AWARD NUMBER: W81XWH-15-1-0055

TITLE: A Counterregulatory Mechanism Impacting Androgen Suppression Therapy

PRINCIPAL INVESTIGATOR: Dr. David Wilson

Contracting Organization: Washington University, The Saint Louis, MO 63130-4862

REPORT DATE: August 2016

TYPE OF REPORT: Final

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.
Androgen deprivation therapy (ADT) with gonadotropin-releasing hormone (GnRH) analogues is a mainstay of prostate cancer treatment. This project explores a novel counterregulatory response that may limit the efficacy of ADT. A key player in this process is HSD17B3, an enzyme required for the conversion of androstenedione to testosterone in testicular Leydig cells (LCs). Normally gonadotropin stimulation of LCs is accompanied by upregulation of genes in the testosterone synthetic pathway. The effect of GnRH analogues on LC function was modeled by conditional deletion of \textit{Gata4}, a transcription factor known to positively regulate multiple genes involved in steroidogenesis. \textit{Gata4} deletion led to decreased expression of several genes in the testosterone biosynthetic pathway (\textit{Cyp11a1}, \textit{Hsd3b1}, and \textit{Cyp17a1}). Unexpectedly, the final gene in the pathway, \textit{Hsd17b3}, was upregulated in the deleted cells. This paradoxical increase in \textit{Hsd17b3} expression was recapitulated when normal LCs were incubated with conditioned medium from \textit{GATA 4}-deficient LCs, implying that a hormone mediates the process. Preliminary results suggest that a loss of LC-derived estrogen in the conditioned media accounts for the effect. If this counterregulatory mechanism also operates in human LCs, it could contribute to inadequate androgen suppression in patients who undergo ADT with GnRH analogues.
# TABLE OF CONTENTS

1. Introduction ...........................................................................................................  4  
2. Keywords ................................................................................................................. 4  
3. Accomplishments .................................................................................................... 4-8  
4. Impact ...................................................................................................................... 8  
5. Changes/Problems .................................................................................................. 9  
6. Products .................................................................................................................. 9-10  
7. Participants & Other Collaborating Organizations ............................................. 10-11  
8. Special Reporting Requirements ......................................................................... 11  
9. Appendices ............................................................................................................. 12-77
1. INTRODUCTION:

Androgen deprivation therapy (ADT) with gonadotropin-releasing hormone (GnRH) analogues is a mainstay of prostate cancer treatment. This project explores a novel counterregulatory response that may limit the efficacy of ADT. A key player in this process is HSD17B3, an enzyme required for the conversion of androstenedione to testosterone in testicular Leydig cells (LCs). Normally gonadotropin stimulation of LCs is accompanied by upregulation of genes in the testosterone synthetic pathway. The effect of GnRH analogues on LC function was modeled by conditional deletion of \textit{Gata4}, a transcription factor known to positively regulate multiple genes involved in steroidogenesis. \textit{Gata4} deletion led to decreased expression of several genes in the testosterone biosynthetic pathway (\textit{Cyp11a1}, \textit{Hsd3b1}, and \textit{Cyp17a1}). Unexpectedly, the final gene in the pathway, \textit{Hsd17b3}, was upregulated in the deleted cells. This paradoxical increase in \textit{Hsd17b3} expression was recapitulated when normal LCs were incubated with conditioned medium from \textit{GATA4}-deficient LCs, implying that a hormone mediates the process. Preliminary results suggest that a loss of LC-derived estrogen in the conditioned media accounts for the effect. If this counterregulatory mechanism also operates in human LCs, it could contribute to inadequate androgen suppression in patients who undergo ADT with GnRH analogues.

2. KEYWORDS:

androgen; estrogen; hydroxysteroid dehydrogenase; gonadotropin; Leydig cell; prostate cancer; steroidogenesis; testosterone

3. ACCOMPLISHMENTS:

What were the major goals of the project?

<table>
<thead>
<tr>
<th>Activity</th>
<th>Completion date or % completed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specific Aim 1</strong>: Assess the impact of \textit{Gata4} silencing on the expression of \textit{Hsd17b3} in BLTK1 murine Leydig tumor cells and in primary cultures of murine adult LCs.</td>
<td></td>
</tr>
<tr>
<td><strong>Major Task 1: Perform a comprehensive biochemical analysis</strong></td>
<td></td>
</tr>
<tr>
<td>Subtask 1: Measurement of steroidogenic transcripts using qRT-PCR</td>
<td>Sept 2015</td>
</tr>
<tr>
<td>Cell lines used: primary murine adult LCs, BLTK1 cells</td>
<td></td>
</tr>
<tr>
<td>Subtask 2: Measurement of enzyme activities</td>
<td>80%</td>
</tr>
<tr>
<td>Cell lines used: primary murine adult LCs, BLTK1 cells</td>
<td></td>
</tr>
<tr>
<td>Subtask 3: Measurement of steroid hormone levels using LC-MS/MS</td>
<td>50%</td>
</tr>
<tr>
<td>Cell lines used: primary murine adult LCs, BLTK1 cells</td>
<td></td>
</tr>
<tr>
<td>\textit{Milestone(s)} Achieved: Measurement of the aforementioned items</td>
<td>80%</td>
</tr>
</tbody>
</table>
Specific Aim 2: Characterize the factor in conditioned medium that is responsible for the paradoxical upregulation of Hsd17b3 expression in response to Gata4 silencing.

Major Task 2: Identify the paracrine or autocrine factor

Subtask 1: Assess the impact of conditioned media on expression of Hsd17b3 and other steroidogenic genes using different combinations of donor and recipient cells.

Cell lines used: primary murine adult LCs, BLTK1 cells

Subtask 2: Subject conditioned medium to simple biochemical purification strategies (dialysis, extraction, ammonium sulfate precipitation, etc).

Cell lines used: primary murine adult LCs, BLTK1 cells

Milestone(s) Achieved: Characterization of the factor in conditioned medium

What was accomplished under these goals?

1) Major activities: The focus of our work has been laboratory research. We have made significant progress on both Specific Aims of this 1-year exploratory grant, as detailed below. This summary includes the 3-month no cost extension.

2) Specific objectives:

   Specific Aim 1: Assess the impact of Gata4 silencing on the expression of Hsd17b3 in BLTK1 murine Leydig tumor cells and in primary cultures of murine adult LCs.

   Specific Aim 2: Characterize the factor in conditioned medium that is responsible for the paradoxical upregulation of Hsd17b3 expression in response to Gata4 silencing.

3) Significant results or key outcomes:

A. Gene silencing of Gata4 in primary LCs leads to a paradoxical upregulation of Hsd17b3 (Major Task 1, Subtask 1). Primary LCs were isolated from adult Gata4<sup>flox/flox</sup> mice. In vitro recombination of the floxed Gata4 alleles was achieved by incubation with adenovirus expressing Cre-GFP; virus expressing GFP alone was used as a negative control. Following infection, cells were washed extensively and then incubated with fresh medium. GFP expression was used to assess infection efficiency. The levels of key steroidogenic transcripts were monitored by qRT-PCR. Early genes in the testosterone biosynthetic pathway (Cyp11a1, Hsd3b1, and Cyp17a1) were downregulated in response to Gata4 deletion (Schrade et al., Endocrinology, 2015). In contrast, Hsd17b3 was significantly upregulated (Figure 1).
Confirmatory analyses using enzyme assays (Major Task 1, Subtask 2) has been completed. Owing to technical challenges with mass spectrometry, we have not completed the steroid hormone measurements (Major Task 1, Subtask 3). We remain optimistic that these will be completed soon.

B. Gene silencing of *Gata4* in BLTK1 cells leads to a paradoxical upregulation of *Hsd17b3* (Major Task 1, Subtask 1). To independently validate the primary LC experiments, siRNA was used to silence *Gata4* in the BLTK1 murine Leydig tumor cell line. BLTK1 cells retain key LC characteristics including expression of *Hsd17b3* and production of testosterone. As in primary LCs, depletion of GATA4 in BLTK1 cells was associated with decreased expression of genes in the proximal testosterone biosynthetic pathway (*Cyp11a1, Hsd3b1*, and *Cyp17a1*) and increased expression of *Hsd17b3*. Because the basal and induced levels of *Hsd17b3* expression were much lower in BLTK1 cells than in primarily LCs, we opted to focus on primary cells for the ensuing experiments. Thus, the stated goals of performing enzyme assays (Major Task 1, Subtask 2) and steroid hormone measurements (Major Task 1, Subtask 3) on BLTK1 cells were not pursued.

C. A soluble factor mediates this counterregulatory response in primary LCs (Major Task 2, Subtask 1). The paradoxical increase in *Hsd17b3* expression was recapitulated when wild-type (WT) LCs (lacking floxed alleles) were incubated with conditioned medium from conditional knockout (cKO) LCs, suggesting that a secreted factor mediates the upregulation of *Hsd17b3* (Figure 2).
Figure 2. Reciprocal “supernatant swap” experiments show that a soluble factor mediates the upregulation of \(\text{Hsd17b3}\). Adult LCs were isolated from \(\text{Gata4}^{\text{floxed/floxed}}\) (\(\text{Gata4}^{\text{F/F}}\)) and WT mice. \textit{In vitro} recombination of floxed alleles was achieved by incubation with adenovirus expressing Cre-GFP or GFP alone. Following infection, cells were washed extensively and then incubated with fresh medium. Conditioned medium was harvested 24 h later from cKO cells later transferred to a separate dish of “reporter” WT cells. GFP expression was used to monitor infection efficiency and to control for cross-contamination of conditioned medium with adenovirus. The reciprocal experiment was conducted with conditioned media from WT cells and cKO reporter cells. Expression levels of key steroidogenic genes in the reporter cells were measured by qRT-PCR after 2 days of exposure to conditioned media (**, \(P < 0.01\)).

\textbf{D. Preliminary results suggest that estrogen is the secreted factor that mediates this response (Major Task 2, Subtask 2).} Mice harboring a null mutation in the estrogen receptor-\(\alpha\) gene exhibit enhanced expression of \(\text{Hsd17b3}\). Therefore, we hypothesized that the paradoxical upregulation of \(\text{Hsd17b3}\) expression could reflect a lack of estrogen production by the knockdown LCs. To test this possibility we repeated the supernatant swap experiment; the addition of 100 \(\mu\)M estradiol to conditioned medium from cKO cells mitigated the upregulation of \(\text{Hsd17b3}\) in WT reporter cells.

\textbf{4) Other achievements: none}

\textbf{5) Future directions:} In upcoming experiments that were not proposed in the original application, we will determine whether treatment of WT primary LCs with an aromatase inhibitor recapitulates the paradoxical upregulation of \(\text{Hsd17b3}\) seen with silencing of \(\text{Gata4}\). To determine whether this upregulation occurs \textit{in vivo}, we plan to inject the interstitial compartment of the testis in an anesthetized mouse with adenovirus expressing Cre-GFP; adenovirus
expressing GFP alone will be injected as a negative control. Testicular mRNA will be harvested 2 days later and subjected to qRT-PCR analysis.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Testosterone, a steroid hormone, plays a key role in the development, growth, and progression of prostate cancer. Testosterone is synthesized mainly by Leydig cells in the testis. The use of drugs to suppress testosterone production, a treatment termed chemical castration, is a mainstay of therapy for advanced prostate cancer. Unfortunately, these drugs do not adequately suppress testosterone production in a subset of men with prostate cancer, and this compromises their survival. Using mouse LCs, which are easier to isolated and manipulate than their human counterparts, we have shown that one of the key genes involved in testosterone synthesis, \textit{Hsd17b3}, is subject to a distinctive form of counterregulation. If this same mechanism is operational in human LCs, it could limit the efficacy of ADT. This possibility justifies the further investigation of this phenomenon using both mouse models and cultured human LCs. The notion that decreased estrogen production by LCs triggers the counterregulatory response is provocative, particularly in light of the use of estrogen as an alternative form of ADT. Estrogen is presumed to act via feedback inhibition of the hypothalamic-pituitary-gonadal axis, but our results suggest that estrogen may have a direct inhibitory effect on expression testosterone biosynthesis. Thus, combination ADT with a GnRH analogue and estrogen might prove more effective than either drug alone.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Not Applicable

Changes that had a significant impact on expenditures

We secured a 3-month no-cost extension to complete the steroid hormone measurements. All the samples have been collected, but the mass spectrometric analyses have not been completed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not Applicable

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

• Publications, conference papers, and presentations

Journal publications


Books or other non-periodical, one-time publications

Nothing to Report

Other publications, conference papers and presentations

Nothing to Report

• Website(s) or other Internet site(s)

Researchgate (a widely used site for dissemination of articles and datasets)

https://www.researchgate.net/profile/David_Wilson33

• Technologies or techniques

Nothing to Report

• Inventions, patent applications, and/or licenses

Nothing to Report

• Other Products

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Dr. David Wilson
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): ORCID ID 0000-0002-1826-7745
Nearest person month worked: 3
Contribution to Project: Dr. Wilson oversaw experimental design and data interpretation.
Funding Support: Institutional Support

Name: Dr. Markku Heikinheimo
Project Role: Unpaid consultant
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 0
Contribution to Project: Dr. Heikinheimo assisted with the gene silencing experiments
Funding Support: Sigrid Jusélius Foundation
Academy of Finland

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Completed grant since last report:
American Heart Association, Grant-in-Aid, 13GRNT16850031
“Regulation of steroidogenic cell differentiation in the mouse”
David Wilson, PI
7/1/2013 - 6/30/2015

New award due to start later this year:
Department of Defense, Ovarian Cancer Research Program Pilot Award OC150105
“Ovarian Granulosa Cell Tumor: New Insights into the Clinical Challenge of Late Relapse”
David Wilson, PI
07/15/2016 – 07/14/2018

What other organizations were involved as partners?

Organization name: University of Helsinki
Location: Helsinki, Finland
Contribution to the project: Collaboration (with Dr. Heikinheimo)

8. SPECIAL REPORTING REQUIREMENTS: NONE

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.
Curriculum Vitae

David B. Wilson, M.D., Ph.D.

Date: 7/13/2016 3:23:26 PM

Personal Information

Birthplace: Berkeley, CA
Citizenship: USA

Address and Telephone Numbers

University: Washington University in St. Louis
School of Medicine
Department of Pediatrics
Hematology and Oncology
660 S. Euclid
Campus Box 8208
St. Louis, MO 63110
Phone: 314-286-2834
Fax: 314-454-2780
eMail: wilson_d@wustl.edu

Present Positions

Professor of Pediatrics, Hematology and Oncology

Education and Training

1976 - 1980 B.A., Chemistry, Kalamazoo College, Kalamazoo, MI
1980 - 1986 M.D., Medicine, Washington University School of Medicine, St. Louis, MO
1980 - 1986 Ph.D., Biochemistry, Washington University School of Medicine, St. Louis, MO
1986 - 1988 Pediatric Resident, Boston Children's Hospital, Boston, MA
1988 - 1991 Hematology/Oncology Fellow, Dana Farber Cancer Inst, Boston, MA

Academic Positions and Employment

1991 - 1992 Instructor of Pediatrics, Harvard University, Boston, MA
1992 - 1997 Assistant Professor of Pediatrics, Hematology and Oncology, Washington University in St. Louis, St. Louis, MO
1997 - 2003 Division Chief, Hematology and Oncology, Washington University in St. Louis, St. Louis, MO
1997 - 2015 Associate Professor of Pediatrics, Hematology and Oncology, Washington University in St. Louis, St. Louis, MO
2016 - Pres Professor of Pediatrics, Hematology and Oncology, Washington University in St. Louis, St. Louis, MO

Appointments and Committees

NIH Study Sections
1995 - 2001 American Heart Association Great American Consortium
1998 - 1999 NHLBI PPG
2000 - 2010 Hope Street Kids Foundation
2000 - 2001 NIH Special Emphasis Panel (CCVS 01)
2007 - 2008 NHLBI PPG
2009 - 2009 Medical Research Council, UK
2014 - Pres Alex's Lemonade Stand Foundation
2014 - 2014 Department of Defense -- Bone Marrow Failure Research Program

Local Appointments
1994 - Pres American Cancer Society, Intramural Grant Program, Study Section member
2008 Data Safety & Monitoring Committee (DSMC) for HSC # 01-0923 “Study of hematopoietic stem cell transplantation (HSCT) in non-malignant disease using a reduced-intensity preparatory regime”
2013 - Pres Cancer Frontier Fund, Siteman Cancer Center, Study Section member

University Affiliations
1996 - 2001 MSTP Admissions Committee
1997 - 2005 Head, American Cancer Society Intramural Grant Program
2006 - 2013 Benefits Committee
2008 - Pres Co-Leader, Developmental Biology and Genetics Research Unit
2010 - Pres Director, Student Exchange Program with Hochschule Mannheim -- University of Applied Sciences
2014 - Pres Co-Director, Pediatric Student Research Program (PSRP)
Hospital Affiliations
1994 - 2002 Medical Director, St. Louis Children's Hospital Blood Bank
2014 - Pres BJC System Anticoagulation Committee

Scientific Advisory Boards
2000 - 2010 Hope Street Kids (Pediatric Oncology Grants)

Thesis Committees (Chair) Advisor
1996 - 1997 Naoko Narita, M.D. D. Wilson, M.D., Ph.D.
2001 - 2003 Alexander Ungewickle P. Majerus, M.D.
2003 - 2005 David Grenda D. Link, M.D.
2004 - 2006 Jesse Lugus K.-C. Choi, Ph.D.
2006 - 2007 Ellen Langer G. Longmore, M.D.
2006 - 2008 Erik Madsen J. Gitlin, M.D.
2008 - 2009 Chin-Chen Liu G. Bu, Ph.D.
2009 - 2010 Antti Kyrölähti, M.D. M. Heikinheimo, M.D., Ph.D
2009 - 2010 Justyna Krachulec D. Wilson, M.D., Ph.D.
2009 - 2011 Daniel Chen P. Jay, M.D., Ph.D.
2010 - 2011 Anja Schrade D. Wilson, M.D., Ph.D.
2010 - 2013 Eric Schoeller K. Moley, M.D.
2011 - 2012 Marjut Pihtlajoki M. Heikinheimo, M.D., Ph.D.
2011 - 2014 Maximilian Schillebeeckx R. Mitra, Ph.D.
2012 - 2013 Elisabeth Gretzinger D. Wilson, M.D., Ph.D.
2012 - 2016 Anja Schrade M. Heikinheimo, M.D., Ph.D.
2013 - 2014 Theresa Röhrig D. Wilson M.D., Ph.D.
2014 - Pres Mark Valentine T. Druley, M.D., Ph.D.
2014 - 2014 Claire Schulkey P. Jay, M.D., Ph.D.
2014 - 2015 Julia Dörner D. Wilson, M.D., Ph.D.

Scholarship Oversight Committees
2008 - 2011 Ghada Kunter, M.D. (Advisor: D. Link, M.D.)
2008 - 2013 Todd Druley, M.D., Ph.D. (Advisor: R. Mitra, Ph.D.)
2009 - 2013 Laura Scheuttpelz M.D., Ph.D. (Advisor: D. Link, M.D.)
2010 - 2012 Edward Dela Ziga (Advisor: J. DiPersio M.D., Ph.D.)
2010 - Pres Todd Druley, M.D., Ph.D. (Advisor: R. Mitra, Ph.D.)
2010 - 2012 Mark Schroeder, M.D. (Advisor: J. DiPersio, M.D., Ph.D.)
2011 - 2012 Leili Dolatshahi, M.D. (Advisor: E. Sadler, M.D., Ph.D.)
2012 - 2014 Alexander Ngwube, M.D. (Advisor: M. Dinauer, M.D., Ph.D.)
2013 - 2015 Mark Levin, M.D. (Advisor: C. Nichols, Ph.D.)
2013 - Pres Monica Hulbert, M.D. (Advisor: R. Hayashi, M.D.)
2013 - 2015 Hoang Nguyen, M.D. (Advisor: P. Jay, M.D., Ph.D.)
2013 - 2016 Alok Kothari, M.D. (Advisor: G. Challen, Ph.D.)

Licensure and Certifications
1993 - Pres MO Physician #103195
1993 - 2008 Pediatrics
1996 - 2020 Pediatric Hematology/Oncology
2015 - Pres IL Physician #036.139375

Honors and Awards
1980 Honors in Chemistry, Kalamazoo College
1980 Phi Beta Kappa
1981 Carter Prize for Achievement in the Medical Curriculum
1982 Lowry Prize in Pharmacology
1986 Alpha Omega Alpha
1992 McDonnell Scholars Award in Oncology
1992 Pfizer Junior Faculty Award in Cardiovascular Biology
1993 March of Dimes Basil O'Connor Award
1995 American Heart Association Established Investigator Award
1998 American Society for Clinical Investigation
Editorial Responsibilities

Editorial Ad Hoc Reviews
1995 - Pres  Development
1999 - Pres  Dev Biol
2004 - Pres  Pediatr Res
2007 - Pres  Mol Cell Endocrinol
2008 - Pres  Eur J Endocrinol
2008 - Pres  Int J Cancer
2008 - Pres  J Mol Endocrinol
2008 - Pres  Ped Blood Cancer
2011 - Pres  Blood
2011 - Pres  Histopathol
2011 - Pres  Pediatr Pulmonol
2012 - Pres  Andrology
2012 - Pres  J Cell Sci
2012 - Pres  PLoS One
2013 - Pres  Endocrinology
2013 - Pres  Histology and Histopathology
2013 - Pres  Biol Repro
2013 - Pres  Tumor Biology
2014 - Pres  Hum Mol Genet
2014 - Pres  Expert Review of Endocrinology & Metabolism
2014 - Pres  Reproduction
2014 - Pres  Cancer Causes & Control
2014 - Pres  J Ped Hematol Oncol
2015 - Pres  Frontiers in Endocrinology
2015 - Pres  Am J Pathol
2015 - Pres  Mol Biol Cell
2015 - Pres  Nat Commun
2015 - Pres  Stem Cells International
2015 - Pres  Veterinary Quarterly
2016 - Pres  Am J Med Genet

Editorial Boards
2012 - 2016 Editorial Board, Mol Cell Endocrinol
2014 - 2016 Guest Editor, Special issue of Mol Cell Endocrinol, "Impact of maternal metabolism on newborn health"

Professional Societies and Organizations
1994 - 2008 American Society for Biochemistry and Molecular Biology
1995 - Pres American Society for Hematology
1995 - Pres Pediatric Oncology Group/Children's Oncology Group
1998 - Pres American Heart Association
1998 - Pres American Society for Clinical Investigation
2012 - Pres Endocrine Society

Major Invited Professorships and Lectures
1993  Keystone Symposium in Cardiovascular Biology, Taos, NM
1995  AHA Symposium on Cardiac Development, New Orleans, LA
1997  Dana-Farber Cancer Institute 50th Anniversary Symposium, Boston, MA
2003  Finnish Endocrine Society, Espoo, Finland
2003  International GATA Factor Meeting, Montreal, Canada
2011  3rd International Conference on Adrenocortical Tumors, Würzburg, Germany
2014  Adrenal 2014 Symposium, Chicago, IL
2015  J. Lester Gabrilove Memorial Lecture, Mt. Sinai Hospital, New York, NY
Research Support

Governmental Support

Ovarian Cancer Research Program Pilot Award OC150105
Department of Defense
Ovarian Granulosa Cell Tumor: New Insights into the Clinical Challenge of Late Relapse
This project uses a mouse model and patient data to explore the role of adjuvant therapy in the management of this tumor.

T32 HD07499 (Alan Schwartz) 4/1/1999 - 3/31/2019
NIH
Training Program in Developmental Hematology
This training grant supports the education of physician-investigators in Hematology.

Completed Support

R01 DK075618 (David Wilson) 4/1/2007 - 3/30/2013
NIH
Regulation of Steroidogenic Cell Differentiation in the Mouse

P50 HL61001 (David Wilson) 1/1/1999 - 12/31/2003
NIH
Regulation and Expression of GATA Transcription Factors

R01 HL52134 (David Wilson) 5/1/1994 - 4/30/1999
NIH
Embryonic Heart Development: Role of GATA-4

Department of Defense
A Counterregulatory Mechanism Impacting Androgen Suppression Therapy

GIA 13GRNT16850031 (David Wilson) 7/1/2013 - 6/30/2015
American Heart Association
Regulation of steroidogenic cell differentiation in the mouse

Pilot Translational Grant (Monica Bessler) 6/1/2009 - 5/31/2010
ICTS
Running the Stop in Bone Marrow Failure

Grant in Aid 0455623Z (David Wilson) 7/1/2004 - 6/30/2006
American Heart Association
Adrenocortical Cell Differentiation and Tumorigenesis

Mallinckrodt Foundation
Molecular Basis of Bone Marrow Failure

Institutional Research Grant IRG-58-010-43 (David Wilson) 7/1/1999 - 6/30/2005
American Cancer Society
Institutional Research Grant

Basic Research Grant (David Wilson) 6/1/2002 - 5/31/2005
March of Dimes
Role of GATA-4 in Stomach Development
Pilot Grant (David Wilson) 1/1/2003 - 12/1/2004
BJH Foundation
Molecular Basis of Familial Myelodysplastic Syndrome

Grant in Aid (David Wilson) 1/1/1998 - 12/31/2000
American Heart Association
Endoderm-Mesoderm Interactions During Mouse Heart Development

Established Investigator Award (David Wilson) 7/1/1995 - 6/30/2000
American Heart Association
Cardiac Transcription Factor GATA-4 and the Disorganization Mouse

Basic Grant (David Wilson) 1/1/1997 - 12/31/1999
Monsanto-WU Biomedical Agreement
Regulation of Vascular Smooth Muscle by Transcription Factor GATA-6

Basic Research Grant (David Wilson) 7/1/1996 - 6/30/1998
March of Dimes
Mammalian Yolk Sac Development

Grant in Aid (David Wilson) 7/1/1994 - 6/30/1997
American Heart Association
Transcription Factors in Embryonic Heart Development

Basic Grant (David Wilson) 1/1/1994 - 12/31/1996
Monsanto-WU Biomedical Agreement
Biochemical Mechanisms of Cardiac Morphogenesis and Tissue Injury

Basil O'Connor Award (David Wilson) 9/1/1993 - 8/31/1995
March of Dimes
Embryonic Heart Development

Scholar's Award (David Wilson) 7/1/1992 - 6/1/1995
McDonnell Foundation
Regulation of Endothelial Cell Gene Expression

Scholars Program (David Wilson) 7/1/1992 - 6/30/1994
Pfizer, Inc.
Regulation of Preproendothelin-1 Gene Expression

R01 CA105312 (Monica Bessler) 5/1/2004 - 4/30/2014
NIH
Molecular Determinants of Bone Marrow Failure

Basic Research Grant MD-II-2009-174 (Monita Wilson) 2/1/2009 - 1/30/2012
Children's Discovery Institute
A Novel Mouse Model Linking Aberrant Inositol Cycling to Neural Tube Defects

Current Trainees
2016  Anja Schrade, Ph.D., Postodtoral Fellow, Role of GATA factors in testicular development and function
Past Trainees

2004 - 2006  Joshua Fields, M.D., M.S., Clinical Research Trainee
Study area: Molecular basis of bone marrow failure and pulmonary hypertension
Present position: Associate Professor, Department of Internal Medicine, Medical College of Wisconsin, Milwaukee, WI

2009 - 2010  Antti Kyronlahti, M.D., Ph.D., Postdoctoral Fellow
Study area: Role of GATA4 in ovarian and testicular development
Present position: Resident in Pediatrics, University of Helsinki Affiliated Hospitals, Helsinki, Finland

2000 - 2003  Elena Genova, Ph.D., Postdoctoral Fellow
Study area: Regulation of transcription factor expression
Present position: Staff Scientist, Abbott Pharmaceuticals, Chicago, IL

2003 - 2004  Leslie A. Andritsos, M.D., Postdoctoral Fellow
Study area: TERC mutations in bone marrow failure
Present position: Assistant Professor of Internal Medicine, Ohio State University, Columbus, OH

1994 - 1996  Naoko Narita, M.D., Postdoctoral Fellow
Study area: Role of GATA factors in endoderm-mesoderm interactions
Present position: Associate Professor, Tsukuba University, Tsukuba, Japan

1994 - 1995  Patricia M. Gearhart, D.V.M., Ph.D., Postdoctoral Fellow
Study area: GATA transcription factor biology
Present position: Veterinary Ophthalmologist, Oakland Veterinary Referral Services, Bloomfield Hills, MI

Study area: Ferret adrenocortical tumors
Present position: Director, Molecular and Ultrastructural Pathology, GlaxoSmithKline, Raleigh-Durham, NC

1997 - 2003  Christina Jacobsen, M.D., Ph.D., Graduate Student
Study area: GATA factors in endoderm development and function
Present position: Instructor of Endocrinology, Boston Children's Hospital, Boston, MA

2012 - 2012  Elisabeth Gretzinger, M.Sc., Graduate Student
Study area: Mouse adrenocortical development
Present position: Staff Scientist, Pharmaceutical Company, Munich, Germany

2014 - 2015  Julia Dörmer, M.Sc., Graduate Student
Study area: Adrenocortical stem cells
Present position: Student, Hochschule Mannheim, Mannheim, Germany

2010 - 2010  Justyna Krachulec, M.Sc., Graduate Student
Study area: Role of GATA4 in post-gonadectomy adrenocortical neoplasia
Present position: Graduate Student, Heidelberg University, Heidelberg, Germany

2011 - 2012  Marjut Pihlajo, M.Sc., Graduate Student
Study area: Role of GATA factors in adrenocortical development and tumorigenesis
Present position: Graduate Student, University of Helsinki, Helsinki, Finland

2011 - 2014  Maximilian Schillebeeckx, Ph.D., Graduate Student
Study area: Epigenetics of adrenocortical neoplasia in the inbred mouse
Present position: Post-doctoral fellow, Washington University

2013 - 2014  Theresa Röhrig, M.Sc., Graduate Student
Study area: Cell fate during adrenal development
Present position: Student, Mannheim University of Applied Sciences

2013 - 2013  Franziska Thol, B.Sc., Rotation Student
Study area: Genes implicated in adrenocortical development in the mouse
Present position: Student, Hochschule Mannheim – University of Applied Sciences, Mannheim, Germany

2009 - 2009  Melanie Vetter, B.Sc., Rotation Student
Study area: GATA factors and steroidogenesis
Present position: PhD Candidate, Max Planck Institute of Biochemistry, Munich, Germany

2014 - 2014  Ricarda Ziegler, B.Sc., Rotation Student
Study area: Adrenocortical stem cell lineage tracing
Present position: Student, Hochschule Mannheim, Mannheim, Germany

2015 - 2016  Ronni Götz, B.Sc., Rotation Student
Study area: Regulation of steroidogenesis
Present position: Undergraduate student, Hochschule Mannheim

2010 - 2011  Rosemarie Euler, B.Sc., Rotation Student
Study area: Role of GATA4 in steroidogenic cell development and function
Present position: Staff scientist, Pharmaceutical Industry, Mannheim, Germany

2008 - 2008  Simone Wagner, B.Sc., Rotation Student
Study area: Cyt b5 in adrenocortical tumors of the ferret
Present position: Staff scientist, Absolvent, Mannheim, Germany

2012 - 2013  Theresa Hiller, B.Sc., Rotation Student
Study area: Novel markers of adrenocortical tumorigenesis in the mouse
Present position: Student, Hochschule Mannheim, Mannheim, Germany

2015 - 2015  Verena Martinez Rodriguez, B.Sc., Rotation Student
Study area: Hedgehog signaling in steroidogenic cells
Present position: Student, Hochschule Mannheim, Mannheim, Germany

1994 - 2006  Markku Heikinheimo, M.D., Ph.D., Other
Study area: Research sabbatical; GATA4 in cardiac development
Present position: Professor, University of Helsinki, Helsinki, Finland
Clinical Responsibilities

1993 - Pres Attending physician, Hematology-Oncology, Pediatrics, Washington University School of Medicine and St. Louis Children's Hospital
2010 - Pres Director, Comprehensive Hemophilia Treatment Center, Pediatrics, Washington University and St. Louis Children's Hospital
2012 - Pres Attending Physician, Sickle Cell Program, Pediatrics, Washington University

Teaching Responsibilities

1995 - Pres Lecturer, "Chemotherapy" (1.5 hr/yr), Hematology-Oncology Pathobiology Course for 2nd year med students
1995 - Pres Teaching Laboratory (3 hr/yr), Hematology-Oncology Pathobiology Course for 2nd year med students
2003 - Pres Course Master, Special Emphasis Pathway in Cancer Biology L41 5196 BIOL (alternate years)
2011 - Pres Discussion Leader – Hematology-Oncology Case Studies (8 hr/yr), Pediatric Clinical Clerkship Students and Residents
2014 - Pres Lecturer, "Transcription and mRNA processing", (1.5 hr/yr), Molecular Foundations of Medicine for 1st year med students

Publications


**Invited Publications**


Books


Book Chapters (most recent editions)


In this issue of *Endocrinology*, Padua et al (1) characterize the phenotype of mice lacking both GATA4 and GATA6 in steroidogenic cells. The double-mutant mice are born with adrenal aplasia. The female pups die from adrenocortical insufficiency, but the males survive owing to ectopic corticoid production by adrenal-like cells in the testis. These mutant mice shed new light on the regulation of steroidogenic cell differentiation and may provide a model for the study of testicular adrenal rest tumors (TARTs).

Steroidogenic cells in the adrenal cortex and testis arise from a common pool of progenitors in the adrenogonadal primordium (AGP), a specialized group of coelomic epithelial cells in the urogenital ridge (Figure 1) (2). Adrenocortical progenitors migrate medially and combine with sympathoblasts, the precursors of the medulla, to form the nascent adrenal gland, which begins to produce glucocorticoids and other steroids (3). Gonadal progenitors migrate laterally and combine with primordial germ cells to form the bipotential gonad. Expression of *Sry* in the male gonad triggers the differentiation of Sertoli cells, which nurture germ cells and secrete paracrine factors that promote the differentiation of steroidogenic Leydig cells (4). In addition to producing T that is crucial for masculinization of the male fetus, fetal Leydig cells secrete insulin like-3, a hormone that promotes testicular descent (5).

The common developmental origin of the adrenal cortex and testis is reflected in overlapping functional profiles for these organs. For example, the testes of newborn mice contain interstitial cells that express adrenocortical differentiation markers (eg, *Cyp21a1*, *Cyp11b1*), and ACTH stimulates androgen and glucocorticoid production by this tissue (6–8). Conversely, the adrenal gland of the adult mouse harbors rare stem/progenitor cells that can differentiate into gonadal-like cells in response to the hormonal changes that accompany gonadectomy (9–11).

Among the plethora of transcription factors implicated in the differentiation of adrenal or testicular steroidogenic cells, a few are indispensable (3, 12). The prototype of these is steroidogenic factor-1 (SF1; also called Ad4BP or NR5A1). *Sf1* is expressed in the AGP, adrenocortical cells, Sertoli cells, and Leydig cells. Mice lacking SF1 exhibit defects in both adrenal and testicular development (13, 14). Because SF1 is expressed in all steroidogenic tissues, it cannot by itself account for functional differences between steroid-producing cells in the adrenal cortex and testis. Two members of the GATA transcription factor family, GATA4 and GATA6, also regulate steroidogenesis and have been shown to impact the balance between adrenal and gonadal differentiation (11). During fetal mouse development, Gata4 and Gata6 are coexpressed in adrenocortical cells, Sertoli cells, and Leydig cells (15, 16). After birth, Gata6 expression persists in the adrenal cortex and testis, but Gata4 expression wanes in the adrenal gland (15, 16).

Mice harboring germline homozygous null mutations in either Gata4 or Gata6 die early in embryonic development, so Cre-LoxP technology has been used to investigate the roles of GATA4 and GATA6 in steroidogenic tissues (15). These studies have shown that GATA4 is required for genital ridge development, testicular morphogenesis, and fetal/adult Sertoli cell function (17–19). In contrast, loss of GATA6 in steroidogenic cells mainly impacts adrenocortical zonation and function (20). As detailed in this issue of *Endocrinology*, combined loss of GATA4 and GATA6...
in SF1\(^{+}\) cells causes striking defects in both adrenal and testicular development (Figure 2) (1). The double-mutant mice lack adrenal glands. The female mutants die due to adrenocortical insufficiency. The males survive and exhibit a dramatic gonadal phenotype that includes severe testicular hypoplasia, cryptorchidism, disorganized seminiferous tubules, germ cell depletion, Leydig cell loss, and impaired T production. Intriguingly, nests of cells that express adrenocortical markers (\textit{Cyp21a1}, \textit{Cyp11b1}, \textit{Cyp11b2}, and the ACTH receptor \textit{Mc2r}) accumulate in the testes of double-mutant mice postnatally and persist into adulthood, suggesting that males escape death because of ectopic adrenocorticoid production in the testis.

The adrenocortical-like cells that accumulate in the testes of the double-mutant males resemble TARTs, neoplastic nodules of hormonally active adrenocortical tissue that develop in men with 21-hydroxylase deficiency (21–23). These neoplasms are benign but can obstruct the rete testis, leading to testicular degeneration and infertility. TARTs are thought to arise from multipotential stem/progenitor cells that reside in the testis (6). RNA analysis has shown that TARTs express adrenocortical differentiation markers (\textit{CYP11B1}, \textit{CYP11B2}, \textit{MC2R}) at 1000–10 000 higher levels than adjacent testicular tissue (23). TARTs also express Leydig cell markers, such as the LH/chorionic gonadotropin receptor (23). The endocrine and paracrine factors that drive TART formation are not fully understood. Elevated ACTH levels can promote TART growth, but a longitudinal analysis of men with 21-hydroxylase deficiency found no association between the presence of TARTs and parameters of disease control with exogenous glucocorticoids ± mineralocorticoids (24). Thus, other signaling molecules such as angiotensin II and gonadotropins are presumed to be involved in tumorigenesis. The presence of LH/chorionic gonadotropin receptor in TARTs may explain why growth of these tumors often coincides with the onset of puberty. \textit{Gata4/Gata6} double-mutant mice afford an experimentally tractable model to investigate the signals underlying TART formation and growth.

The phenotype of the double mutants raises a paradox. If the combination of GATA4 and GATA6 is essential for steroidogenic cell differentiation in the adrenal cortex, how can adrenocortical-like cells arise in the testes of the double mutants? Have the heterotopic cells evaded complete Cre-mediated recombination of \textit{Gata4} and \textit{Gata6}? Does the testis have an alternative route of adrenocortical cell differentiation that circumvents the need for \textit{Gata4} and \textit{Gata6}? Future experiments are likely to address this conundrum and explore the mechanistic basis for the phenotypic abnormalities in the double-mutant mice, including identification of genetic targets for GATA factors in steroidogenic cells. Studies in different cell types have implicated GATA4 and GATA6 in not only steroid production but also a vast array of other cellular processes, such as energy metabolism, apoptosis, polarization, and cytokinesis (25–33). By recruiting functionally distinct classes of transcriptional cofactors to promoter/enhancer sites in DNA, GATA factors can either activate or repress gene expression, depending on the context (34).
In summary, the findings of Padua et al (1), coupled with prior reports (20, 30, 35, 36), show that GATA4 and GATA6 function synergistically during steroidogenic cell development and play key roles in establishing the cellular identities of adrenocortical and gonadal cells. The Gata4/Gata6 double mutant expands the list of mouse models exhibiting ectopic development of steroid-producing cells (11); such models have proven invaluable in elucidating the molecular regulation of steroidogenic cell fate. From a therapeutic perspective, testicular progenitors with the capacity for adrenal-like differentiation represent a population of developmentally primed cells that could be manipulated to reconstitute function in patients with adrenocortical failure.

Acknowledgments
We thank Joshua Rubin, Patrick Jay, and Paul Hruz for helpful comments.

Address all correspondence and requests for reprints to: David B. Wilson, MD, PhD, Box 8208, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110. E-mail: wilson_d@wustl.edu.

This work was supported by American Heart Association Grant 13GRNT16850031, Department of Defense Grant PC141008, the Sigrid Jusélius Foundation, the Academy of Finland, DAAD (German Academic Exchange Service) Fellowship D/12/40505, and Centre for International Mobility Finland Fellowship TM-13-8769.

Disclosure Summary: The authors have nothing to disclose.

References

2. Hatano O, Takakusu A, Nomura M, Morohashi K. Identical origin...
5. Teerds KJ, Huhtaniemi IT. Morphological and functional maturation of Leydig cells: from rodent models to primates. Hum Reprod Update. Published online ahead of print February 27, 2015. DOI: 10.1038/humupd/dnv008.
Review

GATA factors in endocrine neoplasia

Marjut Pihlajoki a, Anniina Färkkilä a,b, Tea Soini a, Markku Heikinheimo a,c, David B. Wilson c,d,*

a Children’s Hospital, Helsinki University Central Hospital, University of Helsinki, 00290 Helsinki, Finland
b Department of Obstetrics and Gynecology, Helsinki University Central Hospital, University of Helsinki, 00290 Helsinki, Finland
c Department of Pediatrics, St. Louis Children’s Hospital, Washington University School of Medicine, St. Louis, MO 63110, USA
d Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA

ARTICLE INFO

Article history:
Received 1 March 2015
Received in revised form 26 April 2015
Accepted 9 May 2015
Available online 28 May 2015

Keywords:
Adrenocortical tumor
Granulosa cell tumor
Parathyroid tumor
Pituitary adenoma
Sertoli– Leydig cell tumor

ABSTRACT

GATA transcription factors are structurally-related zinc finger proteins that recognize the consensus DNA sequence WGATAA (the GATA motif), an essential cis-acting element in the promoters and enhancers of many genes. These transcription factors regulate cell fate specification and differentiation in a wide array of tissues. As demonstrated by genetic analyses of mice and humans, GATA factors play pivotal roles in the development, homeostasis, and function of several endocrine organs including the adrenal cortex, ovary, pancreas, parathyroid, pituitary, and testis. Additionally, GATA factors have been shown to be mutated, overexpressed, or underexpressed in a variety of endocrine tumors (e.g., adrenocortical neoplasms, parathyroid tumors, pituitary adenomas, and sex cord stromal tumors). Emerging evidence suggests that GATA factors play a direct role in the initiation, proliferation, or propagation of certain endocrine tumors via modulation of key developmental signaling pathways implicated in oncogenesis, such as the WNT/β-catenin and TGFβ pathways. Altered expression or function of GATA factors can also affect the metabolism, ploidy, and invasiveness of tumor cells. This article provides an overview of the role of GATA factors in endocrine neoplasms. Relevant animal models are highlighted.

© 2015 Elsevier Ireland Ltd. All rights reserved.

Contents

1. Overview of GATA factors in development and disease ................................................................. 3
  1.1. Members of the vertebrate GATA family ..................................................................................... 3
  1.2. GATA factors as tumor markers .................................................................................................. 4
  1.3. GATA factors interface with signaling pathways involved in both normal development and tumorigenesis .................................................. 5
  1.4. GATA factor dysregulation can affect tumor cell migration, invasiveness, and ploidy .......... 5
  1.5. Focus of this article ....................................................................................................................... 5

2. Ovarian neoplasms .......................................................................................................................... 5
  2.1. Roles of GATA4 and GATA6 in ovarian development and function ........................................ 5
  2.2. Granulosa cell tumors .................................................................................................................. 5
  2.3. Sertoli–Leydig cell tumors of the ovary ...................................................................................... 6
  2.4. Thecoma–fibroma tumors of the ovary ....................................................................................... 7

3. Testicular neoplasms ....................................................................................................................... 7
  3.1. GATA factors implicated in testicular development and function ............................................ 7
  3.2. GATA4 is expressed in large-cell calcifying Sertoli tumors .................................................... 8
  3.3. GATA4 is expressed in canine testicular tumors ...................................................................... 8

MCE Special Edition: Animal Models of Endocrine Neoplasia
* Corresponding author: Washington University School of Medicine, Box 8208, 660 S. Euclid Ave., St. Louis, MO 63110, USA. Tel.: +1 314 286 2834; fax: +1 314 286 2892.
E-mail address: wilson_d@wustl.edu (D.B. Wilson).

http://dx.doi.org/10.1016/j.mce.2015.05.027
0303-7207 © 2015 Elsevier Ireland Ltd. All rights reserved.
1. Overview of GATA factors in development and disease

1.1. Members of the vertebrate GATA family

By regulating cell fate specification and differentiation, GATA transcription factors play important roles in eukaryotic development. The vertebrate GATA family comprises six members (GATA1–6) named in order of discovery (Patient and McChee, 2002). All bind to the consensus DNA element, WGATAA, known as the GATA motif (Bresnick et al., 2012). With the exception of Gata5, homozygous null mutations in genes encoding GATA family members result in embryonic lethality in mice, underscoring the vital roles that these transcription factors play in development (reviewed in Molkentin, 2000 and Viger et al., 2008). Human diseases associated with germline loss-of-function mutations in GATA factors are summarized in Table 1.

All vertebrate GATA proteins contain a pair of conserved zinc finger domains (Patient and McChee, 2002). The C-terminal finger is essential for DNA binding, whereas the N-terminal finger physically interacts with other transcriptional regulators such as the “Friend of GATA” factors FOG1 (ZFPM1) and FOG2 (ZFPM2) (Bresnick et al., 2012). GATA motifs are common in the genomes of vertebrates, but global chromatin immunoprecipitation studies in various cell types suggest that GATA factors occupy less than 1% of the consensus sites (Bresnick et al., 2012). This implies the existence of mechanisms that discriminate among these abundant motifs.

GATA factors can act as either transcriptional activators or repressors depending on the context (Viger et al., 2008). Although all GATA factors bind to the same DNA element, there is surprisingly little functional redundancy among these proteins (Zheng and Blobel, 2010). Individual GATA factors elicit distinctive functions through cooperative interactions with other transcriptional regulators. Functional diversity is also achieved via post-translational modifications (e.g., phosphorylation, acetylation, and sumoylation) that alter the intrinsic activity of different GATA factors (Viger et al., 2008).

During the differentiation of a particular lineage, a GATA motif in the promoter/enhancer of a gene may be occupied sequentially by more than one GATA factor. For example, as erythroid progenitors mature the stem cell factor GATA2 is displaced from specific sites by the terminal differentiation factor GATA1, a phenomenon termed a “GATA switch” (Bresnick et al., 2010). A similar switch is presumed to occur in intestinal epithelium, where GATA6 maintains stem/progenitor cells and GATA4 promotes terminal differentiation into mature enterocytes (Beuling et al., 2011). GATA switches are often

![Table 1](image-url)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human disease phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA1</td>
<td>X-linked dyserythropoietic anemia and thrombocytopenia</td>
<td>Crispino and Weiss (2014)</td>
</tr>
<tr>
<td></td>
<td>X-linked Diamond—Blackfan anemia</td>
<td>Bresnick et al. (2012); Spinner et al. (2014)</td>
</tr>
<tr>
<td>GATA2</td>
<td>Monocytopenia with mycobacterial infections (MonoMAC)</td>
<td>Ali et al. (2007); Nesbit et al. (2004); Van Esch et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Dendritic cell, B, and natural killer (NK) lymphoid deficiency</td>
<td>Loureiro et al. (2011); Prendiville et al. (2014); Shaw-Smith et al. (2014); Yu et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Familial MDS/AML</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Embryer syndrome (primary lymphedema with MDS)</td>
<td></td>
</tr>
<tr>
<td>GATA3</td>
<td>HDR syndrome (hypoparathyroidism, deafness, and renal dysplasia)</td>
<td></td>
</tr>
<tr>
<td>GATA4</td>
<td>Congenital heart disease</td>
<td>Loureiro et al. (2011); Prendiville et al. (2014); Shaw-Smith et al. (2014); Yu et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Congenital diaphragmatic hernia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monogenic diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aberrant testicular development</td>
<td></td>
</tr>
<tr>
<td>GATA5</td>
<td>Congenital heart disease</td>
<td>Shi et al. (2014)</td>
</tr>
<tr>
<td>GATA6</td>
<td>Congenital heart disease</td>
<td>Allen et al. (2012); Bonnefond et al. (2012); Maitra et al. (2010); Prendiville et al. (2014); Yu et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Congenital heart disease and/or monogenic diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pancreatic agenesis and/or monogenic diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biliary tract abnormalities</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Congenital diaphragmatic hernia</td>
<td></td>
</tr>
</tbody>
</table>

3.4. Gata6 is downregulated in an experimental model of Leydig cell adenoma .......................... 9
3.5. Testicular adrenal rest tumors .......................................................................................... 9
4. Adrenocortical neoplasms ....................................................................................................... 9
4.1. GATA6 and GATA4 have been implicated in adrenocortical development and function ........... 9
4.2. Gonadectomy-induced adrenocortical neoplasia .................................................................. 10
4.3. Dysregulation of GATA factors in other genetically-engineered mouse models of adrenocortical neoplasia ........................................................................................................ 11
4.4. Expression of GATA4 and GATA6 in human adrenocortical tumors ..................................... 12
5. Parathyroid neoplasms ............................................................................................................ 12
5.1. Role of GATA3 in parathyroid development ......................................................................... 12
5.2. GATA3 is a marker of parathyroid neoplasms ....................................................................... 12
6. Pancreatic neoplasms ............................................................................................................. 12
6.1. Role of GATA4 and GATA6 in pancreatic development .......................................................... 12
6.2. Gata6 is downregulated in insulinomas triggered by MEN1 deficiency in the mouse .......... 12
6.3. Roles of GATA6 and GATA4 in pancreatic ductal adenocarcinoma ...................................... 12
7. Pituitary neoplasms .................................................................................................................. 13
7.1. Role of GATA2 in pituitary development and function .......................................................... 13
7.2. GATA2 is a marker of pituitary neoplasms .............................................................................. 13
8. Summary and outlook .............................................................................................................. 14
Acknowledgments ....................................................................................................................... 14
References .................................................................................................................................. 14
associated with altered transcriptional output, emphasizing that different GATA factors can exert distinct functions at the same binding site (Bresnick et al., 2012). GATA switches occur not only during normal development but also in disease states, such as endometriosis. The transformation of endometrial stroma into endometriotic stroma is accompanied by an epigenetically-mediated switch from expression of GATA2 to GATA6 (Dyson et al., 2014).

1.2. GATA factors as tumor markers

Since GATA factors regulate genetic networks that can expand stem/progenitor cell populations or drive differentiation, it comes as no surprise that alterations in the expression or function of GATA factors have been linked to neoplastic transformation. Indeed, GATA factor genes have been shown to be mutated, overexpressed, or underexpressed in a wide range of solid tumors and leukemias (reviewed in Ayanbule et al., 2011; Bresnick et al., 2012; Viger et al., 2008; and Zheng and Blobel, 2010).

GATA factors can serve as tumor markers that shed light on the developmental origins, clinical behavior, and pathogenesis of certain neoplasms, as illustrated by studies of GATA3 in breast cancer and GATA2 in prostate cancer. GATA3 is abundantly expressed in luminal cells of the mammary epithelium but not their multipotent progenitors (Chou et al., 2010). Conditional deletion studies in the mouse have shown that Gata3 is required for branching morphogenesis and terminal differentiation of luminal epithelial cells (reviewed in Chou et al., 2010 and Zheng and Blobel, 2010). Interestingly, loss of Gata3 in adult mammary epithelium triggers de-differentiation of luminal cells, increased cell proliferation, and disorganization of ducts, features reminiscent of neoplastic transformation. In primary breast tumors low or absent GATA3 expression is associated with shorter patient survival and a host of negative prognostic indicators (primary tumor size, lymph node metastases, lack of estrogen receptor and progesterone receptor expression, etc.) (Chou et al., 2010). GATA2 is expressed in

Fig.1. Expression of Gata4 and Gata6 in developing mouse ovary. (A–D) Cryosections of E18.5 mouse were subjected to in situ hybridization for GATA4 or GATA6 using [35S]-labeled riboprobes and counterstained with hematoxylin and eosin (H&E). Shown are corresponding brightfield (A,B) and darkfield (C,D) photomicrographs. GATA4 and GATA6 mRNA are evident in fetal ovary (ov) and small intestine (si). Additionally, GATA6 mRNA is seen in the mesovarium (mo) but not in the Mullerian duct (md). Bars – 100 μm. (E,F) Immunohistochemical staining of ovary from a parous female (P90) mouse. GATA4 and GATA6 proteins are evident in granulosa cells (gc) and germinal epithelium (ge). Bars – 50 μm.
both benign prostatic epithelium and prostate cancer, but levels of GATA2 are significantly higher in the latter and correlate with the risk of cancer progression and metastasis (He et al., 2014). Androgen receptor (AR) signaling is a key driver of prostate cancer, and GATA2 has emerged as a critical regulator of AR expression and activity in this malignancy (Chiang et al., 2014; He et al., 2014; Wu et al., 2014).

1.3. GATA factors interface with signaling pathways involved in both normal development and tumorigenesis

Signaling pathways that control stem cell self-renewal, terminal differentiation, and cell survival, such as the WNT/β-catenin and TGFβ pathways, are often co-opted during tumorigenesis. GATA factors have been shown to interface with developmental signaling pathways implicated in oncogenesis, as evidenced by studies of GATA6 in colorectal tumors. Constitutive activation of WNT/β-catenin signaling and inhibition of bone morphogenetic protein (BMP) signaling are the principal genetic alterations associated with colorectal tumor formation (Whissell et al., 2014), GATA6 plays a key role in colorectal tumorigenesis by driving expression of IGR5, which enhances WNT/β-catenin signaling and enables tumor stem cell renewal, and by inhibiting expression of BMPs, which promote terminal differentiation (Tsuij et al., 2014; Whissell et al., 2014). GATA factors have also been shown to regulate genes involved in apoptosis of normal and tumor cells. For example, GATA4 has been shown to protect cardiomyocytes from doxorubicin-induced apoptosis by upregulating anti-apoptotic members of the BCL2 protein family (Aries et al., 2004; Kobayashi et al., 2010) and to protect ovarian tumor cells from an extrinsic apoptosis inducing ligand TRAIL (Kyrölähti et al., 2010) (see Section 2.2).

1.4. GATA factor dysregulation can affect tumor cell migration, invasiveness, and ploidy

GATA factor dysregulation can impact tumor cell biology in other ways. In breast cancer cells GATA3 promotes differentiation, limits cell migration, and suppresses metastasis by inducing expression of a microRNA (miR-29b) that downregulates expression of genes involved in angiogenesis, collagen remodeling, and proteolysis (Chou et al., 2013). Ovarian carcinomas are thought to arise from ovarian surface (germinal) epithelium or neighboring oviduct epithelium (Nezhat et al., 2015). Approximately 50% of ovarian carcinomas lack GATA6 expression entirely, and an additional 40% show abnormal GATA6 immunostaining that is either weak or cytoplasmic rather than nuclear (Cai et al., 2009). Loss of GATA6 in germinal epithelial cells triggers their de-differentiation, manifested as the loss of expression of proteins required for epithelial organization (Cai et al., 2009; Capo-chichi et al., 2009, 2011). Loss of GATA6 expression in these cells also leads to deformation of the nuclear envelop and a failure of cytokinesis, resulting in aneuploidy (Capo-chichi et al., 2009). The link between GATA6 deficiency and aneuploidy appears to be a generalized phenomenon, because mouse peritoneal macrophages lacking Gata6 expression also exhibit changes in ploidy along with metabolic derangements (Gautier et al., 2014; Rosas et al., 2014).

1.5. Focus of this article

This article reviews the role of GATA factors in neoplasias of various endocrine tissues. The expression patterns of GATA factors in developing endocrine organs and in their corresponding neoplasms are described. The use of GATA factors as endocrine tumor markers in both preclinical and clinical settings is discussed. Relevant animal models, such as the mouse, ferret, dog, and goat, are highlighted. Endocrine-related tumors, such as breast and prostate cancer, are not a focus of this review.

Dozens of putative GATA target genes have been identified in endocrine tissues. Unfortunately, space constraints do not allow us to cite all the original research papers characterizing these target genes. Instead, the reader is referred to review articles that summarize GATA target genes in endocrine tissues (LaVoie, 2003; Röhrig et al., 2015; Tevosian, 2014; Viger et al., 2008).

2. Ovarian neoplasms

2.1. Roles of GATA4 and GATA6 in ovarian development and function

GATA4 and GATA6 are the predominant GATA factors expressed in the developing ovary (Fig. 1) (LaVoie, 2014; Viger et al., 2008). At embryonic day (E) 10.5 in the mouse, Gata4 expression is evident in the genital ridge (Hu et al., 2013), and by E13.5 GATA4 is found in most ovarian somatic cells (Anttonen et al., 2003; Eifmenko et al., 2013; Heikinheimo et al., 1997; Kyrölähti et al., 2011b). In the adult ovary, GATA4 is present in theca cells and in granulosa cells of primary, preantral, and antral follicles, but not in primordial follicles or luteal cells (LaVoie et al., 2004; Viger et al., 2008). Like Gata4, Gata6 is expressed in somatic cells of the prenatal ovary and in theca and granulosa cells of large follicles in the adult ovary; however, in contrast to Gata4, Gata6 is expressed in corpora lutea (Heikinheimo et al., 1997; Miyamoto et al., 2008). Both GATA4 and GATA6 are found in ovarian surface epithelium (Capo-chichi et al., 2003).

Ovarian expression of GATA factors is controlled by an array of endocrine and paracrine factors, including gonadotropins and members of the TGFβ superfamily (reviewed in Viger et al., 2008). For example, treatment of juvenile mice with eCG enhances follicular expression of Gata4 and Gata6 (Heikinheimo et al., 1997), while treatment of cultured preovulatory human granulosa cells with hCG upregulates GATA6 expression (Laitinen et al., 2000). In granulosa cell tumors, TGFβ treatment increases GATA4 levels (Anttonen et al., 2006). In ovarian somatic cells and other endocrine tissues, GATA factors serve to integrate input from signaling pathways including the cyclic AMP/protein kinase A (cAMP/PKA) and mitogen-activated protein kinase (MAPK) pathways (reviewed in Viger et al., 2008). Activation of these two signaling pathways in gonadal cells results in phosphorylation of GATA4 at distinctive sites that mediate synergistic interactions with other transcriptional regulators (reviewed in Viger et al., 2008). Promoter analyses have identified a number of GATA-dependent genes in ovarian steroidogenic cells, including Star, Cyp11a1, Cyp19a1, Hsd17b1, and Inha (reviewed in Viger et al., 2008). Gene targeting studies in the mouse have delineated the importance of Gata4 and Gata6 in follicular development and ovarian function (Table 2) (reviewed in Tevosian, 2014).

2.2. Granulosa cell tumors

Granulosa cell tumors (GCTs), the most common sex-cord stromal tumors, are subclassified in two forms: an adult-type (AGCT), that typically occurs in perimenopausal women, and a rare juvenile form that affects mostly children and adolescents (Schumer and Cannistra, 2003). GCTs are steroidogenically active and can cause precocious puberty, disturbances in the menstrual cycle, and endometrial hyperplasia (Jamieson and Fuller, 2012; Schumer and Cannistra, 2003).

AGCTs retain the biological features of normal proliferating granulosa cells of preovulatory follicles (Fuller et al., 2002). Thus, the molecular pathogenesis of AGCT is hypothesized to entail...
disrupted expression of signaling pathways that regulate granulosa cell proliferation and apoptosis. A somatic missense mutation (p.C134W) in FOXL2, a transcription factor required for normal murine granulosa cell differentiation and ovarian maintenance (Schmidt et al., 2004; Uhlenhaut et al., 2009), is present in ~95% of AGCTs, suggesting that it is pathognomonic for this tumor (Jamieson et al., 2010; Kim et al., 2010b; Shah et al., 2009). In contrast, juvenile GCTs lack the p.C134W mutation (Kalfa et al., 2007).

Current evidence suggests that a key event in AGCT pathogenesis is a failure of the mutant FOXL2 to form specific protein—protein interactions, leading to subtle changes in the transcription of target genes (L’Hote et al., 2012). Recent studies suggest that GATA4 cooperates with FOXL2 during granulosa cell tumorigenesis (Fig. 2). The majority of AGCTs express GATA4 at levels comparable to normal preovulatory granulosa cells (Laitinen et al., 2000), and high GATA4 expression in these tumors predicts both increased risk of recurrence and shorter disease specific survival (Farkkila et al., 2014). GATA4 expression in AGCTs also correlates with the intrinsic apoptotic pathway inhibitor BCL2 and proproliferative CCND2 expression, suggesting that GATA4 may act as an anti-apoptotic factor in adult AGCTs (Kyrölähti et al., 2008). GATA4, SMAD3, and FOXL2 physically interact and modulate gene expression, cell viability, and apoptosis in AGCTs (Anttonen et al., 2014). GATA4 is also expressed in juvenile GCTs (Virgone et al., 2012); however, increased expression of GATA4 does not correlate with aggressive behavior as seen in adults. In contrast to GATA4, GATA6 expression in AGCTs is inversely correlated with tumor size, suggesting that GATA6 may suppress proliferation in this cell type (Anttonen et al., 2005).

A number of transgenic mouse models have been generated to examine the pathogenesis of AGCTs (Table 3). Aberrant expression of GATA factors accompanies tumorigenesis in several of these mouse models. Collectively, these models reinforce the importance of SMADs and GATA factors in the genesis of AGCTs.

### 2.3. Sertoli–Leydig cell tumors of the ovary

Sertoli–Leydig cell tumors (STLCs) are rare ovarian sex cord-stromal tumors characterized by proliferation of Sertoli and Leydig cells of varying degrees of differentiation (Zhang et al., 2014). Sertoli cells, not Leydig cells, are thought to constitute the neoplastic component of these tumors (Nouriani et al., 2002). The majority of SLCTs are diagnosed in adolescents or young adults. One-third of patients exhibit hyperandrogenic manifestations (acne, male-pattern baldness, etc.) (Zanotti, 2002; Zhang et al., 2014). A small percentage of SLCTs have estrogenic manifestations, such as menometrorrhagia or postmenopausal bleeding.

SLCTs are one of the characteristic tumors in the pleuropulmonary blastoma familial tumor predisposition syndrome.

### Table 3

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Cre</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata4</td>
<td>Sf1-cre</td>
<td>Impaired granulosa/theca cell proliferation, depletion of primordial follicles, ovarian cyst formation, and fertility</td>
<td>Efmenko et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Amhr2-cre</td>
<td>Cystic ovarian changes, impaired fertility, and a blunted response to exogenous gonadotropins</td>
<td>Kyrölähti et al. (2011b)</td>
</tr>
<tr>
<td></td>
<td>Sf1-cre</td>
<td>No overt phenotype</td>
<td>Pihlajoki et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Amhr2-cre</td>
<td>Early embryonic lethal owing to leaky expression in yolk sac endoderm</td>
<td>Wilson (unpublished observations)</td>
</tr>
<tr>
<td>Gata6 &amp; Gata4</td>
<td>Cyp19-cre</td>
<td>Impaired formation of large pre-ovulatory follicles and corpora lutea, an abnormal estrous cycle, and infertility, impaired expression of Fshr, Lhcgr, Inha, and Inhbb</td>
<td>Bennett et al. (2012, 2013)</td>
</tr>
<tr>
<td></td>
<td>Sf1-cre</td>
<td>Ablerrant folliculogenesis and impaired expression of genes involved in early ovarian development, including Fox2 and Fit</td>
<td>Pihlajoki et al. (2013)</td>
</tr>
</tbody>
</table>

M. Pihlajoki et al. / Molecular and Cellular Endocrinology 421 (2016) 2–17

---

Fig. 2. Interplay among GATA4, FOXL2, and SMAD3 during human granulosa cell tumor development. Sections of formalin-fixed AGCT were subjected to immunohistochemical staining for (A) GATA4, (B) FOXL2, and (C) SMAD3. Bar = 50 µm. Normal granulosa cell growth is modulated by cooperative interactions among wild-type FOXL2, GATA4, and SMAD3 (D). The C134W mutation in FOXL2 disrupts this balance leading to malignant cell growth (E).
which is caused by heterozygous germline mutations in the microRNA maturation gene Dicer1 (Schultz et al., 2014). More than half of SLCTs harbor mutations in Dicer1 (Witkowski et al., 2013). Conditional deletion experiments in mice have established that Dicer1 regulates gonadal somatic cell function (Huang and Yao, 2010; Kim et al., 2010a; Lei et al., 2010; Nagaraja et al., 2008), but sex cord stromal tumors have not been reported in germline or conditional Dicer1 knockout mice.

Based on its established role in testicular somatic cell differentiation (see Section 3.1), GATA4 is hypothesized to have a role in pathogenesis of ovarian SLCTs. A series of studies have shown that GATA4 and its cofactor FOG2 are expressed in some but not all SLCTs (Ketola et al., 2000; Mosbech et al., 2014; Siltanen et al., 1999; Virgone et al., 2012). Currently, however, there is no genetic or epigenetic data linking altered expression or function of GATA factors to SLCTs.

### 2.4. Thecoma–fibroma tumors of the ovary

Thecoma–fibroma is a group of benign ovarian sex cord-stromal tumors. These rare neoplasms are composed of varying amounts of theca cells and fibroblasts (Chen et al., 2003). This group of tumors is subdivided into three categories: (1) thecoma, containing lipid-laden theca cells without fibroblasts, (2) thecoma–fibroma, containing both theca cells and fibroblasts, and (3) fibroma, composed almost entirely of fibroblasts. These tumors can occur at any age, but menopausal and postmenopausal women account for most of the cases (Chen et al., 2003). Patients typically present with an abdominal mass and attendant pain; functional tumors may be associated with irregular menstrual bleeding.

Young women with Gorlin (nevoid basal cell carcinoma) syndrome, a tumor predisposition disorder associated with excessive hedgehog signaling due to heterozygous mutations in PTCH1, develop bilateral ovarian fibromas (Morse et al., 2011). One boy with Gorlin syndrome and a testicular thecoma–fibroma has been reported (Ueda et al., 2010). Loss of heterozygosity at 9q22.3, which harbors the PTCH1 gene, is observed in 40% of sporadic ovarian thecoma–fibroma cases (Tsuiji et al., 2005), supporting a pathogenic association between aberrant hedgehog signaling and thecoma–fibroma development. Abundant expression of GATA4 and its cofactor FOGL2 have been reported in two pediatric cases of thecoma–fibroma, including a case of bilateral ovarian fibroma associated with Gorlin syndrome (Virgone et al., 2012).

### 3. Testicular neoplasms

#### 3.1. GATA factors implicated in testicular development and function

During fetal testicular development Gata4 is expressed in Sertoli cells, fetal Leydig cells, and peritubular myoid cells (Table 4) (Bielinska et al., 2007; Mazaud-Guittot et al., 2014). In the adult testis Gata4 is expressed in Sertoli cells, Leydig cells, and putative stem Leydig cells (Ketola et al., 1999, 2002; Kilcoyne et al., 2014; Landreth et al., 2014; McCoard et al., 2001; Oreal et al., 2002). Like GATA4, GATA6 is found in testicular somatic cells. In the mouse, testicular Gata6 expression begins in Sertoli cells at E14.5 (Robert et al., 2002) and in fetal Leydig cells shortly thereafter (Tevosian, 2014). Postnatally, Sertoli cells continue to express Gata6 through to adulthood (Anttonen et al., 2003; Imaie et al., 2004; Ketola et al., 1999, 2002, 2003; Oreal et al., 2002). Gata1 is also expressed in mouse Sertoli cells, albeit in a stage-specific manner (stages VI–IX of the seminiferous epithelial cycle) that is dependent on the presence of maturing germ cells (Ketola et al., 2002; Yokogawa et al., 1994). Both GATA4 and GATA6 have been detected in adult

---

### Table 3

Genetically-engineered mouse models of GCT.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Tumor penetrance (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inha-TAg</td>
<td>Infertile, GCT formation at age of 5–7 mo, serum inhibin B †, serum gonadotropins ↓</td>
<td>100</td>
<td>Kananen et al. (1995)</td>
</tr>
<tr>
<td>Inha−/−</td>
<td>Bilateral, mixed, or incompletely differentiated sex cord-stromal tumors coupled with cachexia-like syndrome at age of 4 wks</td>
<td>100</td>
<td>Matzuk et al. (1992)</td>
</tr>
<tr>
<td>Smad1/5 double knockout</td>
<td>Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo, histopathological features similar to human juvenile GCT</td>
<td>100</td>
<td>Pangas et al. (2008); Middlebrook et al. (2009)</td>
</tr>
<tr>
<td>Smad1/5/8 triple knockout</td>
<td>Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo</td>
<td>57</td>
<td>Boerboom et al. (2005)</td>
</tr>
<tr>
<td>Catnblox(ex3)/+; Amhr2cre/+</td>
<td>Subfertile, follicle-like lesions that evolve into GCT at 7.5 mo of age</td>
<td>70</td>
<td>Mould et al. (2009)</td>
</tr>
</tbody>
</table>

### Table 4

Testicular phenotypes of Gata4 and Gata6 conditional knockout mice.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Cre</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata4</td>
<td>Sfi-cre</td>
<td>Decreased expression of Dnmt1; germ cell attrition</td>
<td>Manuylov et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Amhr2-cre</td>
<td>Late onset impairment of Sertoli cell function with loss of fertility</td>
<td>Manuylov et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>WT1-creERT2</td>
<td>Conditional ablation at E10.5 leads to a block in testicular differentiation and male-to-female sex reversal</td>
<td>Manuylov et al. (2011)</td>
</tr>
<tr>
<td>Gata6</td>
<td>CAG-creER</td>
<td>Impaired formation of the genital ridge and subsequent licensing of primordial germ cells</td>
<td>Hu et al. (2013, 2015)</td>
</tr>
<tr>
<td></td>
<td>Sfi-cre</td>
<td>No overt phenotype</td>
<td>Phlajakj et al. (2013)</td>
</tr>
<tr>
<td>Gata4 &amp; Gata6</td>
<td>Amb2-cre</td>
<td>Early embryonic lethal owing to leaky expression in yolk sac endoderm</td>
<td>Wilson (unpublished observations)</td>
</tr>
<tr>
<td></td>
<td>Sfi-cre</td>
<td>Impaired spermatogenesis, absence of Leydig cells, and ectopic appearance of adrenocortical-like cells</td>
<td>Padua et al. (2015)</td>
</tr>
</tbody>
</table>
Leydig cells (Bielinska et al., 2007; Ketola et al., 1999, 2003), although GATA4 is the predominant GATA factor in this steroido-
genic cell type.

Promoter analyses and related studies have identified several groups of putative target genes for GATA4 in testis, including genes associated with sex determination (Sry, Sox9, Dmrt1), peptide hormone production (Inha, Inhib, Amh), gonadotropin signaling (Fshr, Lhgr), steroid synthesis (Star, Cyp11a1, Cyp17a1), and cell—cell interactions (Clnp, Clnd1, Cx30.2) (reviewed in Tevosian, 2014; Viger et al., 2008). In both Sertoli and Leydig cells, GATA4 activity is modulated via cooperative interactions with other transcriptional regulators/cofactors including SF1, liver receptor homolog 1 (LRH-1/NR5A2), FOG1, and FOG2 (reviewed in Viger et al., 2008). FOG proteins do not bind directly to DNA, but they can function as either enhancers or repressors of GATA transcriptional activity depending on the cell and promoter context studied; on gonadal promoters, FOG proteins appear to play a strictly repressive role (Tevosian, 2014). As in ovarian cells, GATA4 is a target for post-translational modifications such as phosphorylation (Tremblay and Viger, 2003; Viger et al., 2008).

Analysis of genetically-engineered mice has shown that inter-
actions between GATA4 and its cofactor, FOG2, regulate the differenti-
ation and function of fetal and adult Sertoli cells (reviewed in Tevosian, 2014). Fog2<sup>−/−</sup> mice and Gata4<sup>C0/C0</sup> mice, which bear a knock-in mutation that abrogates the interaction of GATA4 with FOG cofactors, exhibit similar testicular phenotypes including decreased testicular Sry expression, aberrant differenti-
ation of Sertoli cells, and sex reversal (Bouma et al., 2007; Manuylov et al., 2007; Tevosian et al., 2002). More recently, conditional mutagenesis studies have shown that functional GATA4 is required for genital ridge development, testis cord morphogenesis, and Sertoli cell function (Hu et al., 2013, 2015; Kyrölähtti et al., 2011a; Manuylov et al., 2011).

The role of GATA4 in Leydig cell development, however, has remained unclear, because gene targeting experiments in mice have not shown a consistent phenotype (reviewed in Tevosian, 2014). For example, Gata4<sup>−/−</sup> progenitors exhibit an impaired ca-
pacity to differentiate into fetal Leydig cells in the testis of chimeric mice (Bielinska et al., 2007). In contrast, conditional ablation of Gata4 in Leydig cells as early as E12.5 does not cause an overt impairment in the expression of Leydig cell differentiation markers in the fetal or adult testis (Manuylov et al., 2011). Interpreting the results of gene targeting experiments in the mouse testis is chal-
 lenging because of context-dependent effects, compensatory re-
 sponses, alternative pathways of differentiation, and functional 
redundancy (Tevosian, 2014). To circumvent these limitations, the impact of Gata4 deficiency on Leydig cell function has been analyzed in less complicated experimental models: two immor-
talized mouse Leydig tumor cell lines (MA-10, mLTC-1) and primary 
cultures of adult mouse Leydig cells (Bergeron et al., 2015; Schrade et al., 2015). Using siRNA and related knockdown approaches, Gata4 deficiency has been shown to have profound effects on specific metabolic pathways, notably steroidogenesis and glycolysis.

A missense mutation in the human GATA4 gene has been linked to abnor-
mal testicular development in one kindred, although the precise impact of this mutation on somatic cell function is unclear (Lourenco et al., 2011). More recently, mutations in FOG2 have been demonstrated in unrelated individuals with 46,XY gonadal dysgenesis (Rashamboo et al., 2014).

Despite the intriguing, stage-specific expression pattern of 
GATA1 in testis, gene targeting experiments in the mouse suggest that GATA1 is not essential for Sertoli cell function (Lindeboom et al., 2003).

3.2. GATA4 is expressed in large-cell calcifying Sertoli tumors

GATA4 expression has been reported in large-cell calcifying 
Sertoli tumors (LCCSCT), one of the sex cord tumors of testis (Fig. 3) (Ketola et al., 2000). LCCSCTs produce estrogen and are associated with gynecomastia and advanced skeletal maturation (Gourgari et al., 2012). Most cases of LCCSCT are sporadic, but about 40% are associated with several neoplasia syndromes such as Peutz–Jeghers syndrome (PJS) or Carney complex (CNC). PJS is caused by loss-of-

function mutations in the STK11 gene, which inhibit AMP- 
activated protein kinase, resulting in increased activity of the 
mammalian target of rapamycin (mTOR) (Gourgari et al., 2012). Dysregulation of the mTOR pathway has been linked to tumorigenesis in various tissues, including endocrine tissues (de Joussineau et al., 2014). CNC is caused by PRKAR1A mutations, the gene encoding regulatory subunit type 1 of protein kinase A. This leads to excessive cAMP and mTOR signaling (de Joussineau et al., 2014; Sahut-Barnola et al., 2010). Whether signaling activation in these tumor disposition syndromes is associated with altered phosphorylation of GATA4 is unknown. Lending credence to this possibility, increased expression of GATA4 is evident in the adrenal glands of Prkar1a knockout mice signaling (Sahut-Barnola et al., 2010).

3.3. GATA4 is expressed in canine testicular tumors

A comprehensive survey of canine testicular tumors docu-
mented strong GATA4 immunoreactivity in all Sertoli cell tumors and the vast majority of (27/28) Leydig (interstitial) cell tumors (Ramos-Vara and Miller, 2009). Mixed germ cell sex cord-stromal tumors (MGST) in this species also expressed GATA4.
3.4. Gata6 is downregulated in an experimental model of Leydig cell adenoma

Heterozygous loss-of-function mutations in Men1, encoding a chromatin remodeling gene, predispose mice to the development of multiple endocrine tumors, recapitulating the human MEN1 cancer predisposition syndrome. Additionally, Men1<sup>+/−</sup> mice develop gonadal somatic cell tumors, a feature not typical of humans with this cancer predisposition syndrome. Female Men1<sup>+/−</sup> mice develop GCTs that underexpress Gata6 (Table 3), while their male counterparts develop Leydig cell tumors that underexpress Gata6 (Mould et al., 2009). Loss of heterozygosity at the Men1 locus is evident in these gonadal tumors, suggesting a direct link between Men1 gene inactivation and tumorigenesis in this model (Bertolino et al., 2003; Hussein et al., 2007). Decreased expression of Gata6 represents an attractive candidate for mediating gonadal somatic cell tumorigenesis in the Men1<sup>+/−</sup> mouse (Hussein et al., 2008), and (3) targeted mutagenesis of genes involved in TGFβ signaling (e.g. Inha, Amhl, Ambh2) have been linked to testicular and ovarian somatic cell tumors in mice (Behringer et al., 1994; Matzuk et al., 1995; Mishina et al., 1996) (Table 3).

3.5. Testicular adrenal rest tumors

Leydig cells in the adult testis can arise from different populations of stem/progenitor cells, including undifferentiated mesenchymal cells in the testicular interstitium, vascular progenitors, and peritubular cells (Davidoff et al., 2004; Landreh et al., 2014; Mendis-Handagama and Ariyaratne, 2001). Men with disrupted adrenocortical function due to CYP21 or CYP11B1 deficiency develop neoplastic nodules of hormonally-active adrenocortical tissue in the testis (testicular adrenal rest tumors, TARTs), thought to arise from one of these reservoirs of pluripotential stem/progenitor cells (Reisch et al., 2013; Val et al., 2006). TARTs express adrenocortical-specific genes (CYP11B1, CYP11B2, and MC2R) at much higher levels than adjacent testicular tissue (Smeets et al., 2015). In addition, TARTs express the Leydig cell markers such as HSD17B3. These findings reinforce the premise that TARTs may arise from a totipotent embryonic cell type in response to hormonal dysregulation. The endocrine and paracrine factors that drive TART growth are not fully understood. A longitudinal analysis of men with CYP21 deficiency found no association between the presence of TARTs and parameters of disease control with exogenous glucocorticoids ± mineralocorticoids (Reisch et al., 2013). Gata4/Gata6 double knockout mice generated with Sf1-cre develop TART-like cells that produce glucocorticoids (Padua et al., 2015). This GATA-deficient mouse model may be useful for exploring the signals that drive TART formation in humans (Heikinheimo et al., 2015).

4. Adrenocortical neoplasms

4.1. GATA6 and GATA4 have been implicated in adrenocortical development and function

GATA6 is the principal GATA factor expressed in the adrenal cortex. Gata6 is expressed diffusely in the adrenal cortex of the fetal mouse (Kiiveri et al., 2002). Postnatally, expression of Gata4 in the mouse adrenal is limited to capsular and subcapsular cells (Pihlajoki et al., 2013). In primates, GATA6 is expressed in the zona reticularis, where it is thought to regulate androgen biosynthesis (Jimenez et al., 2003; Nakamura et al., 2007, 2009). By comparison, GATA4 has a more restricted pattern of expression during adrenocortical development and is presumed to have a more limited role in the function of this organ (Kiiveri et al., 2002). During human development, GATA4 mRNA is evident in the fetal zone of the adrenal, but there is only weak expression of this transcript in the adrenal cortex postnatally. Similarly, Gata4 is transiently expressed in the mouse adrenal cortex during fetal but not postnatal development.

Consistent with its proposed role in the biosynthesis of adrenocorticoids and androgens, GATA6 has been shown to enhance the transcription of CYP11A1, CYP17A1, CYB5, SULT2A1, and HSD3B2 in cell lines (reviewed in Röhrig et al., 2015). GATA4 can substitute for GATA6 in trans-activation studies of the CYP17A1 promoter (Flück and Miller, 2004), suggesting that GATA4 may serve to augment CYP17A1 expression during fetal development.

The impact of GATA6 on adrenocortical development and physiology has been assessed through conditional gene deletion using Sf1-cre (Pihlajoki et al., 2013). Gata6 conditional knockout mice exhibit a pleiotropic phenotype that includes: (1) a thin, cytomegalic adrenal cortex, (2) decreased expression of the zG differentiation marker Cyp11b2, (3) blunted production of glucocorticoids in response to exogenous ACTH, (4) the spontaneous accumulation of non-steroidogenic cells expressing gonadal-like markers, (5) ectopic chromaffin cells, and (6) the absence of an adrenal X-zone. Based on analogous conditional deletion studies of Gata6 in pulmonary (Tian et al., 2011; Zhang et al., 2008) and intestinal epithelia (Beuling et al., 2011, 2012), GATA6 is hypothesized...
to regulate the balance between stem/progenitor cell expansion and differentiation in the adrenal cortex. Targeted ablation of Gata4 in SF1<sup>+</sup> cells has no significant impact on adrenocortical development, but Gata4/Gata6 double mutant mice exhibit adrenocortical aplasia (Padua et al., 2015).

4.2. Gonadectomy-induced adrenocortical neoplasia

Gonadectomy (GDX) triggers the appearance of gonadal-like neoplasms in the adrenal cortex of mice (Röhrig et al., 2015). This phenomenon, termed GDX-induced adrenocortical neoplasia, is thought to reflect the metaplastic differentiation of stem cells in the adrenal capsule/subcapsule in response to the hormonal changes that accompany GDX (LH, inhibin, etc.). The neoplastic cells express Gata4, Lhcgr, Inha, and enzymes required for sex steroid biosynthesis (Cyp17a1, Hsd17b3, Cyp19a1) (Bielsinska et al., 2006; Schillebeeckx et al., 2015). Prototypical adrenocortical markers, such as Gata6 and adrenocorticoic biosynthetic enzymes (Cyp21a1, Cyp11b1, Cyp11b2), are downregulated in the neoplastic adrenal tissue (Bielsinska et al., 2006). In the mouse GDX-induced adrenocortical neoplasia is strain dependent, and chimera studies suggest that strain susceptibility to GDX-induced neoplasia is cell-intrinsic and resides in the stem/progenitor compartment (Röhrig et al., 2015). The genetic basis of strain susceptibility, however, remains unclear. Linkage analysis of crosses between susceptible (DBA/2J) and non-susceptible (C57Bl/6) mouse strains has shown that GDX-induced adrenocortical neoplasia is a complex trait (Bernichtein et al., 2007).

Loss- and gain-of-function studies have established that GATA4 directly modulates GDX-induced adrenocortical neoplasia. Constitutive and conditional mutations in Gata4 mitigate the accumulation of gonadal-like neoplastic cells and the expression of sex steroidogenic markers in the adrenal cortex of gonadectomized female mice (Krachulec et al., 2012). Transgenic expression of Gata4 in the adrenal cortex using a Cyp21a1 promoter induces adrenocortical neoplasia in a non-susceptible strain (C57Bl/6) (Chrusciel et al., 2013). Fate mapping studies suggest that GATA4<sup>+</sup> neoplastic cells arise from a distinctive pool of WT1<sup>+</sup> progenitors in the adrenal capsule (Bandiera et al., 2013). Under basal conditions, these cells give rise to normal steroidogenic cells in the adrenal cortex; GDX activates these WT1<sup>+</sup> progenitors and triggers their differentiation into gonadal-like steroidogenic tissue. Thus, WT1<sup>+</sup> capsular cells represent a reserve stem/progenitor cell population that can be mobilized in response to extreme physiological demand (i.e., GDX-induced hormonal changes). These WT1<sup>+</sup> capsular cells are presumed to be the progenitors of GDX-induced adrenocortical neoplasms. Whereas GATA4 drives GDX-induced adrenocortical neoplasia, GATA6 appears to inhibit the process. Conditional deletion of Gata6 using Sf1-cre augments the GDX-induced expression of gonadal-like markers in mice (Pihlajoki et al., 2013).

GDX-induced adrenocortical neoplasia is a well documented phenomenon in not only mice but also hamsters, ferrets, goats, and other domesticated species (Beuschlein et al., 2012; Bielsinska et al., 2009). Male Angora goats are routinely gonadectomized to enhance mohair production, and these castrate animals have an increased incidence of adrenocortical adenomas (Altman et al., 1969). GDX-induced adrenocortical neoplasia is a major cause of morbidity and mortality in pet ferrets, affecting up to 20% of these animals.
The neoplastic cells that accumulate in the adrenal glands of gonadectomized ferrets express GATA4 (Fig. 4) and other gonadal-like markers and secrete sex steroids (Bielinska et al., 2006; Schillebeeckx et al., 2015).

There are related mouse models in which GDX triggers the accumulation of gonadal-like cells in adrenal cortex. Following GDX, Inha-Tag mice develop malignant gonadal-like tumors, a process that is thought to entail a feed-forward signaling loop involving Gata4 and Lhgr (Rahman et al., 2004). Similarly, Inha−/− mice develop adrenocortical tumors in response to GDX (Matzuk et al., 1992). The resultant tumors exhibit increased expression of Gata4 and other gonadal-like markers and a reciprocal down-regulation of Gata6 (Looyenga and Hammer, 2006). Enforced expression of LH enhances adrenocortical neoplasia in Inha−/− mice (Beuschlein et al., 2003), whereas ablation of Smad3 attenuates tumor growth in this model (Looyenga and Hammer, 2007).

4.3. Dysregulation of GATA factors in other genetically-engineered mouse models of adrenocortical neoplasia

Several mouse models of adrenocortical neoplasia exhibit abnormal expression of GATA factors, even in the absence of GDX. Mice harboring multiple copies of the steroidogenic factor-1 (Sf1) genetic locus, mimicking the amplification of SF1 seen in childhood adrenocortical carcinoma, develop adrenocortical neoplasms that express gonadal-like markers including Gata4 (Doghman et al., 2007). Activation of WNT/β-catenin signaling is a hallmark of human adrenocortical tumors (Assié et al., 2014; Tissier et al., 2005). Constitutive activation of β-catenin signaling, a hallmark of human adrenocortical tumors, triggers the accumulation of GATA4+ cells in the subcapsule of mice (Berthon et al., 2010). Similarly, overexpression of Igf2, a characteristic of human adrenocortical carcinomas, in the adrenal cortex of mice leads to the accumulation of subcapsular cells that express Gata4 (Drelon et al., 2012). Collectively, these results suggest that deregulation of GATA factors is probably a general feature of adrenal tumorigenesis (at least in rodents), irrespective of whether it is triggered by GDX or genetic alterations also found in patients.

Fig. 6. Immunohistochemical staining of GATA4 and GATA6 in the developing mouse pancreas. Three developmental stages are illustrated: E15.5, newborn (postnatal day 1), and adult (3 months). The expression patterns reveal association of GATA4 (A, G, M) primarily with exocrine and GATA6 (B, H, N) with endocrine cells (arrows). Bar = 150 μm.

Fig. 7. GATA4 immunostaining distinguishes normal exocrine pancreas from a well-encapsulated insulinoma of the ferret. Serial sections of formalin-fixed tissue were stained with (A) hematoxylin and eosin (H&E), or (B) GATA4 antibody, followed by hematoxylin counterstaining. Note that pancreatic acinar cells exhibit GATA4 immunoreactivity, whereas insulinoma cells do not. Bar = 50 μm.
4.4. Expression of GATA4 and GATA6 in human adrenocortical tumors

The vast majority (>90%) of human adrenocortical adenomas and carcinomas express GATA6 (Fig. 5) and approximately one-third express GATA4 (Kiwener et al., 2004). Diminished expression of GATA6, SF1, and their target gene INHA in adrenocortical carcinomas correlates with poor outcome (Parviainen et al., 2013). Over-expression of GATA6 in adrenocortical tumor cells enhances BMP signaling, which inhibits cell proliferation and viability (Johnsen et al., 2009). Although global DNA methylation surveys of human adrenocortical neoplasms have yielded inconsistent results, hypermethylation and downregulation of both GATA6 and GATA4 have been reported (Fonseca et al., 2012; Rechache et al., 2012).

5. Parathyroid neoplasms

5.1. Role of GATA3 in parathyroid development

The parathyroid glands regulate calcium balance in the body through the secretion of parathyroid hormone (PTH). GATA3 expression has been reported in the second and third branchial arches, which harbor the progenitors of the parathyroid glands (Debaker et al., 1999; Grigorieva et al., 2010). The importance of GATA3 in parathyroid function has emerged from characterization of patients with hypoparathyroidism, sensorineural deafness, and renal anomaly (HDR) syndrome, a condition caused by heterozygous loss-of-function mutations in GATA3 (Ali et al., 2007; Nesbit et al., 2004; Van Esch et al., 2000). Using mouse models of HDR syndrome, investigators have shown that GATA3 is involved in the embryonic development of the parathyroid glands and in adult parathyroid cell proliferation (Grigorieva et al., 2010). Gata3+/- and Gata3-/- embryos have smaller parathyroid–thymus primordia, and the parathyroid glands of adult Gata3+/- mice do not show increased proliferation or enlargement in response to hypocalcemia induced by a low calcium/vitamin D diet. These studies identified Gcm2, a gene required for proper parathyroid development, as a target of activation by GATA3.

5.2. GATA3 is a marker of parathyroid neoplasms

In two recent surveys of parathyroid tumors, GATA3 immunoreactivity was demonstrated in all parathyroid adenomas and carcinomas examined (Betts et al., 2014; Ordonez, 2014). As a tumor marker, GATA3 was found to be comparable in sensitivity and specificity to PTH, the marker that has traditionally been used in the diagnosis of parathyroid tumors.

6. Pancreatic neoplasms

6.1. Role of GATA4 and GATA6 in pancreatic development

The pancreas is a mixed endocrine and exocrine gland that arises from the amalgamation of dorsal and ventral buds of foregut endoderm. A number of transcription factor genes, including Pdx1 and Ptf1a, have been shown to be essential for the early stages of pancreatic development in the mouse (Oliver-Krasinski and Stoffers, 2008). GATA4 and GATA6 exhibit overlapping patterns of expression in multipotent progenitor cells within the pancreatic anlagen (Decker et al., 2006; Ketola et al., 2004; Ritz-Laser et al., 2005). As development proceeds, GATA4 expression predominates in pancreatic acinar cells, while GATA6 predominates in the ductal compartment and a subpopulation of endocrine cells (Figs. 6 and 7) (Decker et al., 2006; Ketola et al., 2004).

GATA4 is able to transactivate the glucagon (Gcg) gene promoter in vitro (Ritz-Laser et al., 2005). The same study demonstrated that mutation of the GATA motif in the Gcg promoter reduces its basal promoter activity in glucagon producing cells. GATA6, but not GATA4, has been shown to physically interact with NCKX2.2, an essential islet transcription factor (Decker et al., 2006).

The importance of GATA factors to pancreatic development was underscored when an international consortium of investigators demonstrated that heterozygous mutations in human GATA6 cause a spectrum of pancreatic developmental defects ranging from agenesis to neonatal diabetes and adult-onset diabetes (Bonnefond et al., 2012; De Franco et al., 2013; Lango Allen et al., 2012; Yorifuji et al., 2012). Subsequently deletions or mutations of GATA4 were shown to be a monogenic cause of neonatal and childhood-onset diabetes with variable exocrine phenotypes (Shaw-Smith et al., 2014). The roles of GATA4 and GATA6 in pancreatic development and disease have been modeled in the mouse (Carrasco et al., 2012; Decker et al., 2006; Martinelli et al., 2013; Watt et al., 2007; Xuan et al., 2012). Expression of a Gata6-Engrailed dominant repressor fusion protein in pancreatic progenitors using a Pdx1 promoter caused pancreatic hypoplasia (Decker et al., 2006). Analysis of embryos derived by tetraploid complementation of Gata4+/- ES cells demonstrated a complete absence of the ventral but not the dorsal pancreas; Gata6+/- embryos displayed a similar, albeit less dramatic, phenotype (Watt et al., 2007). Conditional mutagenesis of either Gata4 or Gata6 in multipotent pancreatic progenitors (using Pdx1-cre) has minimal impact on pancreatic development or function, whereas mutagenesis of both genes results in pancreatic agenesis and diabetes (Carrasco et al., 2012; Xuan et al., 2012). The double mutant mice exhibit impaired proliferation of pancreatic progenitor cells, aberrant branching morphogenesis, and a subsequent failure to induce the differentiation of progenitor cells expressing Cpa1 and Neurog3. The prevailing hypothesis, based on the established roles of GATA4 and GATA6 in other foregut derivatives such as the lung and intestine (Beuling et al., 2012; Zhang et al., 2008), is that GATA proteins, in combination with other transcription factors, regulate the balance between stem cell expansion and differentiation in the developing pancreas. Although GATA6 is preferentially expressed in endocrine pancreas, conditional knockout mouse studies using Ptf1a-cre have shown that GATA6 has direct effects on survival of acinar cells in the exocrine pancreas (Martinelli et al., 2013). More recently, investigators have generated β-cell specific knockouts of Gata4 or Gata6 and concluded that these factors have important but nonessential roles in promoting endoplasmic reticulum integrity and β-cell survival, which may contribute to the pathogenesis of type 1 diabetes (Sartori et al., 2014). The precise function of GATA4 in mature pancreatic acinar cells is unclear.

6.2. Gata6 is downregulated in insulinomas triggered by MEN1 deficiency in the mouse

Mice harboring loss-of-function mutations in Men1 develop multiple endocrine neoplasias (see Section 3.4) including insulinomas (Serewko-Auret et al., 2010). Transcriptome analysis of isolated control, normal, hyperplastic, and adenomatous islets showed that Gata6 downregulation accompanies tumor formation. It has been proposed that Gata6 dysregulation plays a fundamental role in tumor formation and progression in this model by modulating TGfβ superfamily or WNT/β-catenin signaling, as in other systems (see Section 1.3).

6.3. Roles of GATA6 and GATA4 in pancreatic ductal adenocarcinoma

Although not an endocrine tumor, pancreatic ductal
adenocarcinoma (PDA) sheds light on the role of GATA factors in organogenesis and oncogenesis. PDA has a complex genomic landscape characterized by frequent point mutations and copy number changes. Common genetic changes include activating mutations of KRAS2 and inactivating mutations in the cell cycle regulator CDKN2A, the tumor suppressor TP53, and SMAD4 (Hong et al., 2011; Jones et al., 2008). GATA6 amplification and overexpression are hallmarks of PDA (Collisson et al., 2011; Fu et al., 2008; Kwei et al., 2008). Enforced expression of GATA6 in pancreatic cancer cell lines enhances proliferation and growth in soft agar, whereas inhibition of GATA6 impairs growth of pancreatic cancer cell lines. GATA6 activates signaling in pancreatic cancer by negatively regulating the WNT antagonist Dickkopf-1 (DKK1) (Zhong et al., 2017). Smoking is a major risk factor for pancreatic cancer, and a recent study showed that nicotine promotes progression of Kras-induced pancreatic adenocarcinoma via Gata6-dependent dedifferentiation of acinar cells (Hermann et al., 2014). GATA4 is frequently overexpressed and infrequently methylated in PDA, whereas GATA5 is generally hypermethylated in these neoplasms (Fu et al., 2007). A separate study documented GATA4 immunoreactivity in a majority of infiltrating pancreatic adenocarcinomas (Karafin et al., 2009).

### 7. Pituitary neoplasms

#### 7.1. Role of GATA2 in pituitary development and function

Among differentiated hormone-secreting cell types found in the pituitary gland, both gonadotrope and thyrotrpoe cells express Gata2 from E10.5 onward in the mouse. The secretory products of thyrotrpoe and gonadotrope cells are heterodimers that share a common α-glycoprotein subunit (αGSU) and a specific β-subunit (FSHβ, LHβ, and thyrotropin-β) (reviewed in Viger et al., 2008). The genes encoding αGSU (Cga) and thyrotropin-β (Tshb) are targets of activation by GATA2 (Gordon et al., 1997; Steger et al., 1994). This transcription factor has also been implicated in the regulation of the GnRH receptor gene (Schang et al., 2013).

Analyses of transgenic and knockout mice have established that GATA2 is involved in both gonadotrope and thyrotrope development (Charles et al., 2006; Dasen et al., 1999; Scully and Rosenfeld, 2002). Interactions between GATA2 and another transcription factor, PIT1, are critical determinants of pituitary cell fate (Dasen et al., 1999). Conditional ablation of Gata2 in the anterior pituitary reduces gonadal-like tumors Inha-Tag and Inha⁻/⁻ gonadal-like tumors (Dasen et al., 1999). In gonadotropes, where GATA2 and PIT1 are coexpressed, thyrotrope-specific genes are up-regulated by the binding of both factors to adjacent DNA cis-elements. PIT1 interacts via its homeodomain with a zinc finger of GATA2, modulating target gene transactivation (Dasen et al., 1999).

#### 7.2. GATA2 is a marker of pituitary neoplasms

Consistent with its established role in gonadotrope and thyrotrpoe development GATA2 is found in most αGSU-positive and thyrotropin-secreting human pituitary adenomas (Umeoka et al., 2002; Wang et al., 2009).
8. Summary and outlook

Studies over the past two decades have established that GATA factors are required for the proper development, differentiation, and function of endocrine tissues. More recently, GATA factors have been implicated in forms of endocrine neoplasia (Table 5), although the molecular mechanisms involved are not fully understood. Altered GATA factor expression or function owing to acquired genetic (mutations, deletions, amplifications) or epigenetic changes (e.g., DNA methylation) has been linked to tumor formation. GATA factors can impact tumorigenesis through modulation of key developmental signaling pathways implicated in oncogenesis, such as the WNT/b-catenin and TGFβ signaling pathways. In addition to affecting signaling pathways, GATA factor dysregulation can have effects on tumor cell metabolism, ploidy, and invasiveness.

Traditionally, transcription factors have been considered poorly druggable, but recent studies offer hope that GATA factors can be targeted pharmacologically in endocrine neoplasms. A small molecule inhibitor of GATA2 has been shown to suppress AR expression and exert anticancer activity against prostate cancer cell lines (He et al., 2014). It may be feasible to adopt similar approaches for inhibition of GATA factors in other tumors.

Acknowledgments

We apologize to investigators whose work was not directly cited due to space limitations. We thank Leila Unkila-Kallio and Jorma Toppari for a critical reading of the manuscript. We thank Ilkka Due to space limitations. We thank Leila Unkila-Kallio and Jorma Toppari for a critical reading of the manuscript. We thank Ilkka Due to space limitations. We thank Leila Unkila-Kallio and Jorma Toppari for a critical reading of the manuscript. We thank Ilkka

References


Transcription factor GATA4 is expressed in somatic cells of the mammalian testis. Gene targeting studies in mice have shown that GATA4 is essential for proper differentiation and function of Sertoli cells. The role of GATA4 in Leydig cell development, however, remains controversial, because targeted mutagenesis experiments in mice have not shown a consistent phenotype, possibly due to context-dependent effects or compensatory responses. We therefore undertook a reductionist approach to study the function of GATA4 in Leydig cells. Using microarray analysis and quantitative RT-PCR, we identified a set of genes that are down-regulated or up-regulated after small interfering RNA (siRNA)-mediated silencing of Gata4 in the murine Leydig tumor cell line mLTC-1. These same genes were dysregulated when primary cultures of Gata4fl/fl adult Leydig cells were subjected to adenovirus-mediated cre-lox recombination in vitro. Among the down-regulated genes were enzymes of the androgen biosynthetic pathway (Cyp11a1, Hsd3b1, Cyp17a1, and Srd5a). Silencing of Gata4 expression in mLTC-1 cells was accompanied by reduced production of sex steroid precursors, as documented by mass spectrometric analysis. Comprehensive metabolomic analysis of GATA4-deficient mLTC-1 cells showed alteration of other metabolic pathways, notably glycolysis. GATA4-depleted mLTC-1 cells had reduced expression of glycolytic genes (Hk1, Gpi1, Pfkp, and Pgam1), lower intracellular levels of ATP, and increased extracellular levels of glucose. Our findings suggest that GATA4 plays a pivotal role in Leydig cell function and provide novel insights into metabolic regulation in this cell type. (Endocrinology 156: 1860–1872, 2015)
eral groups of putative target genes for GATA4 in testis, including genes associated with sex determination (Sry, Sox9, and Dmrt1) (13–17), peptide hormone production (Inha, Inhba, and Ambh) (18), gonadotropin signaling (Fsbr and Lhcgr) (19, 20), steroid synthesis (Star, Cyp11a1, and Cyp17a1) (14, 21–24), and cell-cell interactions (Cmnp, Cldh11, and Cx30.2) (25–27).

Gata4 knockout mice die by embryonic day 9.5 due to defects in ventral morphogenesis and heart development (28, 29), so the role of this transcription factor in gonadal function cannot be determined from these animals. Analysis of other genetically engineered mice has shown that interactions between GATA4 and its cofactor, friend of Gata 2 (FOG2 or ZFPM2), regulate early testis development (14–16). Fog2−/− mice and Gata4+/- mice, which bear a knock-in mutation that abrogates the interaction of GATA4 with FOG cofactors (30), exhibit similar testicular phenotypes, including decreased testicular size and fertility. For example, experiments in mice have not shown a consistent phenotype of male infertility, remains controversial, because gene targeting experiments in the adult mouse.

Establishing that GATA4 plays an essential role in the differentiation and maintenance of Sertoli cells in the fetal and adult mouse.

The role of GATA4 in Leydig cell development, however, remains controversial, because gene targeting experiments in mice have not shown a consistent phenotype (reviewed in Ref. 2). For example, Gata4−/− progenitors exhibit an impaired capacity to differentiate into fetal Leydig cells in the testis of chimeric mice (2, 4). On the other hand, conditional mutagenesis of Gata4 in Leydig cells as early as embryonic day 12.5 does not cause an overt impairment in the expression of Leydig cell differentiation markers in the fetal or adult testis (2, 17).

Interpreting the results of targeted mutagenesis experiments in the mouse testis is challenging because of context-dependent effects, variable degrees of cre-mediated recombination, compensatory responses, alternative pathways of differentiation, and functional redundancy (2). To circumvent these limitations, we have assessed the impact of Gata4 deficiency on Leydig cell function in 2 less complicated experimental models: an immortalized mouse Leydig tumor cell line (mLTC-1) and primary cultures of adult mouse Leydig cells. Using an integrated approach, including transcriptome and metabolome analyses, we show that Gata4 deficiency has profound effects on specific metabolic pathways, especially steroidogenesis and glycolysis.

Materials and Methods

Animals and cultured cells

Experiments involving mice were approved by the institutional committee for laboratory animal care at Washington University. Gata4−/− mice (also termed Gata4+/- mice) were purchased from The Jackson Laboratory and genotyped as described (32, 33). Murine primary Leydig cells were isolated from 3- to 6-month-old animals using Percoll density separation (34). Primary cells were maintained in DMEM/F12 + GlutaMAX media supplemented with 10% fetal bovine serum, 25mM HEPES, and 100-mg/L penicillin/streptomycin (all from Life Technologies). Leydig cell preparations were determined to be 90%–95% pure on the basis of immunostaining for 3β-hydroxysteroid dehydrogenase/isomerase (3B-HSD) and the Sertoli cell marker reproducitive homeobox5 (ROX). mLTC-1 cells were originally purchased from Rebois and Griswold (35) and cultured in Waymouth’s medium supplemented with 5% fetal bovine serum, 10% horse serum, and 100-mg/L gentamicin (all from Life Technologies).

Knockdown of Gata4 in mLTC-1 cells and primary adult Leydig cells

mLTC-1 cells (passages 10–16) were transiently transfected in the absence of antibiotics with a pool of 4 small interfering RNAs (siRNAs) targeting Gata4 (5'-AGAGAUAUGCUUCGAACCA-3', 5'-GGAUAAUGGCUUGCGAGGUU-3', 5'-CUAAUAAUAUCUAGAACCGC-3', 5'-GGACAUAUCAACCGCCGUA-3') or with nontargeting control siRNA (5'-UGGUUUAACUGUCGACUAA-3'; all from Dharmacon) using Lipofectamine RNAiMax transfection reagent in Opti-MEM (Life Technologies) at a final concentration of 0.1µM. Conditioned media and cells were collected 72 hours after transfection for the analyses described below. Primary Leydig cells were cultured in the presence of adenovirus (Ad) (multiplicity of infection, 100) expressing either green fluorescent protein (GFP) (Ad-GFP) or the combination of cre recombinase and GFP [Ad-cre-internal ribosome entry site-GFP (Ad-cre-ires-GFP)] (Vector Biolabs). After infection, the cells were maintained in serum-free DMEM/F12+GlutaMAX (Life Technologies) for 24 hours before RNA extraction.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the Nucleospin RNA/Protein kit (Machrey-Nagel), and reverse transcribed using SuperScript VILO cDNA Synthesis kit (Life Technologies). qRT-PCR was performed using SYBR GREEN I (Invitrogen), and expression was normalized to the housekeeping gene Actb. Primer pairs are listed in Supplemental Table 1.

Western blotting

Protein was extracted from cell cultures with the NucleoSpin RNA/Protein kit (Machrey-Nagel), and 20 µg of protein was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Invitrogen). A list of antibodies used is provided in Supplemental Table 2. Immun-Star WesternC kit (Bio-Rad) was used for detection.

Immunocytochemistry

mLTC-1 cells and primary cells were grown on 8-well glass Lab Tek Chamber Slides (Sigma) and fixed 72 hours after trans-
fection or 24 hours after infection with 4% paraformaldehyde in PBS. Immunoperoxidase and indirect immunofluorescent staining were performed as described (36). See Supplemental Table 2 for a list of antibodies.

**Microarray expression profiling and gene set enrichment analysis**

RNA was isolated from mLTC-1 cells 72 hours after transfection with Gata4 or nontargeting siRNA (n = 3) using NucleoSpin RNA/Protein kit and purified with NucleoSpin RNA Clean-up XS kit (both from Macherey-Nagel). RNA quality was assessed via Bioanalyzer (Agilent). Array hybridization was performed by the Functional Genomics Unit at the University of Helsinki using an Illumina MouseWG-6 v2.0 oligonucleotide BeadChip. Data were background corrected using BeadStudio software (Illumina); quantile normalization and log2 transformation were performed using the BeadArray bioconductor package (37). Differentially expressed genes were identified using linear models for microarray data (38) with Benjamini-Hochberg correction. Expression levels with at least 1.5× difference and a false discovery rate (FDR) below 5% were considered as significantly differentially expressed. Microarray data were subjected to average linkage clustering with uncentered correlation using Cluster (39). A heatmap was generated with R. Gene set enrichment analysis of the differentially expressed genes was performed using GOstats bioconductor package (40). Hypergeometric tests with the Benjamini-Hochberg FDR were performed to adjust the P value.

**Cell viability and apoptosis assays**

Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega). Caspase 3/7 activation was measured using Caspase-Glo 3/7 Assays (Promega). Absolute luminescence was normalized using values obtained from wells containing nontransfected mLTC-1 cells.

**Steroid hormone measurements**

Conditioned media samples from mLTC-1 cells were used to measure steroid levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (41). For steroid separation and quantification, an Agilent 1200 Series HPLC system connected to a mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS) was used.

**Metabolite measurements**

Metabolites were extracted from cell samples, separated using Acquity UPLC and analyzed using XEVO TQ-S Triple Quadrupole LC/MS (Waters Corp). At 72 hours after transfection, approximately 2 million mLTC-1 cells per sample were washed with PBS and deionized water and subsequently quenched in liquid nitrogen. Metabolites were extracted by adding 20 μL of labeled internal standard mix and 1 ml of cold extraction solvent (80/20 acetonitrile/H$_2$O + 1% formic acid). Extracts were vacuum filtered (8 pressure 300–400 mbar for 2.5 min; Hamilton) and injected into the LC system. A detailed description of instrument parameters is given elsewhere (42). A total of 96 metabolite concentrations were measured and data were normalized and analyzed using MetaboAnalyst 2.0 software.

**ATP assay**

At varying time points after siRNA treatment (24, 48, or 72 h) mLTC-1 cells were exposed to 20mM 2-deoxy-D-glucose (2DG) (Caymen Chemical Co) for 4 hours, followed by 1 hour incubation with 20mM dichloroacetate (DCA) (Sigma). Cellular ATP signal levels were measured with ATPlite Luminescence Assay System (PerkinElmer).

**Quantification of glucose, lactate, and ammonium concentrations**

Conditioned cell culture media of mLTC-1 cells was collected 72 hours after transfection, and glucose, lactate, and ammonium concentrations were measured with Konelab Arena 20 XT (Thermo Electron Oy) as previously described (43).

**Statistical methods**

mRNA levels, absorbance values for viability assay, luminescence intensities, and steroid levels were analyzed using the Student’s t test or when appropriate, one-way ANOVA followed by Dunnett’s test. Statistical significance was set at *, P < .05 and **, P < .01.

**Results**

**Changes in gene expression associated with knockdown of Gata4 in mLTC-1 cells**

We used siRNA to inhibit Gata4 expression in mLTC-1 cells. To evaluate the efficiency of gene silencing, RNA and protein were isolated from mLTC-1 cells 72 hours after siRNA transfection. Gata4 mRNA levels were reduced by 80 ± 8% in cells treated with Gata4 siRNA vs nontargeting siRNA-treated cells (n = 3; P < .01) (Figure 1). Western blotting showed no residual GATA4 protein band in the Gata4 siRNA-treated cells (Figure 1B), and immunocytochemistry confirmed markedly reduced GATA4 staining in the nuclei of the targeted cells (Figure 1, C and D).

Microarray hybridization analysis was used to assess the impact of Gata4 silencing on the mLTC-1 transcriptome. A total of 3495 probes were differentially expressed (1740 up-regulated, 1755 down-regulated). Results were ranked according to their log2 fold change (FC) values. Unsupervised hierarchical clustering of the top 50 differentially expressed probes is shown in Figure 2, and FC values for a subset of genes of interest are listed in Table 1. Complete microarray hybridization results are available via Gene Expression Omnibus accession number GSE63320.

Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed with all of the 3495 differentially expressed probes to identify biological processes and pathways affected by the inhibition of Gata4 in mLTC-1 cells (Table 1).
Among the terms identified in the GO analysis were steroid biosynthetic process, sex differentiation, regulation of endocrine process, and response to hormone stimulus, all of which reflect the main function of Leydig cells, biosynthesis of steroids. Other terms identified in the GO analysis were negative regulation of cell proliferation, regulation of cell death, and regulation of apoptotic process. KEGG analysis highlighted alterations in metabolic pathways such as pyruvate metabolism, fatty acid metabolism, and glycolysis/gluconeogenesis (Table 2). The putative disruptions in hormone production and cellular metabolism are explored in detail below.

To validate the microarray results, qRT-PCR analysis was performed on RNA isolated from mLTC-1 cells transfected with Gata4 siRNA vs nontargeting siRNA (Figure 3). Significantly decreased expression levels were observed in genes implicated in Leydig cell function, including steroidogenic factors (Cyp11a1, Hsd3b1, Cyp17a1, Srd5a, Nr5a1 [Sf1/AdBP4], Lhcgr, and Star) (Figure 3, A–G), peptide hormones (Inha and Inhba) (Figure 3, H and I), a gonadotropin-regulated protease inhibitor (SerpinA5) (Figure 3J), a defensin expressed in gonads (Defb19) (Figure 3K), a pseudokinase of the Tribbles family (Trib2) (Figure 3L), and the growth factor receptor and proto-oncogene Kit (44–49) (Figure 3M). Decreases in the protein levels of HSD3B1 and Cytochrome P450 17A1 were confirmed by double immunofluorescence staining (Supplemental Figure 1, A and B) and Western blotting (Supplemental Figure 1C). Indolethylamine N-methyltransferase (Imnt), a gene known to be up-regulated in Wwox−/− mice with impaired steroidogenesis (50), was found to be up-regulated in GATA4-deficient mLTC-1 cells (Figure 3N). Likewise, prostaglandin D2 synthase (Ptgds), a gene implicated in Leydig cell function (51), was confirmed to be up-regulated in the Gata4 siRNA-treated cells (Figure 3O). Importantly, expression of Insl3, which encodes insulin-like 3, a hormone constitutively secreted by Leydig cells, was comparable in the knockdown and control mLTC-1 cells (Figure 3P). This lack of change in the expression of Insl3, an established serum marker of Leydig cell biomass and function (52), suggests that the observed changes in expression of other genes do not reflect cytotoxicity (52).

Changes in gene expression associated with knockdown of Gata4 in cultured primary adult Leydig cells

Immortalized mLTC-1 cells do not fully recapitulate the behavior of all endogenous Leydig cell populations. For example, mLTC-1 cells do not express Hsd17b3, the final enzyme in the testosterone biosynthetic pathway, and consequently do not secrete this sex steroid (53, 54). In this regard, the steroidogenic capacity of mLTC-1 cells more closely resembles that of fetal rather than adult Leydig cells (55). We therefore assessed the impact on Gata4 inhibition on primary cultures of adult Leydig cells. Rather than relying on siRNA, we inhibited Gata4 expression in primary Leydig cells via cre-mediated recombination; primary Leydig cells isolated from Gata4flox/flox mice were infected in vitro with the cre-expressing adenoviral vector Ad-cre-ires-GFP or the control vector Ad-GFP. Based on GFP expression, the infection efficiency of the adenoviral vectors was estimated to be approximately 95% each. qRT-PCR analysis showed that infection of Gata4flox/flox primary Leydig cells with Ad-cre-ires-GFP vs Ad-GFP resulted in 50 ± 7% inhibition of Gata4 at 48 hours after
infection (n = 4; P < .01). The changes in gene expression observed in response to knockdown of Gata4 in primary Leydig cells were strikingly similar to those seen in Gata4 siRNA-treated mLTC-1 cells (Figure 3). These similarities suggest that: 1) the specific metabolic effects associated with GATA4 depletion in mLTC-1 cells (see below) are not merely an artifact of the siRNA transfection system, and 2) mLTC-1 cells are a reasonable model to study the consequences of GATA4 deficiency on Leydig cell function. We therefore focused the ensuing analysis on mLTC-1 cells, because these cells are easier to maintain and experimentally manipulate than primary Leydig cells.

Knockdown of Gata4 in mLTC-1 cells is associated with decreased production of sex-steroid precursors

Leydig cells synthesize testosterone from cholesterol in a pathway catalyzed by series of enzymes (Figure 4). We used LC-MS/MS to quantify sex steroid precursors in conditioned media from mLTC-1 cells treated with Gata4 siRNA vs nontargeting siRNA. Compared with control cells, media from GATA4-depleted mLTC-1 cells contained significantly lower concentrations of pregnenolone and the Δ5-pathway metabolites progesterone, androstenedione, and androstanedione (n = 3; P < .01) (Figure 4B). Notably, the level of androstenedione, the immediate progenitor of testosterone, was reduced markedly in the knockdown cells (Gata4 siRNA: 24.2 ± 4.8nM vs nontargeting siRNA: 172.7 ± 48.1nM, n = 3; P < .01) (Figure 4B). We conclude that the down-regulation of steroidogenic gene expression in Gata4 siRNA-treated mLTC-1 cells (Figure 3, A–D) is accompanied by a concomitant decrease in the production of sex steroid precursors via the Δ5-pathway, the preferred route for androgen biosynthesis in mLTC-1 cells (54).

GATA4 deficiency alters the metabolic profile of mLTC-1 cells

Next, we used LC-MS/MS to assess the impact of GATA4 deficiency on other metabolic pathways. Unsupervised principle component analysis demonstrated a separation of the sample set into 2 groups based on metabolic profiles (Figure 5), confirming that the metabolic profile of mLTC-1 cells was altered by the knockdown of GATA4.
profile of GATA4-depleted mLTC-1 cells differs from that of control cells.

To identify the most significantly changed metabolites, we performed partial least squares discriminant analysis (Figure 5B). Among the top 20 most significantly altered metabolites was nicotinamide adenine dinucleotide (NAD), a key regulator of redox reactions during glycolysis. NAD levels in *Gata4* siRNA-treated cells were decreased by 62 ± 4% (*n* = 3; *P* < .01) as compared with levels in control cells. Note that all the 20 most significant altered metabolites were decreased in GATA4-depleted cells, indicating an overall reduced metabolic activity. Relative differences in the levels of all measured metabolites are illustrated in a heatmap (Supplemental Figure 2).

Quantitative enrichment analysis identified biologically meaningful patterns at pathway levels based on the metabolomics data (Figure 5C). A total of 25 pathways were significantly altered (*n* ≥ 3; *P* ≤ .05) between the 2 sample groups. Based on the combination of *P* value and fold enrichment, glycolysis was identified to be the top hit among all of altered pathways (*P* ≤ .01; *FC* = 4.3).

In keeping with decreased metabolic activity of the GATA4-depleted cells, the concentration of the metabolic waste product ammonium in conditioned media was significantly lower 72 hours after transfection (*Gata4* siRNA: 1.20 ± 0.03 mM vs nontargeting siRNA: 1.39 ± 0.01 mM, *n* = 8; *P* < .01) (Figure 5D).

In light of the GO term analysis and the aforementioned metabolic derangements, we assessed the impact of *Gata4* silencing on viability and survival of mLTC-1 cells (Supplemental Figure 3). Using a MTS-based assay, we found modest decreases in “cell viability” at time points of 48, 72, and 96 hours after transfection (*n* = 4; *P* < .05 for all time points) (Supplemental Figure 3A). These changes could reflect decreased cell proliferation or a reduced pro-

### Table 1. Microarray Expression Values for a Subset of Genes of Interest

<table>
<thead>
<tr>
<th>Gene</th>
<th>FC</th>
<th>Adj. <em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpina5</td>
<td>0.16</td>
<td>3.70E-09</td>
</tr>
<tr>
<td>Trib2</td>
<td>0.19</td>
<td>3.10E-09</td>
</tr>
<tr>
<td>Inha</td>
<td>0.19</td>
<td>6.10E-08</td>
</tr>
<tr>
<td>Kit</td>
<td>0.28</td>
<td>5.20E-06</td>
</tr>
<tr>
<td>Inhba</td>
<td>0.32</td>
<td>7.60E-07</td>
</tr>
<tr>
<td>Hsd3b1</td>
<td>0.33</td>
<td>1.30E-05</td>
</tr>
<tr>
<td>Defb19</td>
<td>0.34</td>
<td>5.00E-05</td>
</tr>
<tr>
<td>Lhcr</td>
<td>0.34</td>
<td>2.50E-07</td>
</tr>
<tr>
<td>Cyp17a1</td>
<td>0.42</td>
<td>3.70E-06</td>
</tr>
<tr>
<td>Srd5a1</td>
<td>0.49</td>
<td>5.80E-06</td>
</tr>
<tr>
<td>Cyp11a1</td>
<td>0.50</td>
<td>8.80E-06</td>
</tr>
<tr>
<td>Gata4</td>
<td>0.50</td>
<td>3.20E-05</td>
</tr>
<tr>
<td>Ldha</td>
<td>0.55</td>
<td>6.00E-08</td>
</tr>
<tr>
<td>Igfbp5</td>
<td>0.56</td>
<td>7.70E-05</td>
</tr>
<tr>
<td>Hk1</td>
<td>0.66</td>
<td>3.40E-04</td>
</tr>
<tr>
<td>Pgam1</td>
<td>0.67</td>
<td>2.40E-06</td>
</tr>
<tr>
<td>Nr5a1</td>
<td>0.70</td>
<td>7.70E-04</td>
</tr>
<tr>
<td>Star</td>
<td>0.71</td>
<td>4.20E-04</td>
</tr>
<tr>
<td>Pdha1</td>
<td>0.77</td>
<td>8.20E-03</td>
</tr>
<tr>
<td>Gpi1</td>
<td>0.79</td>
<td>3.60E-04</td>
</tr>
<tr>
<td>Pfkp</td>
<td>0.86</td>
<td>3.30E-03</td>
</tr>
<tr>
<td>Gata6</td>
<td>1.25</td>
<td>2.20E-03</td>
</tr>
<tr>
<td>Igfbp6</td>
<td>3.05</td>
<td>1.10E-07</td>
</tr>
<tr>
<td>Inmt</td>
<td>8.37</td>
<td>2.50E-09</td>
</tr>
<tr>
<td>Ptgds</td>
<td>11.85 1.10E-08</td>
<td></td>
</tr>
</tbody>
</table>

The table lists differentially expressed genes (DEGs) according to their FCs between the 2 sample groups (mLTC-1 cells transfected with *Gata4* siRNA and nontargeting siRNA; *n* = 3) and adjusted *P* values.

### Table 2. Gene Set Enrichment Analysis of Microarray Data

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Term</th>
<th>Size</th>
<th>Number of Genes</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO</td>
<td>Steroid biosynthetic process</td>
<td>132</td>
<td>5</td>
<td>1.9E-04</td>
</tr>
<tr>
<td></td>
<td>Sex differentiation</td>
<td>212</td>
<td>6</td>
<td>2.1E-04</td>
</tr>
<tr>
<td></td>
<td>Regulation of endocrine process</td>
<td>32</td>
<td>3</td>
<td>2.9E-04</td>
</tr>
<tr>
<td></td>
<td>Reproductive system development</td>
<td>234</td>
<td>6</td>
<td>3.7E-04</td>
</tr>
<tr>
<td></td>
<td>Proteolysis</td>
<td>740</td>
<td>10</td>
<td>4.9E-04</td>
</tr>
<tr>
<td></td>
<td>Response to hormone stimulus</td>
<td>403</td>
<td>7</td>
<td>1.2E-03</td>
</tr>
<tr>
<td></td>
<td>Negative regulation of cell proliferation</td>
<td>472</td>
<td>7</td>
<td>2.9E-03</td>
</tr>
<tr>
<td></td>
<td>Regulation of cell death</td>
<td>1115</td>
<td>11</td>
<td>5.1E-03</td>
</tr>
<tr>
<td></td>
<td>Regulation of apoptotic process</td>
<td>1047</td>
<td>9</td>
<td>2.5E-02</td>
</tr>
<tr>
<td>KEGG pathway</td>
<td>Terpenoid backbone biosynthesis</td>
<td>14</td>
<td>9</td>
<td>1.1E-05</td>
</tr>
<tr>
<td></td>
<td>Pyruvate metabolism</td>
<td>43</td>
<td>16</td>
<td>5.2E-05</td>
</tr>
<tr>
<td></td>
<td>Fatty acid metabolism</td>
<td>48</td>
<td>15</td>
<td>8.0E-04</td>
</tr>
<tr>
<td></td>
<td>Valine, leucine and isoleucine degradation</td>
<td>50</td>
<td>15</td>
<td>1.3E-03</td>
</tr>
<tr>
<td></td>
<td>Fructose and mannose metabolism</td>
<td>37</td>
<td>12</td>
<td>1.8E-03</td>
</tr>
<tr>
<td></td>
<td>Propanoate metabolism</td>
<td>33</td>
<td>10</td>
<td>7.4E-03</td>
</tr>
<tr>
<td></td>
<td>Glycolysis/gluconeogenesis</td>
<td>62</td>
<td>15</td>
<td>1.2E-02</td>
</tr>
<tr>
<td></td>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>19</td>
<td>6</td>
<td>1.9E-02</td>
</tr>
<tr>
<td></td>
<td>Alanine, aspartate, and glutamate metabolism</td>
<td>33</td>
<td>9</td>
<td>2.2E-02</td>
</tr>
<tr>
<td></td>
<td>Arginine and proline metabolism</td>
<td>54</td>
<td>12</td>
<td>4.2E-02</td>
</tr>
</tbody>
</table>

GO and KEGG pathway analysis results are arranged on the basis *P* values. Size describes the overall number of genes related to 1 specific term, and number of genes is the number of genes significant changed in microarray analysis within this group.
duction of reducing equivalents such as NADH or NADPH. Apoptosis of mLTC-1 cells was assayed 72 hours after siRNA transfection by measuring caspase 3/7 activity with a luminescence assay. Gata4 knockdown in mLTC-1 cells increased caspase 3/7 activity by 27 ± 12% when compared with control cells transfected with nontargeting siRNA (n = 4; P < .01) (Supplemental Figure 3B).

**GATA4 deficiency inhibits glycolysis in mLTC-1 cells**

Consistent with the LC-MS/MS and microarray hybridization results qRT-PCR analyses showed significantly reduced expression of the glycolytic genes hexokinase 1 (Hk1), glucose phosphate isomerase 1 (Gpi1), phosphofructokinase (Pfkp), and phosphoglycerate mutase 1 (Pgami1) (Figure 6, A–D).
Given this evidence of impaired glycolysis, we predicted that the amount of intracellular ATP would be decreased in Gata4 siRNA-treated cells. Indeed, treatment of mLTC-1 cells with Gata4 siRNA was associated with a 30% reduction in intracellular ATP levels compared with nontargeted siRNA control cells (Figure 6E). By inhibiting glycolysis 72 hours after siRNA transfection with 2DG and/or DCA, we verified that differences in ATP levels between GATA4-depleted and control cells were predominantly due to effects of gene silencing on glycolysis. In nontargeting siRNA control cells, treatment with DCA or the combination of 2DG and DCA decreased ATP amounts significantly by 45 ± 16% and 54 ± 20%, respectively (n = 4; P < .05). By contrast, in Gata4 siRNA-treated cells, ATP levels were minimally impacted by the addition of 2DG or DCA. Similar results were obtained at 48 hours after transfection, when GATA4 mRNA levels were reduced by 60 ± 5% (n = 4; P < .05) in Gata4 siRNA-treated cells (Supplemental Figure 4B). Supposedly due to statistically not significantly changed GATA4 mRNA levels at 24 hours after transfection, no differences in the response to the addition of glycolytic inhibitors could be observed between the 2 sample groups (Supplemental Figure 4A).

Consistent with decreased glycolysis, glucose concentrations in conditioned media from Gata4 siRNA-treated cells were significantly higher (Gata4 siRNA: 3.57 ± 0.043 g/L vs nontargeting siRNA: 3.28 ± 0.025 g/L, n = 8; P < .001) (Figure 6F), and lactate concentrations were significantly lower (Gata4 siRNA: 0.77 ± 0.01 g/L vs nontargeting siRNA: 1.02 ± 0.025 g/L, n = 8; P < .001) (Figure 6G).

Discussion

Previous studies have provided circumstantial evidence that GATA4 is involved in Leydig cell function. Gata4 is expressed in fetal and adult Leydig cells and in putative stem Leydig cells (3–12). Based on in vitro promoter analyses, this transcription factor has been implicated in the regulation of many genes that are expressed in Leydig cells (reviewed in Ref. 1). Conditional mutagenesis studies have established a role for GATA4 in the control of sex steroidogenic cell function in the mouse ovary (56–58). Gonadectomy of certain mouse strains triggers the ectopic expression of Gata4 and other Leydig cell differentiation markers (eg, Insl3 and Cyp17a1) in the adrenal cortex (59).

Although there is abundant indirect evidence implicating GATA4 in Leydig cell function, there is a dearth of direct (genetic) evidence. A missense mutation in the human GATA4 gene has been linked abnormal testicular...
development in 1 kindred, although the precise impact of this mutation on Leydig cell function is unclear (60). In the mouse, Gata4\textsuperscript{−/−} chimera analysis and conditional mutagenesis experiments using Gata4\textsuperscript{flox/flox} mice have yielded conflicting results. As highlighted in a recent review (2), the interpretation of conditional knockout studies in the mouse testis is fraught with difficulty owing to context-specific effects, compensatory mechanisms, and other factors.

Here, using reductionist approach with simplified experimental models, we provide genetic evidence that GATA4 is a key regulator of Leydig cell function. Silencing of Gata4 in mLTC-1 cells and primary adult Leydig cells led to decreased expression of genes in the androgen biosynthetic pathway (Cyp11a1, Hsd3b1, Cyp17a1, and Srd5a). In mLTC-1 cells this was accompanied by reduced production of sex steroid precursors (steroid levels in conditioned media from primary Leydig cell cultures were too low to be detected by mass spectrometry). Comprehensive metabolomic analysis of GATA4-deficient mLTC-1 cells showed disruption of other metabolic pathways, particularly glycolysis. Consistent with impaired glycolysis, GATA4-depleted mLTC-1 cells had reduced expression of glycolytic genes (Hk1, Gpi1, Pfkp, and Pgam1), lower intracellular levels of ATP, and increased extracellular level-
els of glucose. Our findings suggest that GATA4 plays a pivotal role in Leydig cell function and provide novel insights into metabolic regulation in this cell type.

In steroidogenic cells the binding of hormones to G-coupled receptors leads to activation of the cAMP/protein kinase A (PKA) pathway. PKA phosphorylates cAMP-responsive element binding protein (CREB), which activates transcription of a host of genes. Like CREB, GATA4 is phosphorylated in steroidogenic cells by PKA, and this enhances the intrinsic activity of GATA4 (61). Phosphorylated GATA4 has been shown to activate several cAMP-responsive genes, including those lacking a consensus binding site for CREB (eg, Star, Cyp17a1, Cyp11a1, and Inha) (61). These same genes were significantly down-regulated in GATA4-depleted mLTC-1 cells and primary Leydig cells (see Figure 3), reinforcing the premise that GATA4 is a downstream effector of cAMP/PKA signaling.

Figure 6. GATA4 depletion in mLTC-1 cells is associated with impaired glycolysis. Cells were treated with nontargeting (NT) siRNA or Gata4 (G4) siRNA for 72 hours. A–D, The expression of glycolytic genes was analyzed using qRT-PCR. Results were normalized to Actb mRNA and are presented as relative expression values of the mean ± SD (**, P < .01; *, P < .05; n = 8). NT, NT siRNA; G4, Gata4 siRNA. E, The contribution of the glycolytic pathway to ATP production was examined in Gata4 siRNA-treated mLTC-1 cells and NT siRNA control cells. 2DG and DCA were used to inhibit glycolysis. Values are expressed as the mean ± SD. All bars were compared with NT siRNA control cells in the absence of inhibitors (*, P < .05; n = 4). F and G, Metabolite concentrations in conditioned media derived from mLTC-1 cells 72 hours after transfection was measured using Konelab Arena 20 XT. Values are expressed as the mean ± SD (**, P < .01; n = 8). NT, NT siRNA; G4, Gata4 siRNA.

In addition to regulating of steroidogenesis and glycolysis, GATA4 influences cell survival. We found that Gata4 silencing is associated with a decrease in mLTC-1 cell viability, as measured by an MTS-based assay, and a concomitant increase in apoptosis. Of interest, we previously identified GATA4 as an antiapoptotic factor in human ovarian granulosa cells (62). In rodent cardiomyocytes, inhibition of Gata4 leads to enhanced apoptosis mediated by members of B-cell lymphoma 2 protein family (63). However, no Bcl2 related genes were found to be significantly dysregulated in our transcriptomic analysis of GATA4-deficient mLTC-1 cells. The increased apoptosis in GATA4-depleted mLTC-1 cells may reflect impaired ATP production, which has been shown to trigger apoptosis in other cancer cell lines (64). Disrupted autocrine signaling may also contribute to decreased cell viability in GATA4-depleted Leydig cells. We found that Gata4 silencing of mLTC-1 cells and primary Leydig cells is associated with decreased expression of Kit, a known survival factor for mature Leydig cells and a growth factor for precursor Leydig cells (46).

Genome wide occupancy analyses have shown that GATA4 chromatin occupancy is tissue specific. Interestingly, a study by Zheng et al showed that GATA4 occupies promoter sites of genes associated with glycolysis in the context of hepatocytes but not cardiomyocytes (65). The notion that a single transcription factor can govern cellular growth/survival, steroidogenesis, glycolysis, and other metabolic pathways was shown recently in a study of nuclear receptor subfamily 5, group A, member 1 (AdBP4; SF1) in Y-1 mouse adrenal cortical and MA-10 Leydig tumor cell lines (66). The authors found that NR5A1 regulates genes required to generate ATP and NADPH, 2 essential energy carriers used for the de novo synthesis of steroid hormones. Of note, all natural steroids in animal cells are derived from lanosterol, which is synthesized via cyclization of the triterpenoid squalene (67). It is of interest that the terpenoid biosynthetic pathway appeared to be one of
the most significant changed pathways in our transcriptomic KEGG pathway analysis.

During glycolysis, glucose is catabolized to generate ATP, essential for cellular function, biomass supply, and proliferation. Both transcriptomic and metabolomic analyses suggest that glycolysis is disrupted in GATA4-depleted Leydig tumor cells. Cytosolic NAD functions as a cofactor for key glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase and pyruvate dehydrogenase. The balance between the oxidized and reduced forms of NAD (NAD⁺ to NADH ratio) is described as the redox state of a cell, a measure that reflects both the metabolic activity and general health of a cell (68). The NAD⁺ to NADH ratio impacts not only cytosolic glycolysis but also mitochondrial oxidative phosphorylation requiring for a sufficient supply of NAD. After cytosolic NAD depletion, seen in Gata4 compromised mLTC-1 cells, glucose can no longer be converted to pyruvate, which is needed to fuel mitochondrial oxidative phosphorylation.

Altered energy metabolism is a hallmark of tumor cells; the so-called Warburg effect describes the increased glycolytic capacity of tumor cells in the presence of oxygen (69). Consequently, caution must be exercised in extrapolating metabolic findings in tumor cells to primary tissue. Studies have shown that MA-10 Leydig tumor cells generate a significant proportion of their ATP from glycolysis, whereas primary Leydig cells appear to be almost completely dependent on mitochondrial respiration as their energy source (70). Thus, the changes in glycolysis and ATP production we observed in Gata4-depleted mLTC-1 Leydig tumor cells may or may not be applicable to primary Leydig cells, and future studies will test this. Recently, conditional mutagenesis of Gata6 was shown to alter the expression of metabolic regulators critical for generation of acetyl coenzyme A in murine peritoneal macrophages, proving that GATA factor inhibition can impact intermediary metabolism in nontransformed cells (71).

While this manuscript was under revision, Bergeron et al reported the results of Gata4 knockdown studies in mouse MA-10 Leydig tumor cells (72). As in mLTC-1 cells, inhibition of Gata4 expression in MA-10 cells was associated with reduced expression of genes involved in the synthesis of androgens (eg, Star, Cyp11a1, Hsd3b1, and Std5a) and peptide hormones (eg, Inha and Inhbb). These findings, together with earlier reports, support the notion that GATA4 is a key transcriptional regulator of Leydig cell function.

Acknowledgments

We thank Dr Nafis A. Rahman and Dr Seppo Auriola. We also thank members of the histology core at Washington University and Rebecca Cochran for their expert assistance.

Address all correspondence and requests for reprints to: Markku Heikinheimo, MD, PhD, Pediatric Research Center, Children’s Hospital, University of Helsinki, PO Box 20, 00014 Helsinki, Finland. E-mail: markku.heikinheimo@helsinki.fi.

This work was supported by the Sigrid Jusélius Foundation, the Academy of Finland, National Institutes of Health Grants DK52574 and DK073618, the American Heart Association Grant 13GRNT16850031, the DAAD (German Academic Exchange Service) Fellowship D/12/40505, and the Centre for International Mobility Finland Fellowship TM-13-8769.

Disclosure Summary: The authors have nothing to disclose.

References

14. Tevosian SG, Albrecht KH, Crispino JD, Fujiwara Y, Eicher EM, Orkin SH. Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcrip-


27. Lui WY, Wong EW, Guan Y, Lee WM. Dual transcriptional control of claudin-11 via an overlapping GATA/NF-Y motif: positive regulation through the interaction of GATA, NF-YA, and CREB and negative regulation through the interaction of Smad, HDAC1, and mSin3A. J Cell Physiol. 2007;211:638–648.


GATA4 regulates blood-testis barrier function and lactate metabolism in mouse Sertoli cells

Anja Schrade, Antti Kyrölähti, Oyediran Akinrinade, Marjut Pihlajoki, Simon Fischer, Verena Martinez Rodriguez, Kerstin Otte, Vidya Velagapudi, Jorma Toppari, David B. Wilson, and Markku Heikinheimo

Children’s Hospital, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland; Institute of Applied Biotechnology, University of Applied Sciences Biberach, Biberach, Germany; Metabolomics Unit, Institute for Molecular Medicine Finland, University of Helsinki, Helsinki, Finland; Departments of Physiology and Pediatrics, University of Turku and Turku University Hospital, Turku, Finland; Departments of Pediatrics and Developmental Biology, Washington University, St. Louis, MO, USA

Conditional deletion of Gata4 in Sertoli cells (SCs) of adult mice has been shown to increase permeability of the blood-testis barrier (BTB) and disrupt spermatogenesis. To gain insight into the molecular underpinnings of these phenotypic abnormalities, we assessed the impact of Gata4 gene silencing in cell culture models. Microarray hybridization identified genes dysregulated by siRNA-mediated inhibition of Gata4 in TM4 cells, an immortalized mouse SC line. Differentially expressed genes were validated by qRT-PCR analysis of primary cultures of Gata4flox/flox mouse SCs that had been subjected to cre-mediated recombination in vitro. Depletion of GATA4 in TM4 cells and primary SCs was associated with altered expression of genes involved in key facets of BTB maintenance, including tight/adherens junction formation (Tjp1, Cldn12, Vcl, Tnc, Csk) and extracellular matrix reorganization (Lamc1, Col4a1, Col4a5, Mmp10, Mmp23, Timp2). Western blotting and immunocytochemistry demonstrated reduced levels of TJP1, a prototypical tight junction protein, in GATA4-depleted cells. These changes were accompanied by a loss of morphologically-recognizable junctional complexes and a decline in trans-epithelial membrane resistance. Furthermore, Gata4 gene silencing was associated with altered expression of Hk1, Gpi1, Pfkp, Pgam1, Gls2, Pdk3, Pkd4, and Ldhb, genes regulating the production of lactate, a key nutrient that SCs provide to developing germ cells. Comprehensive metabolomic profiling demonstrated impaired lactate production in GATA4-deficient SCs. We conclude that GATA4 plays a pivotal role in the regulation of BTB function and lactate metabolism in mouse SCs.

Sertoli cells (SCs) provide a microenvironment that facilitates spermatogenesis, the maturation of germ cells within the seminiferous tubules. A key component of this microenvironment is the blood-testis barrier (BTB), a dynamic structure composed of tight junctions, adherens junctions, gap junctions, and other protein complexes that link adjacent SCs (1, 2). The BTB partitions the seminiferous epithelium into two distinct milieus: 1) a basal compartment that is in contact with the systemic circulation and harbors spermatogonial stem cells (SSCs) plus spermatogonia, and 2) an apical compartment that is isolated from the systemic circulation and contains meiotic and postmeiotic germ cells (2, 3). The BTB undergoes remodeling to permit the passage of differentiating germ cells from the basal to apical compartment (1, 4). SC-derived extracellular matrix (ECM) proteins regulate junction dynamics during spermatogenesis (5). These proteins act in concert with proteases, protease inhibitors, focal adhesion proteins, and cytokines to regulate cell-cell interactions and maintain functional barrier integrity (5).

In addition to providing a structural framework for spermatogenesis, SCs afford trophic support for germ cell

Abbreviations:

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA
Copyright © 2016 by the Endocrine Society
Received November 3, 2015. Accepted March 8, 2016.

DOI: 10.1210/en.2015-1927

The Endocrine Society. Downloaded from press.endocrine.org by [Your IP address] on 15 March 2016 at 15:05. For personal use only. No other uses without permission. All rights reserved.
development. SCs secrete growth factors and chemokines that promote SSC self-renewal and maintenance (6–9). SCs also regulate the flow of essential nutrients to germ cells in the apical compartment (10). Whereas spermatogenesis use glucose as a fuel for adenosine triphosphate (ATP) production, more developed germ cells, such as spermatocytes and spermatids, rely on SC-derived lactate as an energy source (11). To ensure adequate lactate production, SCs adopt a metabolic profile typical of cancer cells, the so-called Warburg phenotype, wherein most pyruvate generated through glycolysis is converted to lactate rather than being oxidized via the tricarboxylic acid (TCA) cycle (10, 12–15). SC lactate production is augmented further through catabolism of certain amino acids, notably glutamine (10). For their own energy needs, SCs rely on ATP derived from the β-oxidation of fatty acids (15, 16).

Studies of genetically-engineered mice have implicated GATA4, a transcription factor expressed in SCs and other testicular somatic cells, in the structural and trophic support of spermatogenesis (17–19). Conditional ablation of GATA4 at E10.5 with Wt1-creERT2 impairs SC differentiation and causes male-to-female sex reversal, while deletion of GATA4 at E12.5 using Sfl-cre leads to testis cord defects and decreased expression of another sex determination gene, Dmrt1, in SCs (20). Ablation of GATA4 in fetal and neonatal SCs using Amb-cre disrupts the SSC niche and triggers germ cell depletion by impairing chemokine signaling (9). The gradual deletion of GATA4 in the SCs of adult mice using Amb2-cre leads to increased permeability of the BTB, selective loss of late stage (haploid) germ cells, and late-onset testicular atrophy with loss of fertility (19).

Although mutant mouse studies provide compelling evidence that GATA4 regulates SC development and function, the molecular pathways involved are not well understood, particularly in SCs of the adult. This is due in part to the inherent challenges of interpreting conditional knockout studies in the mouse testis. As reviewed elsewhere (17), cellular heterogeneity, compensatory responses, and other factors confound the analysis of such experiments. To circumvent these limitations, we have assessed the impact of GATA4 deficiency on SC function in less complicated experimental systems: a mouse SC line (TM4) and primary cultures of adult mouse SCs (pSCs). Using complementary methods, including transcriptomic and metabolomic analyses, we show that Gata4 silencing disrupts specific aspects of SC function, notably BTB maintenance and lactate metabolism.

Materials and Methods

Animals and cultured cells

Experiments involving mice were approved by the Animal Studies Committee at Washington University. Gata4flox/flox mice (also termed Gata4<sup>flox/flox</sup>) (21, 22) were purchased from The Jackson Laboratory (Bar Harbor, ME). pSCs were isolated from 3- to 6-month-old Gata4<sup>flox/flox</sup> or wild-type (WT) 129.B6 mice using Percoll cell density separation (23) and maintained in DMEM/F12+GlutaMAX media supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, and 100 µg/mL penicillin/streptomycin (all from Life Technologies, Grand Island, NY). Preparations of pSCs were determined to be 90%–95% pure on the basis of immunostaining for the SC marker RHOX5 and the Leydig cell marker 3βHSD (24). Mouse TM4 cells (25) were cultured in DME/F12+GlutaMAX media supplemented with 10% FBS, 25 mM HEPES, and 100 µg/mL penicillin/streptomycin.

Knockdown of Gata4 in TM4 cells and primary adult Sertoli cells

TM4 cells (passages 12 to 18) and WT pSCs were transiently transfected in the absence of antibiotics with a pool of 4 siRNAs targeting Gata4 (<sup>5′</sup>-AGAAGAUAGCUUCGAACCA-3′, <sup>5′</sup>-GGAAUUGGGUGUCCGGGU-3′, <sup>5′</sup>-UCAGAAUAAUC- UAACAGCGC-3′, <sup>5′</sup>-GGACUAUACCGCGCUAA-3′) or with nontargeting control siRNA (<sup>5′</sup>-UUGGUUUACUGAG- GACUAA-3′; all from Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX transfection reagent in Opti-MEM (Life Technologies) at a final concentration of 0.1 µM. Conditioned media and cells were collected 72 hours post-transfection for the analyses described below. pSCs from Gata4<sup>flox/flox</sup> mice were cultured in the presence of adenosine [multiplicity of infection (MOI) = 100] expressing either green fluorescent protein (Ad-GFP) or the combination of cre recombinase and GFP (Ad-cre-IRES-GFP) (Vector Biolabs, Philadelphia, PA). Following infection, the cells were maintained in serum free DME/F12+GlutaMAX (Life Technologies) for 24 hours before RNA extraction.

Quantitative reverse transcriptase-PCR (qRT-PCR)

Total RNA was isolated using the Nucleospin RNA/Protein kit ( Macherey-Nagel, Düren, Germany) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). qRT-PCR was performed using SYBR GREEN I (Invitrogen, Carlsbad, CA), and expression was normalized to the housekeeping genes Actb and L19. Primer pairs are listed in Supplemental Table 1.

Western blotting

Protein was extracted from cell cultures with the NucleoSpin RNA/Protein kit (Macherey-Nagel), and 20 µg of protein was separated by SDS-PAGE and transferred onto a PVDF membrane (Invitrogen). A list of antibodies used is provided in Supplemental Table 2. The Immuno-Star WesternC kit (Bio-Rad, Hercules, CA) was used for detection. Quantity One 1-D Analysis Software was used to determine quantitative protein signals.
**Immunocytochemistry and immunofluorescence**

TM4 cells and pSCs were grown on 4-well glass Lab Tek Chamber Slides (Sigma, St. Louis, MO) and fixed 72 hours post-transfection or 48 hours post-infection with 4% paraformaldehyde (PFA) in PBS. Immuno-peroxidase and indirect immunofluorescent staining were performed as described (26). See Supplemental Table 2 for a list of antibodies.

**Transmission electron microscopy (EM)**

TM4 cells were grown on 4-well Permanox matrigel coated chamber slides (Sigma) and fixed 72 hours post-transfection with modified Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer) for 1 hour. Samples were postfixed in 2% OsO4 in 0.1M sodium cacodylate buffer for 1 hour. The samples were then dehydrated and embedded in epon. Thick sections (1 μm) were stained with toluidine blue and examined by light microscopy to determine which blocks were to be thin-sectioned (90 nm). Thin sections were stained with uranyl acetate and lead citrate and examined by EM using a Model 1400EX electron microscope (EM) (JEOL, Tokyo, Japan).

**Microarray expression profiling and gene set enrichment analysis**

RNA was isolated from TM4 cells 72 hours after transfection with Gata4 or nontargeting siRNA (n = 3) using NucleoSpin RNA/Protein kit and purified with NucleoSpin RNA Clean-up XS kit (Machrey-Nagel, Düren, Germany). RNA quality was assessed via Bioanalyzer (Agilent, Santa Clara, CA). Array hybridization was performed by the Functional Genomics Unit at the University of Helsinki using an Illumina MouseWG-6 v2.0 oligonucleotide BeadChip. Data was background corrected using BeadStudio software (Illumina, San Diego, CA); quantile normalization and log2 transformation were performed using the BeadArray bioconductor package (27). Differentially expressed genes were identified using LIMMA [linear models for microarray data (28)] with Benjamini-Hochberg correction. Expression levels with at least 1.5x difference and a false discovery rate (FDR) below 5% were considered as significantly differentially expressed. Microarray data was subjected to average linkage clustering with uncentered correlation using Cluster (29). Gene set enrichment analysis of the differentially expressed genes was performed using GOstats bioconductor package (30). Hypergeometric tests with the Benjamini-Hochberg FDR were performed to adjust the P-value.

**Transepithelial resistance measurements**

To assess barrier integrity, TM4 cells, Gata4\textsuperscript{flox/flox} pSCs, and WT pSCs were treated either with siRNA or adenovirus, as described above, and then plated at a density of \(0.5 \times 10^6\) cells/cm\(^2\) (TM4) or \(1.2 \times 10^5\) cells/cm\(^2\) (Gata4\textsuperscript{flox/flox} and WT pSCs) on matrigel-coated bicameral culture units (Merck Millipore, Billerica, MA) (31). Cells were incubated in a humidified CO\(_2\) incubator at 37°C and transepithelial resistance (TER) was measured every 12 hours using the Millicell Electrical Resistance System with Ag/AgCl electrodes as described (31).

**Cell viability assay**

Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega, Madison, WI) at 24 hours, 48 hours, and 72 hours post transfection. Absolute absorbance (490 nm) was normalized using values obtained from wells containing nontransfected TM4 cells. To control for cell number, TM4 cells, Gata4\textsuperscript{flox/flox} pSCs, and WT pSCs that had been subjected to Gata4 gene silencing were trypsinized and counted every 24 hours for 6 days using a hemocytometer.

**Metabolomic profiling**

Metabolites were extracted from cell samples (n = 4), separated using Acquity UPLC, and analyzed using XEVO TQ-S Triple Quadrupole liquid chromatography/mass spectrometry (LC/MS; Waters Corporation, Milford, MA). At 72 hours post-transfection, ~ 2 million TM4 cells per sample were washed with PBS and deionized water, and subsequently quenched in liquid nitrogen. Metabolites were extracted by adding 20 μL of labeled internal standard mix and 1 ml of cold extraction solvent (80/20 acetonitrile/H\(_2\)O + 1% formic acid). Extracts were vacuum filtered (8 pressure 300–400 mbar for 2.5 minutes; Hamilton, Reno, NV) and injected into the LC system. A detailed description of instrument parameters is given elsewhere (42). A total of 110 metabolite concentrations were measured and data were normalized and analyzed using Metaboanalyst 3.0 software.

**Quantification of glucose, lactate, and ammonium concentrations in conditioned media**

Conditioned cell culture media were collected 72 hours post-transfection (TM4 cells) or 48 hours post infection (Gata4\textsuperscript{flox/flox} pSCs), and glucose, lactate, and ammonium concentrations were measured with Konelab Arena 20 XT (Thermo Electron Oy, Helsinki, Finland; n = 4–7) as described (32).

**Lactate dehydrogenase (LDH) activity measurements**

LDH activity in TM4 cells, Gata4\textsuperscript{flox/flox} pSCs, and WT pSCs was determined using the Promega CytoTox96 assay following the manufacturer’s instructions. The assay was calibrated with the positive control included in the kit, and measurements (absorbance at 492 nm) were normalized to the number of cells, with values expressed as fold variation relative to the control group.

**Statistical methods**

mRNA levels, absorbance values for viability assays, cell counts, luminescence intensities, and metabolite concentrations in conditioned media were analyzed using the Student’s t test or when appropriate, one-way ANOVA followed by Dunnett’s test. Statistical significance was set at: * \(P < .05\), and ** \(P < .01\).

**Results**

GATA4-depleted SCs exhibit dysregulation of genes involved the formation and remodeling of junctional complexes

We used siRNA to inhibit Gata4 expression in mouse TM4 cells, an immortalized cell line that retains many of...
the properties of endogenous SCs and is easier to maintain and manipulate in culture than pSCs (33). To determine the efficiency of gene silencing, RNA and protein were isolated from TM4 cells 72 hours after siRNA transfection. 

*Gata4* mRNA levels were reduced by 78 ± 3% in cells treated with *Gata4* siRNA vs. nontargeting siRNA treated cells (n = 4; P < .01) (Figure 1A). Western blotting demonstrated only a trace of residual *GATA4* protein in the *Gata4* siRNA treated cells (Figure 1B), and immunocytochemistry confirmed markedly reduced GATA4 staining in the nuclei of the targeted cells (Figure 1C-D).

Microarray hybridization was used to assess the impact of *Gata4* silencing on the TM4 transcriptome (complete results are available via GEO accession number GSE74471). A total of 2414 probes were differentially expressed (1,230 upregulated, 1184 downregulated). Results were ranked according to their log2 fold change (log FC) values. Unsupervised hierarchical clustering of the top 50 differentially expressed probes is shown in Figure 2. To identify biological processes affected by inhibition of *Gata4* in TM4 cells, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) analysis with all differentially expressed probes (Table 1). Among the terms identified in the KEGG pathway and GO analyses were processes linked to BTB function, such as focal adhesion, ECM receptor interaction, adherens junctions, and extracellular structure organization (1, 4, 5).

To confirm the microarray results, qRT-PCR analysis was performed on RNA isolated from TM4 cells transfected with *Gata4* siRNA or nontargeting siRNA. As further validation, we assessed the impact of *Gata4* inhibition on pSCs from adult mice. *Gata4* expression in pSCs was inhibited either via siRNA or through cre-mediated recombination. For the latter approach SCs isolated from *Gata4* flox/flox mice were infected in vitro with the cre-expressing adenoviral vector Ad-cre-IRES-GFP or the control vector Ad-GFP. Based on GFP expression, the infection efficiency of the adenoviral vectors was determined to be > 90%. qRT-PCR analysis showed that infection of *Gata4* flox/flox pSCs with Ad-cre-IRES-GFP vs. Ad-GFP resulted in 58 ± 10% inhibition of *Gata4* at 48 hours post infection (n = 4; P < .01) (Supplemental Figure 1A). Treatment of WT pSCs with *Gata4* siRNA resulted in 81 ± 6% inhibition of *Gata4* (n = 4; P < .01) (Supplemental Figure 1A). The loss of GATA4 protein in cre-recombined or siRNA-treated pSCs was confirmed by western blotting (Supplemental Figure 1B). As shown in Figure 3, the changes in gene expression observed in response to deletion of *Gata4* in *Gata4* flox/flox pSCs were strikingly similar to those seen in *Gata4* siRNA treated TM4 cells, implying that TM4 cells are a reasonable model to study the consequences of GATA4 deficiency on SC function.

Notably, GATA4 depletion in TM4 cells and *Gata4* flox/flox pSCs was associated with dysregulation of genes involved in the formation and remodeling of junctional complexes. Silencing of *Gata4* was accompanied by decreased expression of two tight junction genes: tight junction protein-1 (*Tjp1*) and claudin-12 (*Cldn12*) (Figure 3A,B). GATA4 depletion also led to aberrant expression of vinculin (*Vcl*), tenasin C (*Tnc*), and c-src tyrosine kinase (*Csk*) (Figure 3C-E), genes involved in formation of the apical ectoplasmic specialization (AES), a distinct actin-based adherens junction restricted to the Sertoli-spermatid interface (1, 34–36). Although not detected in the microarray, connexin 30.2 (*Cx30.2*) (30.2, a component of gap junctions, was downregulated in GATA4-deficient TM4 cells and pSCs (Figure 3F). GATA4 depletion was associated with altered expression of genes encoding ECM proteins, proteases, and protease inhibitors implicated in the regulation of BTB remodeling and integrity. Among these were laminin 1 (*Lamc1*), type IV collagens (*Col4a1, Col4a5*), matrix metalloproteinase 10 (*Mmp10*), matrix metalloproteinase 23 (*Mmp23*), and tissue inhibitor of metalloproteinases 2 (*Timp2*) (Figure 3G-L). The gene en-
coding TNF, a cytokine released when ECM proteins are degraded (37), was upregulated in GATA4-deficient TM4 cells and SCs (Figure 3M). Collectively, these findings implicate GATA4 in the regulation of genes involved in BTB dynamics.

As further confirmation of our gene silencing models, we used qRT-PCR to examine the expression of certain other markers, including established targets of GATA4. In agreement with a recent report linking GATA4 to chemokine signaling in SCs (9), we found that silencing Gata4 in TM4 cells and primary adult SCs led to decreased expression of the chemokines Cxcl12 and Ccl9 (Figure 3N, O). Additionally, the mRNA levels of Ccl25 and Cxcl1 were altered following GATA4 depletion (Figure 3P, Q). Silencing of Gata4 in TM4 cells and pSCs altered the expression of sex determining region Y-box 9 (Sox9) (Figure 3R), a transcription factor known to be regulated by GATA4/FOG2 (17). Krüppel family like protein 4 (Klf4), a transcription factor previously linked to claudin gene expression (19, 38), was also dysregulated in the knockdown cells (Figure 3S). Expression of another transcription factor gene, Rhox5 (reproductive homeobox X-linked protein 5), an established SC marker (39), was not altered by GATA4 depletion in either TM4 cells or pSCs (Figure 3T).

Figure 2. Gene expression profile of GATA4-deficient TM4 cells. Microarray analysis (n = 3 per group) was performed using the Illumina Mouse WG-6 v2.0 BeadChip. After background correction, quantile normalization, and log2 transformation, differentially expressed genes were identified using LIMMA with the Benjamini-Hochberg correction. Only probes with expression levels with at least 1.5-fold difference and a FDR below 5% were considered significantly differentially expressed. A heatmap showing the top 50 DEGs (sorted according to their log2 FCs) was generated with R. Red represents down-regulation and green signifies up-regulation of the particular probes.
Table 1. Gene set enrichment analysis of microarray data. Kyoto Encyclopedia of Genes and Genomes (KEGG) Gene Ontology (GO) pathway analysis results are arranged on the basis of P values. Size describes the overall number of genes related to one specific term, and # of genes represents the number of genes within this group that were significantly changed in microarray analysis (n = 3).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Term</th>
<th>Size</th>
<th># of genes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG Pathway</td>
<td>Focal adhesion</td>
<td>200</td>
<td>42</td>
<td>2.208E-06</td>
</tr>
<tr>
<td></td>
<td>Metabolic pathways</td>
<td>1184</td>
<td>163</td>
<td>3.708E-06</td>
</tr>
<tr>
<td></td>
<td>ECM-receptor interaction</td>
<td>86</td>
<td>19</td>
<td>7.108E-04</td>
</tr>
<tr>
<td></td>
<td>α-Glutamine and γ-glutamate metabolism</td>
<td>3</td>
<td>2</td>
<td>2.82E-02</td>
</tr>
<tr>
<td></td>
<td>Adherens junction</td>
<td>75</td>
<td>13</td>
<td>3.508E-02</td>
</tr>
<tr>
<td></td>
<td>Glycolysis / Gluconeogenesis</td>
<td>62</td>
<td>11</td>
<td>4.27E-02</td>
</tr>
<tr>
<td></td>
<td>Cadherin binding</td>
<td>23</td>
<td>2</td>
<td>4.00E-03</td>
</tr>
<tr>
<td></td>
<td>Extracellular structure organization</td>
<td>161</td>
<td>4</td>
<td>4.60E-03</td>
</tr>
<tr>
<td></td>
<td>α-Lactate-dehydrogenase activity</td>
<td>3</td>
<td>1</td>
<td>1.20E-02</td>
</tr>
<tr>
<td></td>
<td>Lactate Metabolic Process</td>
<td>7</td>
<td>1</td>
<td>2.90E-02</td>
</tr>
<tr>
<td></td>
<td>Protein localization to extracellular region</td>
<td>3</td>
<td>1</td>
<td>1.20E-02</td>
</tr>
<tr>
<td></td>
<td>Extracellular region</td>
<td>1813</td>
<td>14</td>
<td>1.20E-02</td>
</tr>
<tr>
<td></td>
<td>Proteomics extracellular matrix</td>
<td>333</td>
<td>5</td>
<td>1.10E-02</td>
</tr>
</tbody>
</table>

Decreased TJP1 protein levels in GATA4-deficient SCs

Spurred by the results of the transcriptomic analysis, we investigated the expression of Tjp1, a prototypical tight junction marker (40), in more detail. TM4 cells were transfected with Gata4 siRNA or nontargeting siRNA and analyzed 72 hours later. Western blotting (Figure 4A) demonstrated a significant decrease (68 ± 23%) in the level of TJP1 protein in the GATA4-depleted cells (n = 4; P < .01), whereas the level of the housekeeping protein Actin was unchanged. Using the same method a slight reduction in TJP1 protein was also observed in Gata4flox/flox pSCs treated with cre-recombinase when compared to controls (Figure 4B). Immunofluorescence staining showed a concomitant decrease in GATA4 and TJP1 immunoreactivity in TM4 cells treated with Gata4 siRNA vs. nontargeting siRNA (Figure 4C-F). Similarly, immunostaining of pSCs showed a loss of TJP1 protein from the cell surface in response to Gata4 silencing (Figure 4G,H).

GATA4-depleted SCs exhibit impaired junctional complex formation and barrier function

To further probe the role of GATA4 in the formation of junctional complexes, TM4 cells were cultured on matrigel-coated slides, treated with Gata4 siRNA or nontargeting siRNA, and then processed for EM (Figure 5A-D). Junctional complexes with the ultrastructural hallmarks of desmosomes (arrowheads, Figure 5C) were detected readily in cells treated with nontargeting siRNA but not in cells treated with Gata4 siRNA (Figure 5D). The GATA4-depleted TM4 cells contained increased number of vacuoles (Figure 5B,D), a phenotypic feature previously reported in the SCs of Gata4 conditional knockout mice generated with Amhr2-cre (9, 19).

To assess the consequences of GATA4 deficiency on epithelial barrier function, we measured transepithelial resistance (TER), an indicator of the paracellular barrier to ion conductance (31). For this analysis TM4 cells, WT pSCs, or Gata4flox/flox pSCs were grown as monolayers on matrigel-coated bicameral units and treated with siRNA or adenovirus (Figure 5E-G). Beginning 3 days after siRNA transfection, a significantly lower TER was observed in Gata4 siRNA-treated TM4 cells than in TM4 cells treated with nontargeting siRNA (Figure 5E). Gata4 silencing in pSCs was associated with a significantly lower TER at even earlier time points, ie, at day 2.5 in Gata4flox/flox pSCs subjected to adenoviral-mediated cre-recombination (Figure 5F) or day 1.5 in WT pSC treated with siRNA (Figure 5G).

In theory, changes in cell viability or number could account for the observed differences in TER. We found that cell viability, measured with an MTS-based assay, was not significantly altered in either Gata4 siRNA-treated TM4 cells (Supplemental Figure 2A) or GATA4-deficient Gata4flox/flox pSCs subjected to cre-mediated recombination (Supplemental Figure 2B). Gata4 silencing led to significantly reduced cell numbers only after day 5 (TM4 cells) or day 6 (Gata4flox/flox pSCs and WT pSCs) (Supplemental Figure 2C-E). Thus, the early (day 1–4) differences in TER between GATA4-depleted SCs and controls cannot be attributed to reduced cell viability or number.

GATA4-depleted SCs exhibit aberrant expression of genes involved in lactate metabolism

The production of lactate via glycolysis and glutaminolysis, to fulfill the energy needs of developing spermatocytes and spermatids, is a crucial function of SCs (11). KEGG pathway and GO analyses of GATA4-depleted TM4 cells identified significant changes in pathways controlling lactate and glutamine metabolism (glycolysis, LMG-2-lactate-dehydrogenase activity, lactate metabolic process, as well as D-glutamine and D-glutamate metabolism) (Table 1). Subsequent qRT-PCR analysis of GATA4-depleted
Figure 3. Impact of Gata4 silencing on gene expression in TM4 cells and primary mouse Sertoli cells. Each panel shows the relative mRNA expression results for a specific gene, as determined by 3 different methods: (method 1, Array) microarray analysis of mRNA derived...
TM4 and Gata4\textsuperscript{flox/flox} pSCs confirmed the downregulation of glycolytic enzymes hexokinase 1 (Hk1), glucose phosphate isomerase 1 (Gpi1), phosphofructokinase (Pfkp), and phosphoglycerate mutase 1 (Pgamp1) (Figure 6A-D).

Two other genes of importance in SC lactate metabolism, Pdk3 and Pkd4, encode kinases that modulate the activity of pyruvate dehydrogenase complex (PDC), determining whether pyruvate is converted to lactate or alternatively oxidized via the TCA cycle (41–43). In SCs, the follicle stimulating hormone (FSH) induced upregulation of Pdk3 with concomitant downregulation of Pdk4 was recently shown to increase lactate production (44). Interestingly, depletion of GATA4 in TM4 and Gata4\textsuperscript{flox/flox} pSCs elicited the reciprocal changes of decreased Pdk3 and increased Pdk4 expression (Figure 6E, F). Glutaminase 2 (Gls2), which catalyzes the first step in the conversion of glutamine to lactate via glutaminolysis, was downregulated in Gata4 siRNA treated TM4 and Gata4\textsuperscript{flox/flox} pSCs (Figure 6G). In addition, altered expression of LDH B (Ldhb) was also evident in the GATA4-deficient cells (Figure 6H). Taken together the above mentioned changes in gene expression suggest that GATA4 influences the production of lactate.

**Metabolomic analysis of GATA4-depleted TM4 cells demonstrates impaired lactate production**

The physiological consequences of treatment of TM4 cells with Gata4 siRNA vs. nontargeting siRNA were determined by comprehensive metabolomic profiling using LC-MS/MS. Unsupervised principle component analysis demonstrated a separation of the sample set into 2 groups based on the metabolic profiles (Figure 7A), thus confirming that the metabolic profile of GATA4-depleted TM4 cells differed from that of control cells.

To identify the most significantly changed metabolites, we performed a partial least squares discriminant analysis based on the metabolic profiles (Figure 7A), thus confirming that the metabolic profile of GATA4-depleted TM4 cells differed from that of control cells.

The top 20 changed metabolites are presented in Figure 7B. A heatmap of all the analyzed metabolites and a list of the pathways identified by QEA are shown in Supplemental Figure 3 and 4, respectively. The metabolite exhibiting the largest change was glutamine, which was enriched 2.3-fold in the GATA4-depleted TM4 cells (Figure 7B), suggesting an impaired conversion of this amino acid to lactate via glutaminolysis (10). Glutamine is known to inhibit the incorporation of alanine into protein (45), and indeed alanine levels were increased in the GATA4-deficient cells (Figure 7B). Gata4 silencing also led to increased levels of several other amino acids (serine, glycine, threonine, asparagine, and aspartate) that, like alanine, normally function as alternative fuel sources in SCs (10, 45) (Figure 7B).

The concentration of the metabolic waste product ammonium, a byproduct of glutaminolysis, was lower in conditioned media from GATA4 siRNA-treated TM4 cells and cre-treated Gata4\textsuperscript{flox/flox} pSCs when compared to controls (Figure 7C).

In keeping with the aforementioned metabolic changes, the concentration of lactate was significantly lower in conditioned media from GATA4-deficient SCs as compared to controls (Figure 7D), whereas the concentration of glucose was significantly higher (TM4 cells) or unchanged (Gata4\textsuperscript{flox/flox} pSCs) (Figure 7E).

Finally, LDH activity was measured in all three Gata4 knockdown cell preparations (Figure 7F,G). Reduced enzyme activity was evident in cells lacking GATA4.

Collectively, these findings are indicative of both an overall reduced metabolic activity and significantly impaired lactate production in the GATA4-depleted cells. Thus, silencing of Gata4 in SCs cells was associated with a loss of the Warburg phenotype that normally typifies the metabolism of this cell type. Although there are known differences between the biology of immortalized SC cell lines and pSCs (46) the results obtained from the TM4 cell line and pSCs are strikingly similar, reinforcing the notion that TM4 cells are a useful model to study SC metabolism.

**Discussion**

Multiple lines of evidence support the notion that GATA4 plays a key role in the differentiation and function of SCs
GATA4 is expressed in SCs throughout fetal and adult life (18, 48–56). Promoter analyses and related studies have identified groups of putative target genes for GATA4 in SCs, including genes involved in sex determination (Sry, Sox9, Dmrt1) (20, 57–60), FSH signaling (Fshr) (61, 62), cell-cell interactions (Clmp, Cldn11, Cx30.2) (19, 38, 63), and peptide hormone production (Inha, Inhba, Amh) (64). Studies of genetically-engineered mice demonstrate that GATA4 is required for early testicular development, germ cell licensing, maintenance of the SSC niche, and spermatogenesis (9, 19, 20, 38–60, 63, 66). Mouse fibroblasts can be efficiently reprogrammed into embryonic Sertoli-like cells using Gata4 in combination with Nr5a1, Wt1, Dmrt1, and Sox9 (67). Although genetic studies in the mouse provide strong evidence that GATA4 is essential for SC development and function, the molecular pathways regulated by this transcription factor have not been fully elucidated, especially in SCs of the adult animal. The results described herein provide new insights into the downstream targets of GATA4 in adult SCs. Specifically, our findings suggest that GATA4 plays a pivotal role in the regulation of BTB function and lactate metabolism in mouse SCs.

Silencing of Gata4 in TM4 cells and pSCs was associated with decreased expression of the tight junction genes Tip1 and Cldn12. Tip1 encodes phosphoprotein that localizes to the cytoplasmic membrane surface at sites of cell-cell contact (68), and Cldn12 encodes a transmembrane protein implicated in barrier function (69). The downregulation of Tip1 and Cldn12 in GATA4-depleted TM4 cells was accompanied by a loss of morphologically recognizable junctional complexes. TM4 cells and pSCs deficient for GATA4 further showed a decline in transepithelial membrane resistance. Prior studies have implicated GATA factors in the regulation of other genes important for BTB integrity, including the tight junction associated genes Cldn2 (70) and Cldn11 (63).

Cx30.2, encoding a gap junction protein hypothesized to mediate interactions between SCs and germ...

Figure 4. Reduced TJP1 protein levels in GATA4-depleted Sertoli cells. Cells were treated with either siRNA for 72 hours (A; C-H) or adenovirus for 48 hours (B) and grown in the absence of matrigel. A–B, Western blot analysis of tight junction protein 1 (TJP1) protein levels with ACTIN antibody as control. Quantitative protein signal was determined using Quantity One 1-D Analysis Software (Biorad, Hercules, CA). The data is presented as mean relative expression values ± S.D. (**, P < .01, n = 4). C–H, TM4 cells and wild-type primary Sertoli cells (WT pSC) were fixed in 4% PFA and subjected to double immunofluorescence staining. GATA4 (red) and TJP1 (green); DAPI (blue) was used as nuclear stain. Note the disappearance of TJP1 protein from the cell surface after GATA4 loss in WT pSCs. Bars = 2 μm.
cells (71), was downregulated in GATA4-deficient TM4 cells and pSCs. Cx30.2 is a known target of GATA4 in the heart (72), and decreased expression of Cx30.2 has been reported in whole testis extracts from conditional knock-out mice lacking GATA4 in adult SCs (19).

GATA4 depletion in TM4 cells and pSCs also led to dysregulated expression of genes involved in the AES (Vcl, Tnc, Csk). One of the functions of the AES is to prevent the release of immature spermatozoa into the lumen of the seminiferous epithelium (34). Interestingly, mice harboring a conditional deletion of Gata4 in SCs exhibit premature release of spermatocytes and spermatids into the tubule lumen (19).

As reviewed in detail elsewhere (5), SC-derived ECM proteins have been shown to regulate tight junction remodeling during spermatogenesis, and genes encoding basement membrane components (Lamc1, Col4a1, Col4a5) were downregulated in GATA4-depleted TM4

Figure 5. Decreased junctional complexes and epithelial barrier resistance in GATA4-depleted SCs. A–D, Shown are representative electron micrographs of TM4 cells grown on matrigel coated chamber slides. Cells were transfected with Gata4 siRNA (G4) or nontargeting siRNA (NT), and then processed for EM 72 hours later. Higher magnification views of the boxed areas in panels A & B are shown in panels C & D, respectively. Note the presence of large vacuoles in the GATA4-deficient cells (B, D). Even though two adjacent cells are aligned over a long distance and their membranes are juxtaposed, junctional complexes with ultrastructural hallmarks of desmosomes are absent in GATA4-depleted cells (B,D). No morphological features of apoptosis, such as homogenous chromatin condensation within nuclei, were evident after GATA4 depletion. Abbreviations: D, Desmosome; v, vacuole. Bars, 500 nm. E–G, Indicated cells were either transfected with siRNA or infected with adenovirus (gray line = Gata4 knockdown cells; black line = control cells) and cultured at a density of 0.5 × 10⁶ cells/cm² (TM4) or 1.2 × 10⁶ cells/cm² (Gata4F/F and WT pSC) in matrigel coated bicameral culture units. The establishment of a tight junction permeability barrier was assessed by measurement of transepithelial resistance (TER). Values are expressed as the mean ± S.D. (*, P < .05; **, P < .01; n = 3).
cells and pSCs. Providing independent evidence that GATA4 impacts the expression of ECM genes, decreased levels of laminin and type IV collagen have been reported in embryoid bodies derived from Gata4-/- embryonic stem cells (73).

Metalloproteinases, protease inhibitors, and cytokines are known to impact BTB integrity and remodeling (5, 74). Mmp10 was upregulated in GATA4-deficient TM4 cells and pSCs, whereas Mmp23 and Timp2 were downregulated. These findings are consistent with a prior report showing that tissue-remodeling genes, including Mmp23, are dysregulated in the ovaries of Gata4/6 double conditional knockout mice (75).

Taken together, these marked derangements in the expression profile of genes important for BTB integrity and SC–ECM interactions underline the crucial role of GATA4 for physiological SC function. One additional upregulated factor in GATA4-depleted SCs is TNF, a cytokine released when ECM proteins are degraded (37). Earlier studies revealed that TNF perturbs SC tight junction formation in a dose-dependent manner and that Gata4 conditional knockout mice, suffering from a leaky BTB, have elevated TNF mRNA levels (19, 76). Interestingly, TNF is known to stimulate lactate production in SCs (77), suggesting a link between deranged ECM-SC interactions and lactate production in these cells.

The profound metabolic changes observed in GATA4-depleted TM4 cells are summarized in Figure 8 and a probable attempt to maintain lactate production. Glutamine, the most elevated metabolite in GATA4-deficient TM4 cells, is one of the most important substrates for the production of lactate. Indeed, conversion of this amino acid to lactate via glutaminolysis has been reported to yield much of the energy required by SCs (13, 45). The decreased expression of Gls2 in GATA4-deficient TM4 cells and pSCs offers a plausible explanation for the elevation of intracellular glutamine levels. Glutamine is known to inhibit the incorporation of alanine during protein anabo-

Figure 6. Altered expression of genes impacting lactate metabolism in GATA4-depleted SCs. A–H, Each panel shows the relative mRNA expression results for a specific gene, as determined by 3 different methods: (method 1, Array) microarray analysis of mRNA derived from TM4 cells 72 hours post siRNA treatment with nontargeting (NT) or Gata4 (G4) siRNA (n = 3), (method 2, TM4) qRT-PCR analysis of mRNA derived from TM4 cells 72 hours post siRNA treatment with NT or G4 siRNA (n = 4), and (method 3, pSC) qRT-PCR analysis of mRNA derived from primary Gata4flox/flox Sertoli cells (Gata4F/F pSC) 48 hours post infection with adrenovirus expressing either cre/GFP (Cre) or GFP alone (GFP) (n = 4). Microarray results are presented as relative fold changes in mRNA expression. qRT-PCR results, normalized to Actb and ribosomal protein L19 mRNA, are presented as relative expression values of the mean ± S.D. **, P < .01; * P < .05. A, hexokinase 1 (Hk1); (B) glucose phosphate isomerase 1 (Gpi1); (C) phosphofructokinase, platelet (Pfkp); (D) phosphoglycerate mutase 1 (Pgaml); (E) pyruvate dehydrogenase kinase, isozyme 3 (Pdk3); (F) pyruvate dehydrogenase kinase, isozyme 4 (Pdk4); (G) glutaminase 2 (Gls2), (H) lactate dehydrogenase (LDH) B (Ldhb).
Figure 7. Altered metabolic profile and lactate metabolism in GATA4-depleted SCs. (A, B) For metabolic profiling of TM4 cells a total of 110 metabolites were analyzed 72 hours post siRNA transfection using mass spectrometry (n = 4). A, Principle component (PC) analysis revealed the unsupervised separation between of the 2 sample groups [Gata4 (G4) siRNA vs. nontargeting (NT) siRNA treated] based on differences in their metabolic profiles. B, Partial least squares discriminant analysis identified the 20 most significantly changed metabolites and their relative abundance in control and GATA4-depleted cells. The components were sorted according to the variable of importance in the projection (VIP) for the first component. Relative magnitudes of each metabolite disturbance are listed on the right as high (red) and low (green).

C–E, Concentrations of ammonium, lactate, and glucose in conditioned media from TM4 cells and Gata4flox/flox pSCs (Gata4F/F pSC) were quantified with Konelab Arena 20 XT analyzer (n = 4–7) 72 hours post siRNA transfection or 48 hours post infection with adenovirus, respectively.

(F, G) 48 hours and 72 hours post transfection or infection cells of all three Gata4 knockdown experiments were trypsinized and counted; 5000...
lism (45). This is of importance in SCs since alanine can be converted into pyruvate, thus serving as a substrate to maintain lactate production. GATA4-deficient TM4 cells accumulated other amino acids (serine, threonine, asparagine, glycine, proline, aspartate) normally used as alternative fuel sources to support lactate production (10). These changes are indicative of an overall reduced metabolic activity in TM4 cells lacking GATA4. Consistent with this notion of reduced metabolic activity, conditioned media from GATA4-depleted TM4 cells and pSCs contained decreased concentrations of lactate and the metabolic waste product ammonium.

The three major steps in shunting glucose to lactate within SCs are regulated by glycolytic enzymes (converting glucose to pyruvate), PDC (regulating the entry of pyruvate into the TCA cycle), and LDH (converting pyruvate to lactate) (Figure 8A). The glycolytic activity of GATA4-depleted SCs was attenuated as evidenced by reduced expression of key glycolytic genes (Hk1, Gpi1, Pfkp, Pgam1) and attenuated glucose utilization. This is in accordance with our recent findings in Leydig cells, which exhibit diminished glycolytic activity in response to GATA4 depletion (24).

PDC activity is tightly regulated through a reversible phosphorylation/dephosphorylation mechanism (41). Phosphorylation of PDC by PDK isoforms leads to an inactive state of PDC, whereas dephosphorylation by pyruvate dehydrogenase phosphatase (PDP) leads to an active state. Consequently, the relative activities of PDK and PDP determine the flux of pyruvate into the TCA cycle (42, 43). A recent study showed that FSH increases lactate production in rodent SCs by regulating this balance; following FSH exposure, Pdk3 mRNA levels increased, while Pdk4 mRNA levels decreased (44). We observed the opposite effect (↓Pdk3, ↑Pdk4) in GATA4-depleted pSCs and an attendant decrease in lactate production. Hence, Gata4 silencing in SCs mimics the phenotypic changes associated with FSH withdrawal. GATA4-deficient TM4 cells have elevated intracellular levels of succinate (Supplemental Figure 4), consistent with increased flow of pyruvate into the TCA cycle.

The final step of lactate production, the conversion of pyruvate to lactate (Figure 8), is catalyzed by LDH, a tetramer composed of LDHA and/or LDHB subunits. The random combination of these subunits into tetramers results in 5 LDH isoenzymes: LDH-1 (B4), −2 (A1 B3), −3

**Legend to Figure 7 Continued...**
cells of each sample were lysed and LDH activity was determined. Results are presented as fold variation to control. C–G, Values are expressed as the mean ± S.D. (*, P < .05; **, P < .01).

![Figure 8. Impact of GATA4 depletion on lactate metabolism in SCs.](image-url)

A, normal Sertoli cell

B, GATA4-depleted Sertoli cell

The figure is adapted from Oliveira et al, 2015 (10).
(A2 B2), –4 (A3 B1), and –5 (A4) (78). Recent reports suggest that LDHA may be the most important LDH subunit in SCs (15), although LDHB is also present in this cell type (78, 79). We observed reduced Ldhb expression accompanied with a decreased LDH activity in GATA4-depleted TM4 cells and pSCs.

SC-derived lactate is the principal energy source for mature germ cells, and lactate has a stimulating effect on RNA and protein synthesis in spermatids (80). Impaired lactate production in SCs can have profound effects on spermatogenesis. Concentrations of lactate are low in the testes of the cryptorchid rat, and intratesticular infusion of lactate into these animals improves spermatogenesis (81). In addition to serving as an energy source, lactate functions as an antiapoptotic factor in mature testicular germ cells (82). We surmise that the apoptosis of haploid germ cells seen in Gata4 conditional knockout mice generated with Ambr2-cre (19) reflects in part an insufficient supply of SC-derived lactate.

In summary, we propose that GATA4 functions physiologically to regulate the integrity of blood-testis barrier and apical ectoplasmic specializations and to maintain SCs in a Warburg-like state, thereby ensuring a robust supply of lactate for nourishment of germ cells. GATA4 exerts its metabolic effects by transmitting signals of the FSH pathway and regulating the expression and activity of key enzymes involved in lactate production. The concept that transcription factor GATA4 can govern intracellular metabolism was recently demonstrated in another testicular somatic cell type – the Leydig cell (24, 83, 84). Manipulation of Gata4 expression in SCs affords a means to study the Warburg effect in a noncancerous cell type. These findings come on the heels of a report demonstrating that GATA4 plays a central role in senescence; in a variety of cell types the DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4 (85). Thus, GATA4 has emerged as a pivotal regulator of a diverse array of biological processes, including stem cell maintenance, cell-cell interactions, intermediary metabolism, inflammation, and senescence.

**Acknowledgments**

We thank Dr. Hélder A. Santos, Head of the Pharmaceutical Nanotechnology and Chemical Microsystems Research Unit, Faculty of Pharmacy, University of Helsinki, Finland for providing the equipment to measure TER. We thank members of the EM core, the histology core, and Rebecca Cochran from the Washington University in St. Louis, St. Louis, MO for their expert assistance. We also thank Dr. René Handrick from the Institute of Applied Biotechnology, University of Applied Sciences Biberach, Germany for technical assistance.

Address all correspondence and requests for reprints to: Correspondence: Markku Heikinheimo, M.D., Ph.D., Children’s Hospital, University of Helsinki and Helsinki University Central Hospital, 00 290 Helsinki, Finland, Email: markku.heikinheimo@helsinki.fi.

Disclosure Summary: The authors have nothing to disclose.

This work was supported by Grant Support: This work was supported by the Sigrid Jusélius Foundation (MH), the Academy of Finland (MH), NIH grants DK52574 (DBW), DK075618 (DBW), American Heart Association grant 13GRNT16850031 (DBW), Department of Defense grant PC141008 (DBW), the Jalmari and Rauha Ahokas Foundation (AS), the Biomedical Helsinki Foundation (AS), Orion Pharma Foundation (AS), the Oskar Oflund Foundation (AS), Doctoral Programme in Biomedicine (DPBM), University of Helsinki (AS), Finnish Cultural Foundation grant 00 150 083 (OA), and the Ida Montin Foundation (OA).

**References**

11. Boussouar F, Benahmed M. Lactate and energy metabolism in male


