫學院臨床醫學研究所

計畫主持人：賴明陽

報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 5 月 25 日

基因體醫學國家型科技計畫

National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.

計畫名稱-慢性C型肝炎之藥物基因體學研究

計畫名稱-Pharmacogenomics of Chronic Hepatitis C

報告類別：☐ 新進研究計畫 (New Proposal) ☐ 修正後計畫書 (Revised Proposal) ☑ 年度成果報告 (Progress Report)

計畫類別：☑ 個別型計畫 (Individual Project) ☐ 整合型計畫 (Program Project)

計畫編號：GM099

計畫主持人 (Principle Investigator)：賴明陽（Ming-Yang Lai）

共同主持人 (Co-Principle Investigator)：蔡有光（Yeou-Guan Tsay）
高嘉宏（Jia-Horng Kao）
呂勝春（Sheng-Chung Lee）

執行單位 (Institution)：國立台灣大學醫學院臨床醫學研究所

中華民國　92年12月31日

National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.
Progress Report—Research Project

基因體醫學國家型科技計畫

國科會延續性計畫進度報告

個別型計畫 (Individual Project)

Program Classification:

☑ **Genomic Medicine** ☐ **Bioinformatics**

☐ **Proteomics & Structural Genomics** ☐ **ELSI**

Project Number: **GM099** (計畫編號)

NSC Funding Number:_____ (93 年度國科會預核編號)

Title of Project 計畫名稱	(in Chinese) 中文 慢性 C 型肝炎之藥物基因體學研究
	(in English) 英文 Pharmacogenomics of Chronic Hepatitis C
Institution 研究（執行）單位	(in Chinese) 中文 國立台灣大學醫學院臨床醫學研究所
	(in English) 英文 Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine
Principle Investigator 計畫主持人	(in Chinese) 中文 賴明陽
	(in English) 英文 Ming-Yang Lai

FY	2002	2003	2004	Total
Budget				

(in NT dollars: 1USD = 34 NTD)

Signature of the PI：_____Date：_____

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Progress Report

1. Response to previous reviewers' critiques

Please describe the previous reviewers' critiques and how based on the critiques, you made modifications to specific aims, experimental design, or resource allocation etc.

(1) Critiques of Reviewer # 1: " Dr.Lai has identified a protein induced by ribavirin in primary adult hepatocytes, which are not observed in control hepatocytes by 2-D gel electrophoresis. This protein is a homologue of mouse SF20/IL25. Dr. Lai noted that interferon induced the receptor of SF20/IL25, thus allowing him to make a hypothesis that this might be the mechanism of the synergistic effect of IFN-ribavirin combination therapy. However, he did not explain why this particular protein was selected for his studies. It appears that many other proteins are also induced by ribavirin *in vitro*". "Nevertheless, studies on this protein are worth pursuing".

Response: We studied this protein (RBV-30) because its expression was distinctively increased in primary hepatocytes treated with ribavirin compared to ribavirin-untreated hepatocytes in 2-D gel electrophoresis. We confirm this finding by western blots afterwards. Another reason is that beause intrahepatic failure of CD8+ T lymphocytes is probably one of the important contributing factors of chronic hepatitis C. SF20/IL25 was claimed to be able to support proliferation of lymphoid cell lineages, including CD8+ T cells. It thus has the potential to counteract the acquired immune defect of chronic hepatitis C. Furthermore, interferon alfa can induce its receptor (9804). Therefore, we are very interested to pursue the hypothesis that ribavirin can induce RBV-30 and may explain or contribute to the remarkably enhancing effect of ribavirin when combining interferon alfa in the treatment of chronic hepatitis C. To prove the importance of RBV-30 expression and its relationship with treatment outcome, chronic hepatitis C patients treated with IFN plus ribavirin will be examined in the next granting period.

(2) Critiques of Reviewer # 2: "The progress has been excellent. …..The budget are deemed appropriate"

Response: We sincerely thank the reviewer's support.

(1) Specific Aims

Please state the overall goals of the project, and specific aims, as reviewed and approved by the Study Section and actually awarded. If these specific aims as actually funded did not differ in scope from those actually pursued during the grant period, and if the aims have not been modified, state this. If they have been modified, give the revised aims.

The **long-term goal** of our research is to use genomic-wide approaches to find new biomarkers for predicting treatment outcome with interferon alfa plus ribavirin in patients with chronic hepatitis C, to understand the molecular mechanisms of the enhancing effects of ribavirin, and ultimately, to optimize the treatment effect and stop disease progression. The **hypothesis** to be evaluated is that there is a genetic basis for treatment outcomes with interferon plus ribavirin in hepatitis C. The **specific aims** are as follows:

(1) To identify the genetic determinants of interindividual differences in response to interferon plus ribavirin by microarray analysis in liver biopsy samples from different patient groups based on their response to interferon plus ribavirin therapy.

(2) To search for the serum proteins differentially expressed in the patient groups with sustained response or failure to interferon plus ribavirin therapy.

(3) To elucidate the molecular mechanisms of the remarkable enhancing effect of ribavirin against hepatitis C by applying genome-wide microarrays and proteomic analysis in hepatocyte cultures and animal experiments.

(4) To establish a hepatoma cell line capable of supporting HCV replication to help confirm the anti-HCV mechanisms of ribavirin revealed by microarrays and proteomic studies.

The goal and specific aims of this study have been kindly granted. We do not wish to modify these specific aims.

2. Progress Summary

Summarize concisely the results obtained for **each specific aim during the past year (or reporting period)**. Negative results, if any, should also be included and approaches taken to improve the prospects of the project discussed. (Do not exceed **5 pages**, not including figures and references.)

The following are the summaries of results in each specific aim:

(1) **Specific aim 1** :**To identify the genetic determinants of interindividual differences in response to interferon plus ribavirin by cDNA microarray analysis in liver biopsy samples from different patient groups based on their response to interferon plus ribavirin therapy.**

Because the microarray core lab in NTUH still has contamination problems in some clones, we begin to use Affimatrix gene chips for analyzing intrahepatic gene expressions which correlate with response to IFN plus ribavirin. After assuring the quality of isolated mRNA from pretreatment liver biopsy samples of 5 sustained responders and 5 nonresponders, experiments with Affimetrix gene chips were carried out. A distinct gene expression pattern is preliminarily found in the nonresponder group by cluster analysis **(Figure 1, right upper)**, but not yet found in the sustained responder group (**Figure 1, left).**

(2) **Specific aim 2** : **To search for the proteins that are differentially expressed in the patient groups with different response to interferon plus ribavirin.**

We first tried to find differential biomarkers in the pretreatment serum samples of chronic hepatitis C patients with different response to IFN plus ribavirin therapy by traditional 2-D gel electrophoresis. We did not succeed. With the help of our co-investigator, Dr. Yeouguang Tsay, we then use the 2D DIGE (Two-Dimensional Difference Gel Electrophoresis) technique (Amersham Pharma Biosciences). **Figure 2** are our preliminary results of the 2D DIGE. The Normal and HCV samples were labeled with Cy5 and Cy3 dyes respectively per instructions of the manufacture (Amersham-Pharmacia). The samples were combined together and resolved first on one single pH 4-7 isoelectric focusing (IEF) gel (Bio-Rad). After equilibration in 1X SDS sample dye, the IEF gel was then placed on a 10 to 15% polyacrylamide gradient gel. The gel image was developed in a FLA-5000 fluorescence scanner (Fuji). The grey-scale figures show the protein contents in serum from normal **(Figure 2A)** and chronic hepatitis C **(Figure 2B)** group respectively. The colored image **(Figure 2C, normal & HCV)** shows the direct comparison of protein contents in these two groups. The yellow spots represent those found in both groups, while green and red spots represent those only found in

Normal and HCV samples respectively. Based on our experience, this DIGE method is probably the best method available to identify difference between samples. Thus, with the same rationale, we should be able to identify the difference in serum from responsive and non-responsive chronic hepatitis C patients.

(3) **Specific aim 3** : **To elucidate the molecular mechanisms of the remarkable enhancing effect of ribavirin against hepatitis C by applying genome-wide cDNA microarrays and proteomic analysis in hepatocyte cultures and animal experiments.**

In the past granting period, we have found a ribavirin-induced protein (RBV-30) which is identified to be a homologue protein of mouse SF20/IL-25. The SF20/IL-25 is a novel bone marrow stroma-derived growth factor which may support proliferation of cell in the lymphoid lineage. including CD4 + and CD8 + T cells. We have cloned the RBV-30 gene into an expression rector and the RBV-30 protein has been used to generate rabbit polyclonal antibodies. When RBV-30 expressing retroviral vector DNA was transfected into various human hepatocyte cell lines in serum free media, RBV-30 was found to be secreted in the supernatant by western blots analysis **(Figure3A)**. A RBV-30-secreting stable cell line has been established by transfecting RBV-30 expressing retroviral vector DNA into 293 cells. We can confirm that RBV-30 protein expression is increased by adding ribavirin in both the 293 cell lines **(Figure3B)** and ribavirin-treated primary human hepatocytes **(Figure3C)**. We are now doing studies correlating RBV-30 expression in the PBMC extract to the IFN plus ribavirin treatment outcomes in chronic hepatitis C patients, and also plan to study its physiological function.

(4) **Specific aim 4** : **To establish a hepatoma cell line capable of supporting HCV replication to help confirm the anti-HCV mechanisms of ribavirin revealed by microarrays and proteomic studies.**

We have not yet established a hepatoma cell line which can support HCV replication. The effort with a protocol provided by Professor M Lai at USC is still ongoing. Meantime, we have obtained a culture of G418-resistant Huh-7 cells supporting the replication of the Nneo/3-5B HCV replicon from Professor Stanley Lemon at UT. Currently we are using this HCV replicon system to test some of our hypothesis.

4. Projected Timeline & Brief Summary of Plans for Next Year

Provide a short paragraph to describe the plans for next year including a realistic timetable and appropriate milestones for the project, based on the progress reached so far. (Do not exceed **1 page**.)

In the next year, we plan to accomplish our project according to our specific aims:

1. To accomplish microarray analysis of more liver biopsy samples and correlate the expression pattern of particular genes with the treatment outcome.
2. To use Two-Dimensional Difference Gel Electrophoresis (DIGE) technique to find out differential serum biomarkers associated with different treatment outcome.
3. To establish immunohistochemical studies of RBV-30 and 9804 in pretreatment liver biopsy samples, and ELISA assay of RBV-30 and 9804 in serum; to correlate their expressions in the liver, serum and PBMC with the outcome of ribavirin plus interferon therapy; RBV-30 protein will be purified from the 293 cell line constitutively expresses RBV-30, and will be further studied to characterize its function in animal experiment and treated hepatitis C patients.
4. To establish a hepatoma cell line capable of supporting HCV replication.

5. Personnel

Summarize the **personnel involved in the project during the grant period**. List the personnel in accordance to the following categories: (1) senior investigators, including visitors; (2) postdoctoral fellows; (3) graduate students; (4) technicians or research assistants. Specify for each individual the period of involvement and the percentage commitment of effort.

Name		Position Title	Education Degree	% of personal effort on this project	Job Description or Responsibilities
In Chinese	In English				
賴明陽	Ming-Yang Lai	Principal investigator (professor)	MD, PhD	30%	Original research idea, writing proposal, research design, supervision and coordination
蔡有光	Yeou-Guan Tsay	Co-investigator (lecturer)	MD, PhD	5%	Advising proteomic study
陳健尉	Jeremy J.W. Chen	Co-investigator (Assistant professor)	PhD	5%	Advising microarrays study
高嘉宏	Jai-Horng Kao	Co-investigator (Assoc. professor)	MD, PhD	10%	Assiting the clinical and virological studies of HCV
呂勝春	Sheng-Chung Lee	Co-investigator (professor)	PhD	10%	Advising molecular study
伍安怡	Betty A. Wu-Hsieh	Co-investigator (professor)	PhD	10%	Advising immunology study
蘇文正	Wen-Cheng Su	Graduate student	PhD candidate	80%	Study molecular mechanism of ribavirin, esp. the function of RBV-30 and its receptor
程靜暐	Ching-Wei Cheng	Research assistant	Master	100%	Executing the microarrays and proteomic experiments
洪克璿	Ko-Hsuan Hung	Research assistant	Master	100%	Executing the microarrays and proteomic experiments

6. Publications and/or Patents

6a. Publications

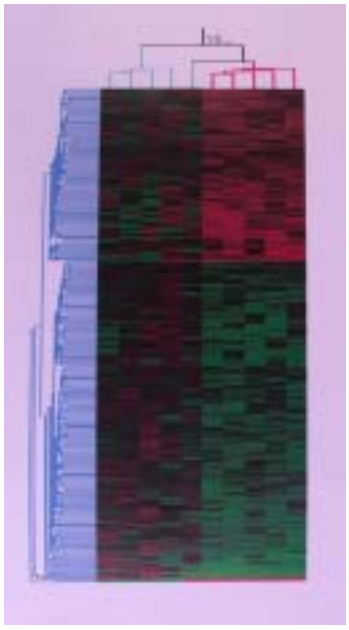
List the title and complete references (author(s), journal or book, year, page number) of all publications **directly resulting from studies supported by the project (i.e., with citation of this grant in the acknowledgement section)**. List the publications for the project in accordance to the following categories: (1) manuscripts published and accepted for publications; (2) manuscripts submitted; and (3) conference proceedings. Provide one copy of each publication not previously reported to the National Science Council in the Appendix.

6b. Patents

List all inventions disclosed, patents filed, and patents granted. Please note the inventors, assignee, title of patent, country or area where patent applied for, filing or issued number and date.

Appendix

Fig 1.



Affymetrix gene chip analysis of gene expression pattern in liver biopsy samples from 5 sustained responders (left) and 5 nonresponders with chronic hepatitis C receiving IFN plus ribavirin therapy. A distinct pattern is preliminarily identified in the nonresponders.

Fig 2A.

Normal serum

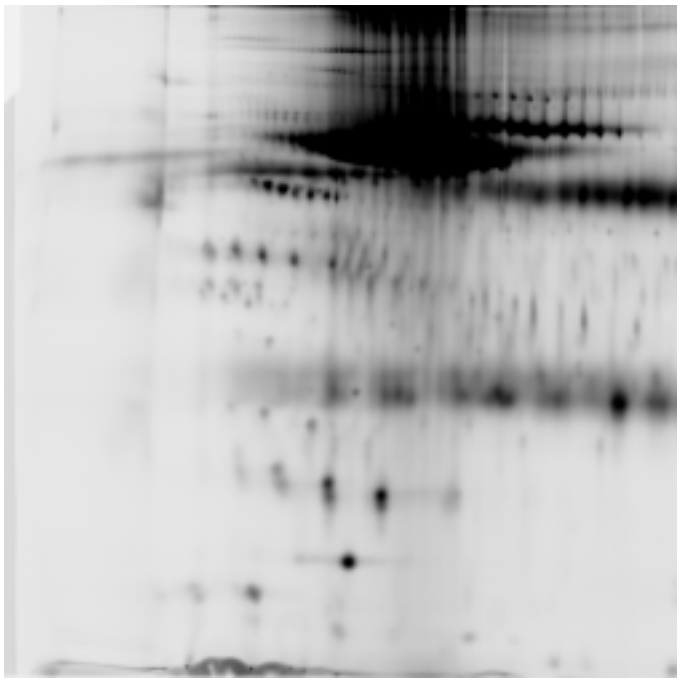


Fig 2B.

Chronic hepatitis C patient’s serum

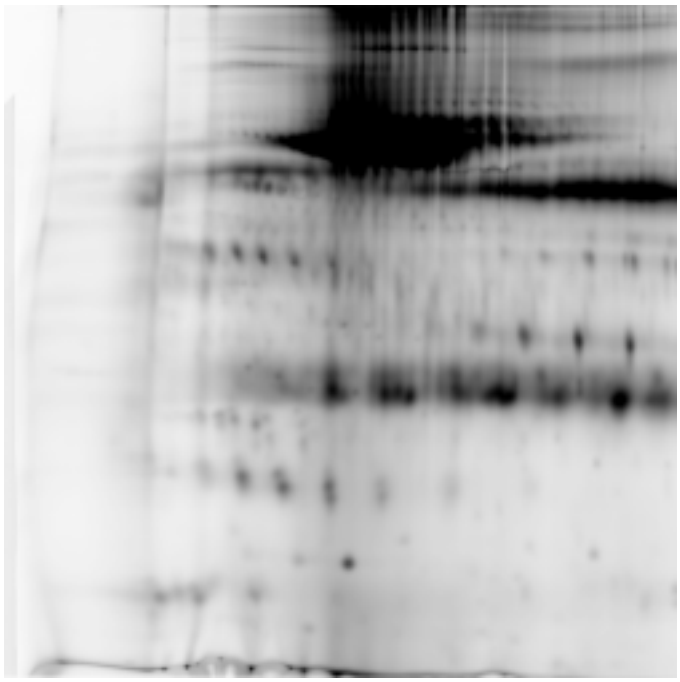


Fig 2C.

Colored image shows the 2D DIGE (Two-Dimensional Difference Gel Electrophoresis) from normal and chronic hepatitis C patient’s serum.

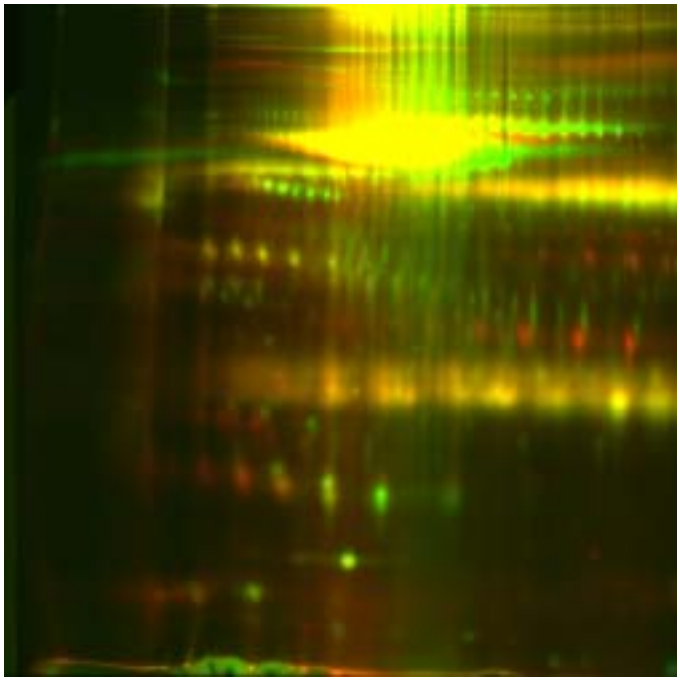
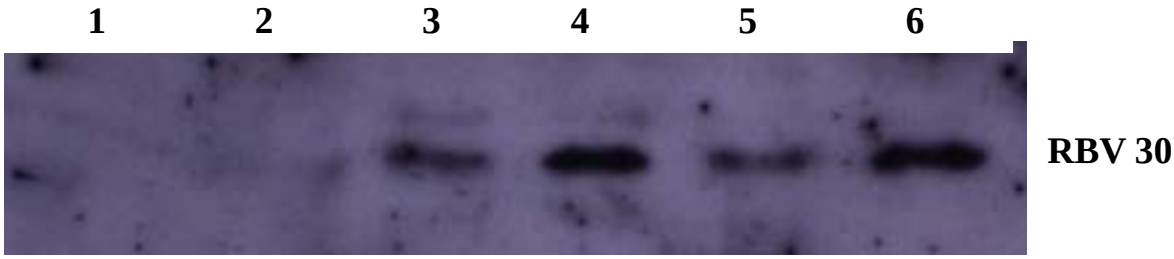


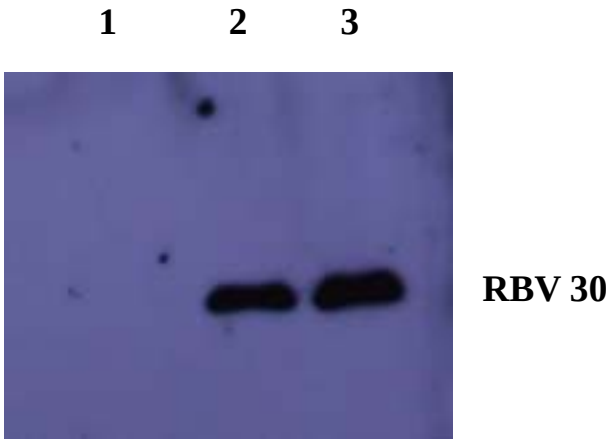
Fig 3A.



RBV-30 expressing retroviral vector DNA was transfected into various human hepatocytes. After 48 hours of transfection, serum free supernatant was collected and concentrated via Amicon Ultra-4 (Millpore Inc.). 100 g of concentrated supernatant was loaded onto 15% SDS-PAGE and performed the Western blot analysis using rabbit anti-RBV-30 polyclonal antibody.

(Lane 1: vector control/Huh-7 cells, Lane2: RBV-30/HepG2 cells, Lane 3: RBV-30/Hep3B, Lane 4: RBV-30/Huh-7 cells, Lane 5: RBV-30/N-Neo cells, Lane 6: RBV-30/293 cells)

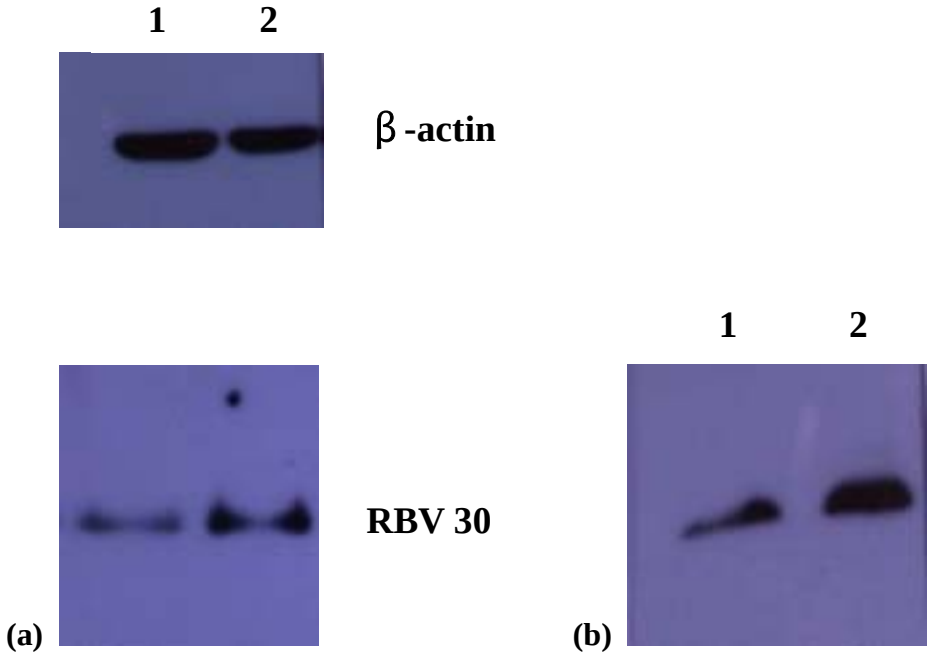
Fig 3B.



RBV-30 expressing retroviral vector DNA was transfected into 293 cells. Hygromycin-B selected individual stable clones were subjected to serum starvation for 48 hours while the cells grow to 80~90% of confluence. Cultural supernatant was collected and concentrated. 80 μg of concentrated supernatant was loaded onto 15% SDS-PAGE and performed the Western blot analysis using rabbit anti-RBV-30 polyclonal antibody.

(Lane 1: vector control, Lane2: RBV-30/293 clone a, Lane 3: RBV-30/293 clone b)

Fig 3C.



The primary adult hepatocytes which recovered from the liver tissues of HCC patients treated by operations were separated via perfusion method. After treatment of ribavirin for 48 hours in serum free medium, cell lysate and cultural supernatant were collected and subjected to Western blot analysis by rabbit anti-RBV-30 polyclonal antibody.

(a) cell lysate: Lane 1→ without treatment of ribavirin, Lane 2→ treatment of ribavirin

(b) cultural supernatant: Lane 1→ without treatment of ribavirin, Lane 2→ treatment of ribavirin