

Antifibrotic Therapy in the Liver by Adenovirus-mediated Expression of a TGF- β 1 Antisense mRNA

**Von der Fakultät für Mathematik, Informatik und
Naturwissenschaften der Rheinisch-Westfälischen Technischen
Hochschule Aachen zur Erlangung des akademischen Grades einer
Doktorin der Naturwissenschaften genehmigte Dissertation**

vorgelegt von

Biochemikerin und Pharmazeutin

Mónica Arias-Kuhn

aus

Córdoba, Argentinien

**Berichter: Herr Univ.-Prof. Dr.rer.nat Fritz Kreuzaler
Herr Univ.-Prof. Dr.med. Axel M. Gressner**

Tag der mündlichen Prüfung: 11. Dezember 2003

Berichte aus der Biologie

Mónica S. Arias-Kuhn

**Antifibrotic Therapy in the Liver by
Adenovirus-mediated Expression
of a TGF- β 1 Antisense mRNA**

D 82 (Diss. RWTH Aachen)

Shaker Verlag
Aachen 2004

Bibliographic information published by Die Deutsche Bibliothek

Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the internet at <http://dnb.ddb.de>.

Zugl.: Aachen, Techn. Hochsch., Diss., 2003

Copyright Shaker Verlag 2004

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior permission of the publishers.

Printed in Germany.

ISBN 3-8322-3054-8

ISSN 0945-0688

Shaker Verlag GmbH • P.O. BOX 101818 • D-52018 Aachen

Phone: 0049/2407/9596-0 • Telefax: 0049/2407/9596-9

Internet: www.shaker.de • eMail: info@shaker.de

To my parents

To my husband

A	Table of Contents	I
B	Index of Abbreviations	V
1	Introduction	1
1.1	Liver Fibrosis	3
1.2	Transforming Growth Factor- β 1	4
1.3	Gene Therapy	5
1.4	Antisense Therapy	6
1.5	Gene Delivery	7
1.6	Adenoviral System	8
2	Material	9
2.1	Products	9
2.2	Chemicals	10
2.3	Enzymes	11
2.4	Antibodies	13
2.5	Primer	14
2.6	Reagent System and Radioactive Nucleotides	14
2.7	Other Biomolecular Reagents	15
2.8	Cell and Bacteria Culture Mediums	15
2.9	Biologic Materials	16
2.9.1	Bacteria	16
2.9.2	Cells	16
2.9.3	Plasmids	17
2.10	Solutions and Buffers	17
3	Methods	28
3.1	Isolation of Genomic DNA from Mammalian cells	28
3.2	Isolation of Plasmid DNA	28
3.2.1	Small-Scale Preparation of Plasmid DNA	29
3.2.2	Large-Scale Preparation of Plasmid DNA	29

3.2.3	Preparation of Plasmid DNA by Ultracentrifugation	30
3.3	RNA Isolation	30
3.4	Measurement of Nucleic Acids Concentration	31
3.5	DNA Quantification (SYBR[®] Green I)	31
3.6	DNA Cutting by Restriction Endonucleases	31
3.7	Protein Isolation	32
3.8	Protein Quantification (BSA Protein Assay Reagent Kit)	32
3.9	Separation of Molecules by Gel Electrophoresis	32
3.10	Electrophoresis of DNA	33
3.11	Electrophoresis of RNA	33
3.12	Protein Electrophoresis	33
3.13	Cloning Strategies	34
3.14	Recovery of DNA from Agarose Gel by Electroelution	34
3.15	Filling up with Klenow Fragment	34
3.16	Dephosphorilation of Linearized DNA	35
3.17	Ligation of DNA Fragments	35
3.18	Sequence Analysis	35
3.19	Northern Blot Analysis	36
3.20	Southern Blot Analysis	36
3.21	Western Blot Analysis	36
3.22	Radioactive Labeling by Random Priming	37
3.23	Hybridization	37
3.24	Polymerase Chain Reaction (PCR)	37
3.25	Real-Time PCR (RT-PCR)	38
3.26	Preparation of Competent Bacteria (Hanahan Method)	39
3.27	Transformation: Uptake of DNA by Competent Cells	39
3.28	Isolation and Culturing of Rat HSCs and MFBs	39
3.29	Cell Subculturing	40
3.30	Cell Freezing and Storage	41
3.31	Transfection and Infection in Cell Culture	41
3.32	Construction of a Replication-defective Recombinant Adenovirus	42

3.33	Purification, Concentration and Titration of Adenoviruses	42
3.33.1	Purification by Cesium Chloride Gradient Centrifugation	42
3.33.2	Purification by Ion Exchange	43
3.34	Cell ELISA	43
3.35	TGF- β 1 ELISA	44
3.36	Metabolic Labeling, Immunoprecipitation and Fluorography	45
3.37	Microarray Analysis	45
3.38	Proliferation Assay (Thymidine Incorporation)	46
3.39	Animal Models	46
3.40	Immunohistochemistry	47
4	Results	48
4.1	Construction of Antagonist Construct and Respective Viruses	48
4.1.1	Construction of Chimaeric pCMV-asTGF- β 1 Antagonist Construct	48
4.1.2	Construction of E1-Replacement Vectors	50
4.1.3	Examination of Functionality of Antagonist Construct	52
4.1.4	Testing of the Presence of E1a-E1b Genes in HEK293 Cells	53
4.1.5	Construction of Recombinant Adenoviruses	54
4.1.6	Proof of Functionality of Adenoviruses	55
4.1.7	High-Level Expression of the Transgene	56
4.2	Testing of Functionality of the Antisense Construct <i>in vitro</i>	56
4.2.1	Blockage of TGF- β 1 Synthesis	58
4.2.2	Effects of TGF- β 1 in Cell Proliferation	58
4.2.3	Biological Activity	60
4.2.4	Expression of Matrix Related Proteins	61
4.2.5	Expression of TGF- β 1 Related Genes	62
4.3	Testing of Functionality of the Antisense Construct <i>in vivo</i>	62

4.3.1	Fibrosis Induced by Ligature of the Common Bile Duct	63
4.3.2	Analysis of Adenoviral Expression in Normal Tissue	65
4.3.3	Expression of Adenovirus in Experimentally Induced Liver Fibrosis	67
4.3.4	Expression and Antifibrotic Capacity of the asTGF-β1 Device	68
4.3.4.1	Histological Scoring of Liver Fibrosis in Rats	70
4.3.4.2	Suppression of Liver Fibrosis by Transferring the Antisense Construct	70
4.4	Antifibrotic Potential by Selective Expresssion in HSC	73
4.4.1	Expression of the TIMP-1-promoter	73
4.4.2	Construction of TIMP-1-Recombinant Adenoviruses	74
4.4.3	Testing of Functionality of Adenoviral Expression	76
5	Discussion	78
6	Summary	82
7	References	84
8	Publications	93
9	Acknowledgements	95
10	Curriculum Vitae	96