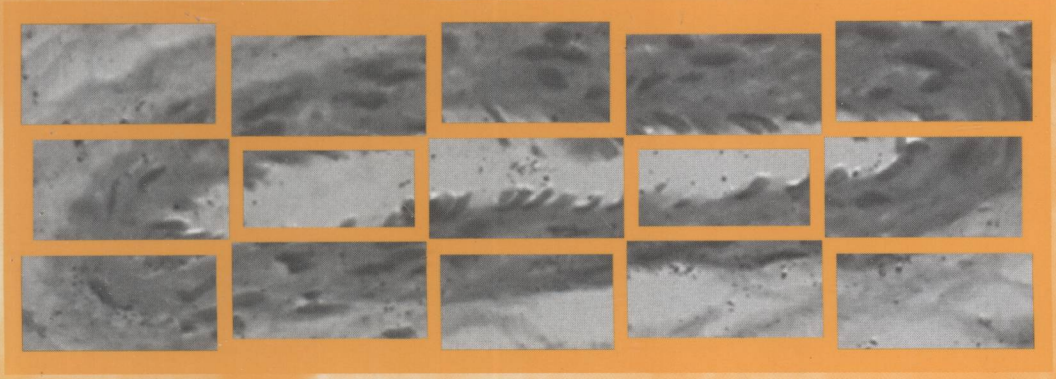
METHODS IN MOLECULAR BIOLOGY™ 366

Cardiac Gene Expression

Methods and Protocols

Edited by

Jun Zhang
Gregg Rokosh



HUMANA PRESS

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Edited by

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HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American Standards Institute)

Permanence of Paper for Printed Library Materials.

Cover illustration: From Fig. 5D (Background) and Fig. 6D (Inset) of Chapter 9, "*In Situ* Hybridization: *A Technique to Study Localization of Cardiac Gene Expression*," by Thierry P. Calmels and David Mazurais.

Cover Design by Nancy K. Fallatt

Production Editor: Jim Geronimo

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN 1-59745-030-8

Library of Congress Cataloging in Publication Data
Cardiac gene expression : methods and protocols / edited by Jun Zhang and Gregg Rokosh.
p. ; cm. — (Methods in molecular biology ; v. 366)
Includes bibliographical references and index.
ISBN 1-58829-352-1 (alk. paper)
1. Myocardium—Laboratory manuals. 2. Gene expression—Laboratory manuals. 3. Genetic regulation—Laboratory manuals. I. Zhang, Jun, 1968- II. Rokosh, Gregg. III. Series: Methods in molecular biology (Clifton, N.J.) ; v. 366.
[DNLM: 1. Gene Expression Regulation—Laboratory Manuals. 2. Gene Expression Profiling—Laboratory Manuals. 3. Myocardium—Laboratory Manuals. QU 25 C267 2007]
QP113.2C364 2007
612.1'7—dc22 2006008866

Cardiac Gene Expression

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Preface

The past decade has ushered in enormous changes in how we perceive and study changes in gene expression in the heart. Early in the 1990s, the human genome project was just getting underway and establishing methods with the sensitivity to measure changes in the expression of genes with low copy number was an accomplishment. We all experienced some trepidation when the first news of microarrays arrived espousing the ability to measure changes in expression of hundreds to tens of thousands of genes (the whole genome) at once. This high throughput method was an astonishing jump in our approach to biological science. At the same time Steve Fodor and Pat Brown published papers describing two completely different approaches to measuring the expression changes of large numbers of genes at the same time. Thus began the microarray era and as a consequence the beginning of an era with a host of new approaches in pursuit of understanding the role and regulation of gene expression in cell biology and pathology including driving forward the field of bioinformatics.

The array, no pun intended, of contributions contained in *Cardiac Gene Expression: Methods and Protocols* an edition of Humana's Methods in Molecular series, address both new and established methods that researchers in the cardiac field will certainly find useful as a reference for the development of projects and training. Our aim in this compilation was to provide insight and details for a comprehensive range of methods that will serve both startup and sophisticated users alike. Sections cover expression profiling by microarray Section I, targeted analysis of gene expression (Section II), transcription factor DNA binding and regulation of promoter activity (Section III), *in silico* approaches to identifying functional *cis* regulatory elements and regulation of cardiac gene expression (Section IV), *in silico* and mass spectrometry methods to identify sequence nucleotide polymorphisms (SNPs) (Section V), and to bring findings from the above studies to the next level overexpression of genes in vivo and isolated myocytes and cardiac-specific targeted gene deletion (Section VI).

Section I, Cardiac Gene Expression Profiling: the Global Perspective. Five chapters describe several different approaches to examining and identifying changes in gene expression in the transcriptome as well as analytic approaches. Methods and analysis have improved significantly as many investigators have strived to increase array reliability and reproducibility. Section II, Cardiac Gene Regulation: Gene-Specific mRNA Measurement in the Myocardium, follows accordingly with chapters outlining more sensitive and gene targeted expression methods that are more conducive for follow up studies to verify and fur-

ther characterize those important findings from array experiments or those of your favorite gene. Underlying mechanisms of gene regulation can be studied using methods that focus on the interaction of transcription factors with their cognate *cis* binding elements and how these *cis* elements impact overall promoter activity in Section III, Cardiac Gene Regulation: Promoter Characterization in the Myocardium. Changes in gene expression reflect the combined effects of transcriptional enhancers and repressors that serve to precisely control the level of expression of thousands of genes from conception to death. Studies that focus on how the interaction between transcription factors and their cognate *cis* DNA elements regulate gene expression were provided some assistance recently with the completion of the Human Genome Project in 2003 in addition to several other genomes with more coming available at a rapid pace. New analytical approaches to decipher the functional elements buried in the 3 billion nucleotides of the human genome and other model genomes are described in Sections IV, *In Silico* Assessment of Regulatory *cis*-Elements and Gene Regulation, and V, Cardiac Single Nucleotide Polymorphisms. One important aspect of understanding the importance of available sequence is being able to sift through sequence and reliably identify and distinguish functional regulatory elements from nonfunctional elements. Pennachio and colleagues at Lawrence Livermore Labs have simplified this task by using a comparative approach. By using available genome sequence for several different species across evolution this approach was able to reliably predict the functionality of elements according to their evolutionary conservation. Resources for the analysis of gene regulation data and SNPs will provide essential functionality for the understanding of changes in gene expression and effects of SNPs on gene function and expression. With the identification of exciting new targets one begins to think of the functional aspects and begins to plan experiments to validate hypotheses. Section VI, Gene Overexpression and Targeting in the Myocardium, highlights methods that facilitate overexpression or cardiac specific targeted deletion of your favorite gene in the heart

Thus, this array of contributions provides an array of methods that will take the investigator through screening, analysis, characterization, and functional confirmation of novel genes or old genes with a new function serving as a template for a solid research program.

Jun Zhang
Gregg Rokosh

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Contents

I

Cardiac Gene Expression

The Global Perspective

1

Microarray Analysis of Gene Expression in Murine Cardiac Graft Infiltrating Cells

Yurong Liang, Xin Lu, and David L. Perkins

Summary

Microarray technology can rapidly generate large databases of gene expression profiles. Our laboratory has applied these techniques to analyze differential gene expression in cardiac tissue and cells based on mouse heart transplantation. We have analyzed the different gene expression profiles such as stress or injury including ischemia following transplantation. We also have investigated the role of infiltrating inflammatory cells during graft rejection by purifying subsets of infiltrating cells using GFP transgenic mice and detailed all technical experiences and issues. The purpose of this study is to assist researchers to simplify the process of analyzing large database using microarray technology.

Key Words: Gene expression; microarray, bioinformatics; heart transplantation; mouse.

1. Introduction

The analysis of gene expression profiles using microarray technology is a powerful approach to investigate the functions of specific tissues or cells. Our laboratory has applied these techniques to analyze differential gene expression in cardiac tissue and cells in a model of murine heart transplantation *(1–4)*. Specifically, we have analyzed the response by cardiac cells to various forms of stress or injury including ischemia following transplantation *(5)*. In addition, we have investigated the role of infiltrating inflammatory cells during graft rejection by purifying subsets of infiltrating cells. Using current microarray technology, it is possible to analyze approx 45,000 probe sets representing known mouse genes or expressed sequence tags (ESTs). The ability to perform global analyses of gene expression creates the potential to analyze

From: *Methods in Molecular Biology, vol. 366: Cardiac Gene Expression: Methods and Protocols*
Edited by: J. Zhang and G. Rokosh © Humana Press Inc., Totowa, NJ

complex biological systems. These methods could be applied to other questions of cardiac development or disease.

2. Materials

1. Collagenase II (Gibco) and pancreatin (Sigma).
2. D-phosphate-buffered saline (PBS; Gibco).
3. Tri Reagent (Gibco-BRL Life Technologies, Rockville, MD).
4. Dnase I (Invitrogen).
5. SuperScript II (Invitrogen).
6. ALTRA flow cytometer (Beckman Coulter).
7. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA).
8. GeneAmp 5700 Sequence Detection System (Applied Biosystems).
9. SuperScript Choice system (Gibco-BRL Life Technologies) and T7-(dT) polymerase (Gensetoligos, La Jolla, CA).
10. BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY).
11. RNeasy mini kit (Qiagen, Valencia, CA).
12. Affymetrix GeneChip Software.

3. Methods

3.1. Vascularized Heterotopic Cardiac Transplantation

1. Murine hearts are transplanted as previously described *(6,7)*.
2. Briefly, hearts are harvested from freshly sacrificed donors and immediately transplanted into 8- to 12-wk-old recipients that are anesthesized via intraperitoneal injection with 100 mg/kg of ketamine and 20 mg/kg of xylazine.
3. The donor aorta is attached to the recipient abdominal aorta by end-to-side anastamosis, and the donor pulmonary artery is attached to the recipient vena cava by end-to-side anastomosis.
4. All surgical procedures should be completed in less than 45 min from the time that the donor heart is harvested to ensure similar ischemia times. Donor hearts that do not beat immediately after reperfusion or that stop within 1 d following transplantation should be excluded (>98% of all grafts function at 1 d following transplantation).

3.2. Single Cell Suspension

1. Donor grafts are harvested at the indicated time following transplantation and processed to prepare a single-cell suspension using collagenase and pancreatin digestion.
2. The graft heart is harvested following cold saline perfusion.
3. Hearts are minced to fine fragments with a scalpel or razor blade.
4. The heart tissue is digested four times with 0.5% collagenase II (Gibco) and 2.5% pancreatin (Sigma) in 37°C water for 7 min (*see* **Note 1**).

5. The cell suspension should be filtered and washed twice with 2% FCS D-PBS solution.
6. Resuspend cells, add 2 mL 2% FAS solution, and perform flow cytometry analysis.

3.3. Cell Sorting

Graft infiltrating cells have been shown to play important roles in triggering immune responses during graft rejection after transplantation and other inflammatory diseases such as myocarditis. To determine whether gene expression differences were expressed in infiltrating inflammatory or stromal cells, we used microarray technology to analyze the gene expression profile. To purify cell populations of infiltrating or stromal cells, we purified cell subsets by fluorescence-activated cell sorting (FACS) based on expression of green fluorescent protein (GFP) or fluorescent labeled monoclonal antibodies (*see* **Note 2**). Gene expression can be analyzed by DNA microarrays or real-time polymerase chain reaction (PCR) in the purified cell populations.

3.3.1. Analysis of Graft Infiltrating Cells

Because of technical difficulties, methods of purifying infiltrating cells often isolate a small percentage of the total population of infiltrating cells. To improve specificity and yield, we have developed a protocol using donor or recipient mice containing a transgene that constitutively expresses the GFP in all cells. These cells have greater than three logs of green fluorescence, making purification by FACS efficient and quantitative. As previously reported, we can purify sufficient infiltrating cells to perform microarray analysis from small numbers of mice. For example, our typical yield is from 10^6 (at early time points) to 10^7 (at late time points) infiltrating cells per graft (*see* **Note 3**). Thus, we can harvest sufficient cells from a single mouse at d 7 following transplantation to obtain sufficient RNA for microarray analysis. An advantage of this approach is that infiltrating cells can be analyzed without requiring amplification of RNA.

3.4. RNA Extraction

Total RNA is isolated from tissues or purified cell populations using TRIZOL reagent (Gibco-BRL Life Technologies). RNA purity is determined initially by 260/280 = 1.85 to 2.01 and by scanning with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip®. RNA samples not meeting these basic parameters of quality should be excluded from the study.

3.5. DNA Microarrays

1. The initial step of cDNA synthesis is performed using Affymetrix protocols with the T7 dT Primer (100 pM) 5'-GGCCAGTGAATTGTAATACGACTCACTATA GGGAGGCGG-(dT) 24-3'.