Université de Montréal

Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat

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Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat

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RÉSUMÉ

De plus en plus d'études démontrent que l'hyperglycémie et l'activation du système rénine-angiotensin (RAS) sont des facteurs de risque majeurs dans la pathogénèse de la néphropathie diabétique (DN). L'existence d'un RAS local intrarénal est maintenant bien acceptée. Toutes les composantes du RAS sont exprimées dans les cellules du tubule proximal du rein. L'angiotensinogène est le seul substrat du RAS et est exprimé principalement dans les RPTC; il est ensuite converti en Ang II (forme biologiquement active), par la rénine rénale et l'enzyme convertrice d'angiotensine (ACE). Les niveaux d'Ang II et l'expression génique du RAS sont augmentés dans le diabète, indiquant que le RAS intrarénal joue un rôle important dans la progression de DN. Le(s) mécanisme(s) de régulation de l'expression génique d'AGT dans le rein par un milieu élevé en glucose ainsi que l'insuline, demeurent cependant mal compris.

Afin d'identifier et des cloner ces protéines ainsi que définir leur action sur l'expression génique d'AGT, les protéines nucléaires extraites des IRPTC ont été séparées par électrophorèse en deux dimensions. Les protéines positives ont été hybridées par Southwestern Blot, identifiées par spectrométrie de masse et enfin confirmées par Western Blot. Nous avons identifé deux protéines nucléaires de 48-kD et de 70-kD identiques aux ribonucléoprotéines hétérogènes F (46-kDa hnRNP F) et hnRNP K (65-kDa hnRNP K). Les cDNA de hnRNP F et hnRNP K ont été clonés des IRPTC par RT-PCR et ensuite exprimés dans des clones bactériens. Les recombinants bactériens de hnRNP F et hnRNP K sont liés à l'élément de réponse à l'insuline (IRE)-AGT du rat, comme démontré par essai de retardement sur gel (GMSA) et immunoprécipitation de la chromatine

(ChIP). L'addition d'anticorps polyclonaux contre hnRNP F résulte en un supershift dans le GMSA. La transfection transitoire de cDNA de hnRNP F ou hnRNP Kdans des IRPTC a inhibé l'expression AGT au niveau mRMA et protéine. D'autre part, le knockdown de hnRNP F ou hnRNP K par small interference RNA augmente l'expression de AGT au niveau mRNA (et protéine?) dans les IRPTC. De plus, hnRNP F interagit avec hnRNP F dans les essais de pulldown et de Co-IP La co-transfection de hnRNP F et hnRNP K inhibent l'expression génique d'AGT au niveau mRNA et protéine. Un mileu élevé en glucose stimule hnRNP F ou hnRNP K tandis que l'insuline inhibe leur expression dans les IRPTC in vitro et des RPTC de rat in vivo. Nous avons établi des tranfectants stables d'IRPTC surexprimants hnRNP F et des souris transgéniques surexprimant hnRNP F de façon spécifique dans les RPTC in vitro et in vivo. Nous avons démontré que la surexpression de hnRNP dans les RPTC peut prévenir la stimulation par le glucose (25mM) d'AGT ainsi que de growth factor-β1 (TGF-β1) au niveau mRNA et protéine, ainsi que l'hypertrophie cellulaire (ie : contenu total en protéine de la cellule, incorporation [^{3}H]-Leucine et expression protéique de $p27^{kip1}$). De plus, les souris transgéniques surexprimants HnRNP F démontrent une atténuation de l'expression d'AGT et du récepteur TGF-β1 II au niveau mRNA ainsi qu'une diminution de l'expression de la protéine $p27^{kip1}$) et du ratio protéine/ADN dans les RPTC de souris diabétiques induite au streptozotocin. Ces observatioms suggèrent qu'AGT est modulée par HnRNP F et HnRNP K via la liaison avec l'IRE, action aussi régulée par le glucose et l'insuline. Nous résultats démontrent aussi que HnRNP F ou K pourrait jouer un rôle protecteur ou modulateur dans la prévention de l'hypertrophie des RPTC dans le diabète et ses mécanismes

sont médiés via l'atténuation de l'expression génique d'AGT intrarénal et de la voie de signalisation TGF-β1 *in vitro* and *in vivo.*

Mots-clé: angiotensinogène, hnRNP F, hnRNP K, glucose, Insuline, élément de réponse à l'insuline, souris transgéniques, rein, hypertrophie

ABSTRACT

Accumulating evidence has demonstrated that hyperglycemia and renin-angiotensin system (RAS) activation are major risk factors in the pathogenesis of diabetic nephropathy (DN). The existence of a local intrarenal RAS has now been well accepted. All components of the RAS are expressed in renal proximal tubular cells (RPTCs). Angiotensinogen (AGT) is the sole substrate in the RAS and is expressed predominantly in RPTCs and converts into biologically-active Ang II by renal renin and angiotensin converting enzyme (ACE). Intrarenal Ang II levels and RAS gene expression are elevated in diabetes, strongly indicating that intrarenal RAS activation plays an important role in the progression of DN. The mechanism(s) of regulation of intrarenal AGT gene expression by high glucose and insulin remain, however, incompletely understood. Previously, our lab has demonstrated that an insulin-responsive element (IRE) in rat AGT gene promoter that binds to two nuclear proteins with apparent molecular weights of 48 and 70 kD from rat immortalized RPTCs (IRPTC). The expression of two nuclear proteins in IRPTCs was up-regulated and down-regulated by high glucose and insulin, respectively. To identify and clone these proteins and to define their action on AGT gene expression, nuclear proteins from IRPTC were separated by 2-dimentional gel electrophresis, positive proteins were detected by Southwestern blotting and identified by mass spectrometry and subsequently confirmed by Western blotting. We identified that the 48-kD and 70-kD nuclear protein were identical to 46-kD and 65-kD heterogenous ribonucleoprotein F (hnRNP F) and hnRNP K. HnRNP F and K cDNAs were then cloned from IRPTC by reverse transcriptase-PCR and expressed in bacteria. Bacterially

expressed recombinant hnRNP F and K bound to rat AGT-IRE, as revealed by gel mobility shift assay (GMSA) and chromatin immunoprecipitation assay. The addition of polyclonal antibodies against hnRNP F yielded a supershift in GMSA. Transient transfection of hnRNP F or hnRNP K cDNA in IRPTC inhibited AGT mRNA and protein expression. In contrast, knockdown hnRNP F or hnRNP K gene expression by small interference RNA enhanced AGT mRNA expression in IRPTC. Moreover, hnRNP F interacted with hnRNP F in pulldown and co-immunoprecipiation assays. Co-transfection of hnRNP F and hnRNP K further suppressed AGT mRNA expression. Hyperglycemia stimulated and insulin inhibited hnRNP F and hnRNP K expression in IRPTCs in vitro and rat RPTCs in vivo. We have established stable IRPTC transfectants overexpressing hnRNP F and transgenic mice overexpressing hnRNP F specifically in RPTCs *in vitro* and *in vivo*. We demonstrated that overexpression of the hnRNP F in RPTCs prevented the high-glucose stimulation of AGT and transforming growth factor-β1 (TGF-β1) mRNA and protein expression as well as cellular hypertrophy, i.e., total cellular protein contents, [^{3}H]-Leucine incorporation and $p27^{kip1}$ protein expression). HnRNP F transgenic mice displayed an attenuation of AGT and TGF-β1-receptor II mRNA expression as well as a decrease of cell and nuclear volume, $p27^{kip1}$ protein expression and protein/DNA ratio in RPTCs in streptozotocin-induced diabetic mice. Taken together, these data suggest that AGT was modulated by hnRNP F and hnRNP K through the binging with IRE and this was also regulated by glucose and insulin. Our findings demonstrate that hnRNP F or K may play a protective or modulatory role in preventing RPTC hypertrophy in diabetes and its underlying mechanism is mediated via attenuation of intrarenal AGT

expression and TGF-β1 signaling *in vitro* and *in vivo.*

Key words: Angiotensinogen, hnRNP F, hnRNP K, glucose, Insulin, insulin-responsive element, transgenic mice, kidney, hypertrophy.

Table of Contents

Résumé..... iii

Abstract.....vi

Table of Contents.....ix

List of Tables xiv

List of Figures xv

List of Abbreviations.....xvi

Acknowledgments.....xxi

Chapter 1 – Introduction

1.1 Anatomy and Function of the Kidneys.....2

1.2 End-Stage Renal Disease (ESRD) 3

1.2.1 Epidemiology of ESRD..... 3

1.2.1.1 Incidence of ESRD..... 3

1.2.1.2 Prevalence of ESRD..... 4

1.2.2 Health Care Cost of ESRD..... ..5

1.2.3 Causes of ESRD..... 5

1.2.4 Diabetes and Renin-Angiotensin System (RAS).....7

1.2.5 Diabetic Nephropathy 8

1.2.6 Cellular Hypertropathy in Diabetic Nephropathy.....8

1.2.7 Hyperglycemia and Kidney Injury.....10

1.3 The RAS.....11

1.3.1 Biosynthesis of RAS Members... 12

1.3.2 Historical Views of RAS.....17

1.3.3 General Properties of RAS Members.....18

1.4 Local RAS.....21

1.4.1 Discovery of local RAS.....22

1.4.2 Local RAS in the Kidneys and Its Function.....23

1.5 Activation of the Local Renal RAS in DN.....25

1.5.1 Ang in DN.....25

1.5.2 Ang Receptors in DN.....27

1.5.3 Ang II-Induced DN.....27

1.6 Clinical Trials of RAS Blockages in Diabetic Patients.....28

1.6.1 Clinical Trails of ACEi in Diabetic Patients.....29

1.6.2 Clinical Trails of ARBs in Diabetic Patients.....33

1.7 Gene Regulation of AGT.....34

1.7.1 AGT Regulated by Hormonal Factors.....35

1.7.2 AGT Expression in Diseases.....36

1.7.3 Transcriptional Regulation of AGT.....37

1.8 Insulin and AGT.....38

1.8.1 Insulin Signaling.....38

1.8.2 Insulin Effect on Gene Regulation.....41

1.9 Heterogenous Nuclear Ribonucleoprotein Family (hnRNP).....45

1.9.1 Structure of the hnRNP Family.....46

1.9.2 Transcriptional Function of the hnRNP Family.....47

1.9.3 hnRNP F.....49

1.9.4 hnRNP K 50

1.10 Mouse Models in Diabetes Research.....52

1.10.1 Characterization of Diabetic Nephropathy

in Humans and Mice.....53

1.10.2 Advantages and Disadvantages of Mouse Models

in Research of Diabetic Nephropathy.....55

1.11 Hypothesis and Aims of These Studies.....58

Chapter 2 – Article 1

Heterogenous nuclear ribonucleoprotein F modulates angiotensinogen gene expression in rat kidney proximal tubular cells.

2.1 Abstract.....63

2.2 Introduction.....64

2.3 Materials and Methods 66

2.4 Results 76

2.5 Discussion 81

2.6 Acknowledgements.....87

2.7 References 88

2.8 Figures.....106

Chapter 3 – Article 2

Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells.

3.1 Abstract.....119

3.2 Introduction 120

3.3 Materials and Methods.....122

3.4 Results.....133

3.5 Discussion.....137

3.6 Acknowledgements.....142

3.7 References.....143

3.8 Figures.....157

Chapter 4 – Article 3

Heterogenous nuclear ribonucleoprotein F overexpression attenuates angiotensinogen gene expression and renal proximal tubular cell hypertrophy in transgenic mice.

4.1 Abstract.....169

4.2 Introduction.....170

4.3 Materials and Methods 171

4.4 Results.....177

4.5 Discussion.....182

4.6 Acknowledgements.....187

4.7 References.....187

4.8 Figures.....192

Chapter 5 – Discussion

5.1 Protein-DNA interaction.....203

5.1.1 General prospecting technologies to elucidate Protein-DNA interaction 204

5.1.2 Advantages and disadvantages of 2D gel electrophoresis combined with MS 207

5.2 Advantages and disadvantages of IRPTCs.....210

5.3 Molecular Mechanisms of hnRNP F and hnRNP K on AGT Gene

Expression.....212

5.4 Renopathology Significance of Suppression AGT Gene Expression via hnRNP F or hnRNP K in Hyperglycemia.....216

5.5 Physiological Significance of Suppressing Local RAS Gene Expression via hnRNP F and hnRNP K in Diabetes.....220

5.6 Conclusion.....223

Chapter 6 –Perspectives of Research

6.1 Correlation between hnRNP F/hnRNP K and RAS Gene Expression in Diabetic Animals *in vivo*.....226

6.2 Identification of Transcriptional Factors that Interact or Associate with hnRNP F and hnRNP K *in vitro*.....227

6.3 Gene Chip Microarrays to Identify the Downstream Genes that are Differentially Regulated by hnRNP F and hnRNP K in RPTCs.....228

6.4 Potential Gene Therapy with hnRNP F and hnRNP K.....229

Chapter 7 – References

References.....231

List of Tables

Table 1	Proposed mechanisms of angiotensin II effects in diabetic nephropathy
Table 2	Receptors for angiotensin related peptides
Table 3	Clinical evidence for prevention of diabetes with ACE inhibitors
Table 4	Clinical evidence for prevention of diabetes with ARBs
Table 5	Insulin response sequences/elements
Table 6	Some mouse models of diabetes studied for DN

List of Figures

Figure 1	Incident ESRD RRT patients, selected countries, from 1998-2003 (Crude Rate PMP)
Figure 2	Prevalent ESRD patients at year-end, Canada 1990-2004 (number)
Figure 3	Distribution of incident ESRD patients by primary diagnosis, Canada, 1993-2004
Figure 4	Enzymatic pathway of bioactive angiotensins generation
Figure 5	Structure of AT1 and AT2 receptors
Figure 6	Tubular and interstitial formation, secretion and uptake of angiotensin
Figure 7	Insulin signaling pathway and its cellular function
Figure 8	General structural domains of the hnRNP family
Figure 9	Structure of hnRNP F
Figure 10	Interaction of hnRNP K with TATA-Box Binding Protein (TBP)
Figure 11	HnRNP K overexpression prevents cellular hypertrophy in high glucose
Figure 12	Endogenous hnRNP F and K mRNA expression in diabetic animal models
Figure 13	PAS staining of the kidneys

List of Abbreviations

2D gel	2-dimentional gel
ACC2	Acetyl-Co-A Carboxylase-2
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
AGCE1	AGT core promoter element 1
AGCF1	AGT core promoter binding factor 1
AGT	Angiotensinogen
ALLHAT	The Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack trial
ALPINE	The Antihypertensive treatment and Lipid Profile In a North of Sweden Evaluation
Ang	Angiotensin
AP-1	Activator protein 1
APA	Aminopeptidase A
APN	Aminopeptidase N
Apo-CIII	apolipoprotein CIII
ARBs	Angiotensin II receptor blockers
ASCOT	The anglo-scandinavian cardiac outcomes trial
AT1	Angiotensin II receptor type 1
AT2	Angiotensin II receptor type 2
CAPPP	The Captopril Prevention Project
Cbl	Casitas B-lineage lymphoma
CHARM	The Candesartan in Heart failure—Assessment of Reduction in Mortality and morbidity
CIHI	Canadian Institute for Health Information
CVD	Cardiovascular disease
DAG	Diacylglycerol
DREAM	The Diabetes Reduction Approaches with ramipril and rosiglitazone Medications trial
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
FAS	Fatty acid synthesis
FOXO1	Forkhead transcription factor box O1
G6Pase	Glucose-6-Phosphatase
GLUT4	Glucose transporter 4
GPCR	G-protein-coupled receptor

Grb2	Growth factor receptor binder-2
Gsk3	Glycogen synthase kinase 3
GSK3β	glycogen synthesis kinase 3β
HCTZ	Hydrochlorothiazide
HNF1	Hepatic nuclear factor 1
HNF3	Hepatic nuclear factor 3
hnRNP	Heterogeneous nuclear ribonucleoproteins
HOPE	The Heart Outcomes Prevention Evaluation
IDDM	Insulin-dependent diabetes mellitus
IEF	Isoelectric focusing
IGF	Insulin-like growth factor
IGF1R	IGF receptor 1
IP3	1,4,5-inositol triphosphate
IPG	Immobilized pH gradient
IR	Insulin receptor
IRE	Insulin-responsive element
IRPTC	Immortalized renal proximal tubular cell
IRR	IR-related receptor
IRS	Insulin receptor substrates
JNK	Jun (N)-terminal-kinase
LIFE	The Losartan Intervention For Endpoint reduction in hypertension study
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
MMP-1	Matrix metalloproteinase-1
MS	Mass spectrometry
mSOS	Mammalian son of seven less
mTOR	Mmmalian target of Rapamycin
NIDDM	Non-insulin-dependent diabetes mellitus
$P70^{S6K}$	p70 ribosomal S6 kinase
PDE3b	Phosphodiesterase 3b
PDK	Posphoinositide-dependent kinase
PEPCK	Phosphoenoolpyruvate carboxykinase
PH	Pleckstrin homology
PI	Posphatidylinositol
pI	Isoelectric point
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Posphatidylinositol 3, 4, 5 triphosphate

PKC	Protein kinase C
PRA	Plasma renin activity
PTK	Protein tyrosine kinase
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-Angiotensin System
RBD	RNA binding domain
RPTC	Renal proximal tubular cell
RRM	RNA recognition motif
RRT	Renal replacement therapy
SCOPE	The Study on COgnition and Prognosis in the Elderly
Ser	Serine
SH2	Src homology 2
SHC	Src homology collagen
SHR	Spontaneously hypertensive rats
SOLVD	The studies of left ventricular dysfunction
SRE	Sterol response element binging protein-1
SREB1	Sterol response element binging protein-1
STZ	Streptozotocin
TAT	Tyrosine aminotransferase
TBP	TATA box binding protein
TGF-β	Transforming growth factor-β
TOF	Time-of-flying
TTF2	Tyroid transcription factor-2
VALUE	The valsartan antihypertensive long-term use evaluation
ZF	Zinc finger

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Chapter 1: Introduction

1.1 Anatomy and Function of the Kidneys

In humans, the kidneys are two bean-shaped organs, each about the size of a fist and located in the posterior part of the abdomen, on each side of the spine. The right kidney sits just below the liver, and the left kidney, below the diaphragm, adjacent to the spleen. In a normal human adult, each kidney weighs 150 grams and is about 10 cm long, 5.5 cm wide and 3 cm thick (1; 2).

Both kidneys are surrounded by 3 layers. 1. The r*enal capsule* covers the outer surface of the entire organ. 2. *Fat* keeps them in place and surrounds the renal capsule. 3. The *renal fascia* is a dense, fibrous outer layer that also secures the kidneys to the posterior abdominal wall and surrounding structures (3). The kidneys themselves are made up of 2 layers, the cortex and the medulla. The cortex is the outer layer, and the medulla, the inner layer. Within the medulla are 8-18 triangular structures, the renal pyramids. The tips of the pyramids are referred to as renal papillae. The renal cortex and the pyramids together make up the parenchyma, which consists of approximately 1.25 million nephrons, the functional units of the kidneys that produce urine and help regulate blood composition (1; 4).

The kidneys filter wastes (such as urea) from the blood and excrete them, along with water, as urine. The nephrons consist of renal tubules and renal corpuscles. The tubules are approximately 50 mm in length and comprise the convoluted tubules (proximal and distal) and loop of Henle (4). The proximal tubule, as a part of the nephron, can be divided into an initial convoluted portion and a following straight (descending) portion. Fluid in

filtrates entering the proximal convoluted tubule is reabsorbed into the peritubular capillaries, including approximately two-thirds of filtered salt and water (65%) and all (100%) filtered, organic solutes (primarily glucose and amino acids). These functions of the renal tubules are performed by a number of transporters and channels expressed at the apical and basolateral membranes of the tubular cells (5).

1.2 End-Stage Renal Disease (ESRD)

ESRD (or kidney failure) is a slow, progressive loss of kidney functions caused by inherited disorders, prolonged medical conditions or the long-term use of medications (6; 7). If untreated, it is irreversible and fatal. Renal replacement therapy (RRT), which includes hemodialysis, peritoneal dialysis and renal transplantation, is the major treatment available since the 1960s for ESRD patients (8). ESRD has become a serious medical and economic health problem in developed countries, including Canada (9; 10).

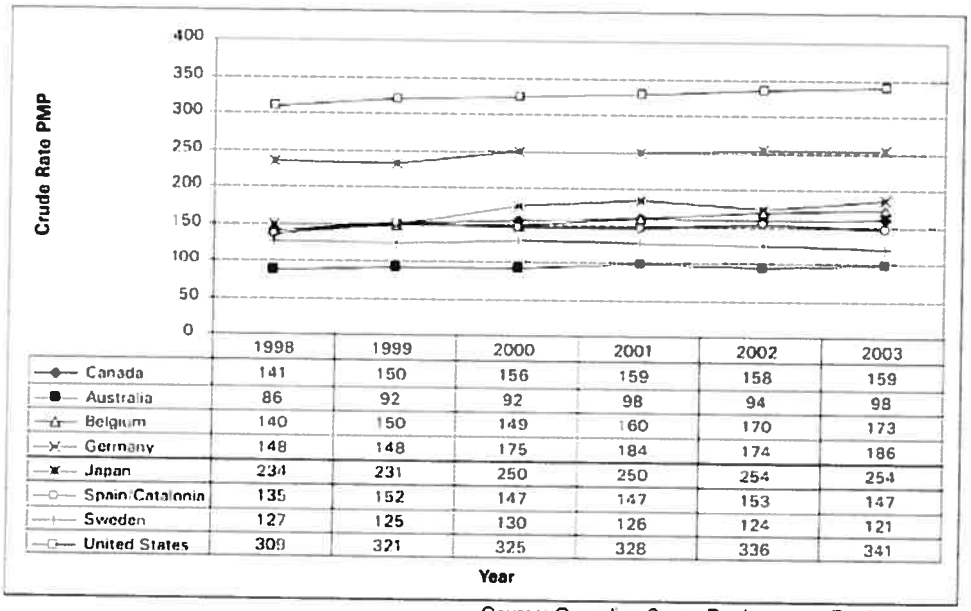
1.2.1 Epidemiology of ESRD

The Canadian Institute for Health Information (CIHI) is the national information system that provides timely, accurate and comparable health information on ESRD and other diseases in Canada.

1.2.1.1 Incidence of ESRD

The incidence of ESRD represents the number of persons newly diagnosed with the disease in a specific population in a given time period. It is useful in medical and epidemiological research examining the causes of disease and the differences in subpopulations affected by these causes. The incidence of ESRD in Canada and other countries during 1998 to 2003 is shown in Figure 1. Over 150 new cases have been reported since 1999,

and this number continues to rise every year. If we examine the data in more detail by age group, the rate among persons aged 19 years or younger was relatively stable, and decreased by 5% in young adults (20 to 39 years old). However, it increased by 32% among those between the ages of 40 to 59 years, and by 17% in people over 60 years (11).



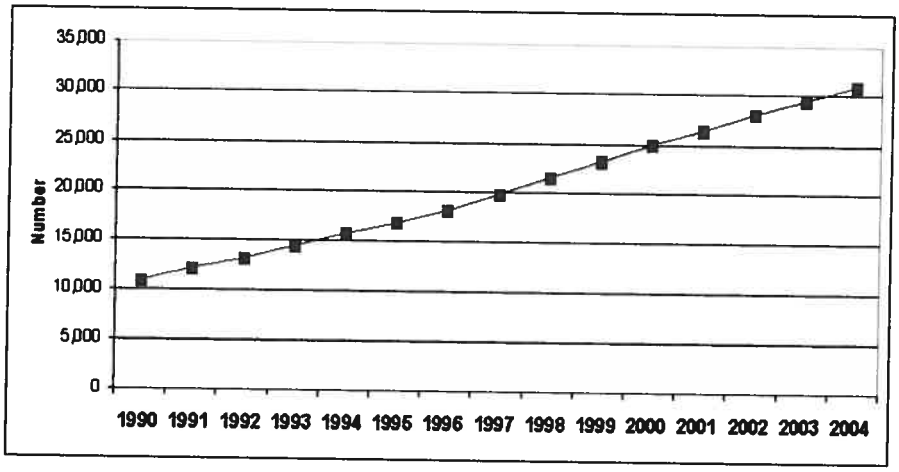
	1998	1999	2000	2001	2002	2003
Canada	141	150	156	159	158	159
Australia	86	92	92	98	94	98
Belgium	140	150	149	160	170	173
Germany	148	148	175	184	174	186
Japan	234	231	250	250	254	254
Spain:Catalonia	135	152	147	147	153	147
Sweden	127	125	130	126	124	121
United States	309	321	325	328	336	341

Source: Canadian Organ Replacement Register, CIHI

Figure 1. Incident ESRD RRT patients, selected countries, from 1998-2003 (Crude Rate PMP)

1.2.1.2 Prevalence of ESRD

ESRD prevalence represents the number of persons in a specific population who have ESRD at a given time point. Figure 2 shows that ESRD prevalence increased dramatically, by almost 2-fold from 1990 to 2004 in Canada. At the end of 2004, 18,827 patients were on dialysis and 12,099 were living with a functioning kidney transplant, for a total of 30,924 Canadians with kidney failure registered in the CIHI (11).



Source: Canadian Organ Replacement Register, Canadian Institute for Health Information (2006)

Figure 2. Prevalent ESRD patients at year-end, Canada,1990-2004 (Number)

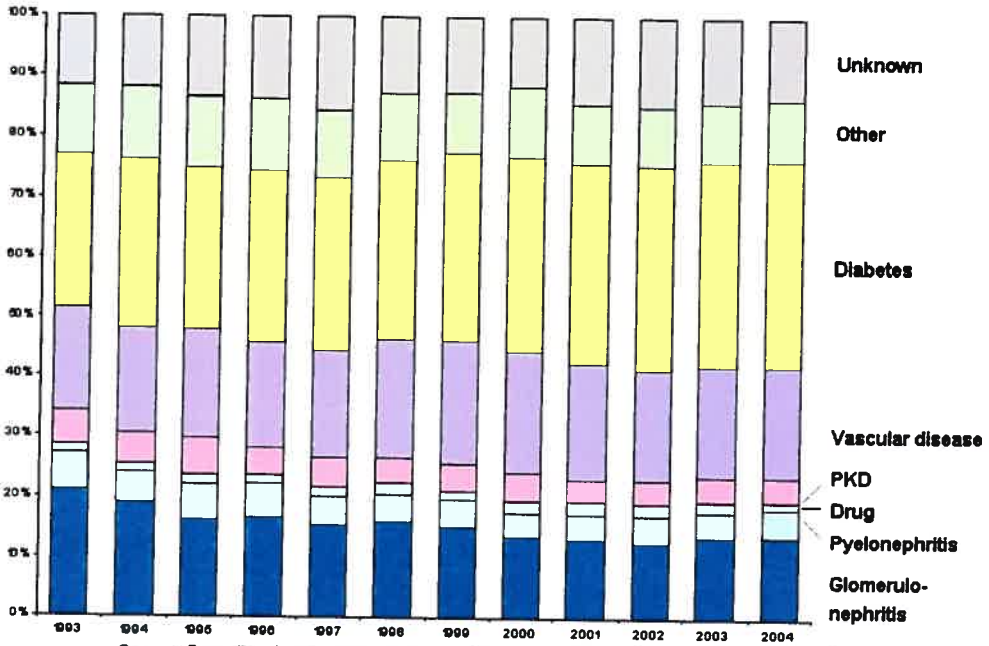
1.2.2 Health Care Costs of ESRD

The health care costs incurred by dialysis and transplantation are enormous. The average cost of peritoneal or hemodialysis is $50,000-75,000 per patient year (12; 13). At least 1 billion Canadian dollars were spent to maintain the quality of life of ESRD patients in last 5 years. Since its incidence and prevalence have increased every year in Canada, the economic burden of ESRD in Canada has become a serious problem. Effective and economic treatment as well as prevention of ESRD onset are major ways of reducing its clinical incidence and could result in considerable cost savings for the Canadian public healthcare system.

1.2.3 Causes of ESRD

According to the CIHI, there are several major causes of ESRD (Figure 3). Diabetes and hypertension are the number 1 and 2 causes of

kidney disease, respectively, in the USA and Canada. Besides this, kidney failure patients aged 65 and older had the highest overall rate of diabetes between 1995 and 2004, more than doubling from 124 per million in 1995 to 270 per million in 2004.



Source: Canadian Organ Replacement Register, Canadian Institute for Health Information (2006)

Figure 3. Distribution of incident ESRD patients by primary diagnosis, Canada, 1993-2004

Diabetes is the leading cause of ESRD, accounting for more than 40% of all registered ESRD patients in Canada, up from 25% 10 years ago, a pattern that is also being encountered in the USA. In addition, it is noteworthy that diabetes types account for these increases. While the number of ESRD patients with type 1 diabetes declined from 526 in 1995 to 303 in 2004 (down 42% in 10 years), the number of patients with type 2 diabetes more than tripled over the same period, from 540 to 1,836. Investigating effective ways of reducing the prevalence of diabetes could

help decrease the devastating health consequences associated with it and ESRD.

1.2.4 Diabetes and the Renin-Angiotensin System (RAS)

Diabetes is a chronic disease characterized by hyperglycemia and complications of multiorgan dysfunction, i.e. the kidneys, heart, brain and eyes. In Canada, over 2 million people have diabetes, of which there are 3 main types: type 1 (insulin-dependent diabetes mellitus, IDDM), type 2 (non-insulin-dependent diabetes mellitus, NIDDM), and gestational diabetes (caused by the hormones of pregnancy). The most common types are type 1 and type 2, with 10 to 21% of diabetes patients developing kidney disease (11).

Considerable evidence suggests that the intrarenal RAS plays an important role in diabetic nephropathy (DN). Landmark studies by Anderson et al. (14) have revealed the superiority of angiotensin-converting enzyme (ACE) inhibitors (ACEi) in halting the progression of renal disease. Conversely, chronic infusion of angiotensin (Ang) II produces marked glomerular and tubulointerstitial injury in the kidneys (15-17). These studies suggest that Ang II is pivotal in the pathophysiology of chronic renal disease. However, it is difficult to separate the effects of blood pressure (BP) reduction from these agents in whole animals and humans. This is because patients with overt DN generally develop systemic hypertension, an adverse factor in all progressive renal diseases and especially so in DN. To elucidate the non-hemodynamic effects of Ang II on DN, finding relevant animal models has become a

challenge for researchers. Furthermore, there is close interaction between the RAS and transforming growth factor-beta (TGF-β) signaling (18-20). In fact, many profibrotic effects of Ang II are mediated by TGF-β stimulation.

1.2.5 Diabetic Nephropathy

DN, a chronic renal disease characterized by glomerulosclerosis, fibrosis of the tubulointerstitial microenvironment, and tubular atrophy (21), is due to longstanding DM. The deposition and decreased turnover of extracellular matrix (ECM) proteins, including fibronectin, collagen types I, III, and IV, are important components of the scarring observed during the evolution of glomerulosclerosis and tubulointerstitial fibrosis (22). TGF-β serves as a paradigm of profibrogenic cytokines (23). Its function in the renal pathophysiological process has been investigated by Border et al. (24).

DN is one of the most significant long-term complications in terms of morbidity and mortality for individual patients with diabetes. Although both type 1 and type 2 DM lead to ESRD, the majority of patients are those with NIDDM. In a prospective study in Germany, the 5-year survival rate was less than 10% in the elderly population with type 2 diabetes, and no more than 40% in the younger age group with type 1 diabetes. The exact cause of DN is unknown, but various postulated mechanisms are hyperglycemia (causing hyperfiltration and renal injury), advanced glycosylation products, and the activation of cytokines (8; 25; 26).

1.2.6 Cellular Hypertropathy in Diabetic Nephropathy

Diabetic nephropathy is typified by renal hypertrophy, increased glomerular filtration rate (GFR), and increased urinary albumin excretion (27; 28). It has been presumed that early hypertrophic changes in the renal cells are integral to the subsequent development of more severe renal histopathologic changes. Nevertheless, the mechanisms initiating and regulating the functional changes in renal cells remain incompletely characterized. The proximal tubule is uniquely susceptible to a variety of metabolic and hemodynamic factors associated with diabetes. Renal function and prognosis correlate better with structural lesions in the tubuli and cortical interstitium than with classical glomerular changes of diabetic nephropathy (29). The proximal tubules show a variety of poorly characterized changes, which have led to the notion that tubular injury represents a "final common pathway" for proteinuric renal injury. However, tubular hypertrophy, reduced organic ion transport, and other tubular changes are already apparent before the onset of proteinuria in diabetes. It seems reasonable to assume that cytokines and polypeptide growth factors including inhibitory factors such as TGF-beta induce in concert, rather than as single factors, tubular hypertrophy (30).

Tubular cells can undergo hyperplasia or hypertrophy, two totally different growth responses. Hyperplasia with mitogenesis of tubular cells plays a central role in the regeneration of functional tubular epithelium subsequent to acute tubular necrosis. The molecular mechanisms how mitogenic signals are transduced to the nucleus are relatively well characterized. In contrast to the hyperplasia, cellular hypertrophy is less well understood. Hypertrophic cells are arrested in the G1-phase of the cell

cycle and increase their size, protein and RNA content, but do not replicate their DNA normally. Such an enlargement of tubular cells often occurs in more chronic situations of renal damage in which remnant nephrons adapt their function to the increasing need. However, evidence exists that hypertrophic tubules are finally joined into the process of maladaptation of renal function leading to tubular atrophy, interstitial scarring, and progression of renal disease (31).

1.2.7 Hyperglycemia and Kidney Injury

The critical role of hyperglycemia in the genesis of DN has been established by cell culture studies, experimental animal models, and clinical trials. Certain cytokines and growth factors have been identified as likely mediators of the effects of high ambient glucose on the kidneys, and prominent among them is TGF-β, a prototypical hypertrophic and fibrogenic cytokine. TGF-β overexpression has been demonstrated in the glomerular and tubulointerstitial compartments of experimental diabetic animals (32). The TGF-β receptor signaling system is also triggered, as evidenced by the upregulation of TGF-β type 2 receptors and activation of the downstream Smad signaling pathway (33). Treatment of diabetic mice with neutralizing anti-TGF-β antibodies prevents the development of renal hypertrophy, mesangial matrix expansion, and the decline in renal function. Antibody therapy also reverses the established lesions of diabetic glomerulopathy (34). TGF-β may also contribute to the cellular hypertrophy found in persons with DN. Cell culture studies suggest that high-glucose concentrations stimulate the hypertrophy of proximal tubular and mesangial cells (35; 36) as well as the production of matrix molecules, such as

fibronectin and collagens in these cells, and even in epithelial, endothelial, and interstitial-fibroblastic cells (37-39). Such investigations strongly support the hypothesis that overactivity of the TGF-β system in the kidneys is a crucial mediator of diabetic renal hypertrophy and mesangial matrix expansion.

Hyperglycemia may activate protein kinase C (PKC) (40; 41), which may contribute to renal disease and other vascular complications of diabetes. Hyperglycemia may also activate Ang II, which is the bioactive peptide of the RAS, and has hemodynamic and non-hemodymic effects in the kidneys, as shown in Table 1.

Table 1. Proposed mechanisms of Ang II effects in DN

Hemodynamic effects	Non-hemodynamic effects
Systemic hypertension	Induction of renal hypertrophy and cell proliferation
Systemic and renal vasoconstriction	Stimulation of extracellular matrix synthesis
Increased glomerular capillary pressure and permeability	Inhibition of extracellular matrix degradation
Mesangial cell contraction leading to reduction of the filtration surface area	Stimulation of cytokine (e.g., TGF-β, VEGF) production
	Stimulation of superoxide production

Modified from Ref. 21

1.3 The RAS

The RAS, or the renin-angiotensin-aldosterone system, is an extracellular hormonal system that plays an important role in regulating blood volume and systemic vascular resistance, which together influence cardiac output, arterial pressure, and electrolyte homeostasis (42). In this tightly-regulated system, physiological regulators of BP and fluid balance induce the production of potent vasoactive Ang peptides by the sequential proteolysis of angiotensinogen (AGT) prohormone. AGT is the only known

precursor of Ang peptides, whose circulating concentrations influence the tonic activity of the RAS (43). AGT abundance is controlled at the transcriptional level by hormonal and cell type-specific regulators (44; 45).

AGT has been identified as a non-inhibitory member of the serine proteinase inhibitor (serpin) family. Although the most abundant source of plasma AGT is the liver, Northern blotting and reverse transcription-polymerase chain reaction (RT-PCR) techniques have confirmed AGT mRNA expression in a wide range of tissues, including the kidneys, brain, vascular tissue, adrenal glands, placenta, and leucocytes (46; 47). The sequencing of rat (r) and human (h) AGT genes has increased our understanding of this protein and its role in the physiology and pathogenesis of human disease (48; 49). Early observations on the regulation of AGT are now explicable at the molecular level, with identification of the core promoter, the hormone, acute phase responsive elements and tissue-specific enhancers (45). The role of AGT in DN has been studied.

Ang II, the most important biologically-active end product of the RAS, is an extremely potent vasoconstrictor and a major determinant of salt and water homeostasis. It is thought to act as both a circulating hormone and paracrine factor. However, the physiological significance of paracrine Ang II is still not well-understood.

1.3.1 Biosynthesis of RAS Members

The RAS is composed of the precursor molecule AGT, a peptide related to the family of serpins. A unique substrate for the circulating

protease renin (EC 3.4.23.15), AGT is derived mainly from juxtaglomerular cells of the kidneys, and also from many other tissues and organs (46; 47; 50). Figure 4 illustrates the biosynthesis pathway of the RAS.

Prorenin, the inactive precursor of renin, circulates in human plasma in excess of renin, at up to 100 times higher concentrations (51). Studies in transgenic (Tg) animals displaying prorenin expression show that prorenin activation at tissue sites might involve proteolytic removal of its prosegment (52-54). Alternatively, activation could occur in a non-proteolytic manner, for instance, through binding to a receptor. Indeed, Ichihara et al. have proposed recently that human prorenin has so-called gate and handle regions for non-proteolytic activation through its putative receptors (55). Nguyen et al. have cloned a prorenin receptor, as prorenin binding to it allows prorenin to become fully active enzymatically in a non-proteolytic manner (56). It has been reported recently that prorenin binding to the prorenin receptor plays a pivotal role in the development of DN by a mechanism that involves the receptor-associated prorenin system (55; 57). AGT hydrolysis by renin is rate-limiting for the whole system and results in production of the peptide Ang I, which is converted to the peptides Ang II and Ang III by ACE (EC 3.4.15.1) and aminopeptidase A (APA) (EC 3.4.11.7), respectively. These peptides act on 2 membrane-bound Ang II receptors (AT1 and AT2) belonging to the 7-transmembrane G-protein-coupled receptor family.

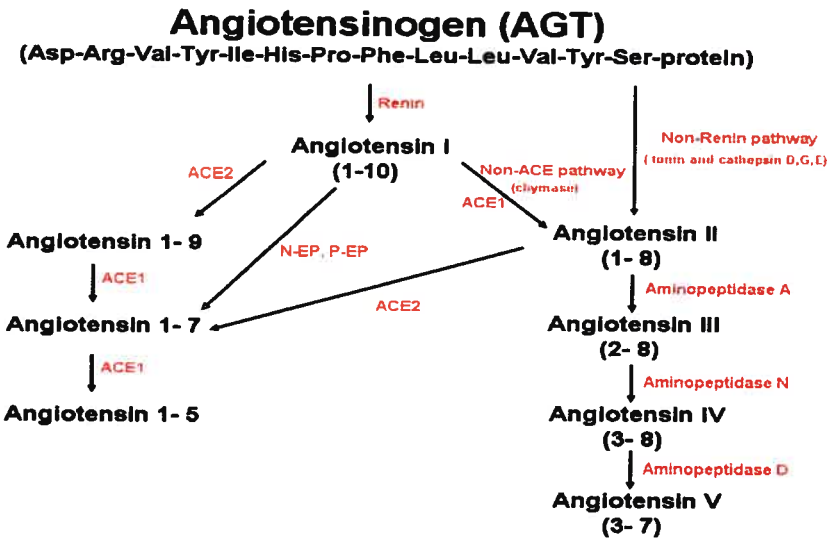


Figure 4. Enzymetic pathway of bioactive angiotensins generation.

Besides the circulating renin of renal origin, there are at least 2 categories of tissue renin-like enzymes (isorenins) (58). The first category is represented by tissue cathepsins (G, D, and E), which are lysosome serine-proteases, ensuring local capacity for the lysis of AGT to Ang I, as the common precursor of bioactive Angs. While cathepsin G is present in human neurotrophils and activates circulating prorenin (59; 60), cathepsin D has been found in the heart and vascular smooth muscle and stimulates myocyte growth (61). In turn, cathepsin E is involved in Ang I and endothelin biosynthesis (62). The second category of tissue renin-like enzymes is represented by tonin (59), a cytosolic serine-protease that allows direct Ang II synthesis from tissue AGT.

Classical ACE1, a zinc metallopeptidase located predominantly at the luminal surface of the pulmonary vascular endothelium, cleaves the histidylleucine dipeptide from inactive Ang I, producing active Ang II (63). Being a dipeptidylcarboxypeptidase, ACE1 is rather nonspecific in the way

it hydrolyzes several substrates, including bradykinin and opioid peptides (64; 65).

Ang II is detected in plasma and tissues, even when ACE1 is inhibited, indicating a role for other enzymes in the conversion of Ang I to Ang II. In the human heart, for instance, chymase is the major Ang II-forming enzyme (66). It belongs to the family of myocardial cathepsins with Ang-forming activity. Unlike the converting enzyme present mainly in the atria and endothelial cells, chymase has a predominantly ventricular localization and does not inactivate bradykinin (67).

Recently, a new homologous ACE, ACE2, has been discovered in the heart, kidneys, liver, and intestine (68). This new converting enzyme induces hydrolysis of the last amino acid of Ang I, producing Ang 1-9, an inhibitor of classical ACE1 and a precursor of vasodilating Ang 1-7. Ang 1-9 thus limits the formation and actions of Ang II, contributing to autoregulation of the local circulation (69). In addition, the proteolytic activity of ACE2 is 400 times higher with Ang II as substrate than Ang I (70). ACE2 may function as a tissue-specific, negative feedback regulator of the activated RAS, e.g. by vasodilation mediated by Ang 1-7 and bradykinin (71). The actions of both ACE1 and its homologue ACE2 seem to be more complex than originally believed. The precise interplay between ACE1, ACE2 and their substrates and by-products is still unclear.

Angiotensinases are peptidases that generate active or inactive Ang peptides, altering the ratio between their bioactive forms. Ang II is converted to Ang III by APA, whereas Ang III is inactivated by aminopeptidase N (APN) (72; 73). Endopeptidases use Ang I as a

substrate to form Ang 1-7 (74; 75). Tissue carboxypeptidases and proteolytic enzymes (pepsin, trypsin, chymotrypsin, etc.) also contribute to the degradation of bioactive Angs to inactive fragments (76).

The major biological actions of Ang II are mediated by 2 well-characterized receptors, type 1 (AT1) and type 2 (AT2) (Table 2) (77). In adult tissues, the AT1 receptor is widely distributed in the vasculature, kidneys, adrenal glands, heart, liver, and brain (50; 78). The AT2 receptor is widely distributed in the fetus and then expressed minimally in adults. In healthy adults, the AT2 receptor is present only in the adrenal medulla, uterus, ovaries, vascular endothelium, and distinct brain areas (50; 78).

Table 2. Receptors for angiotensin related peptides

Type	Ligand	Distribution	Function
AT1	Ang II	Widespread, (kidneys, blood vessels, etc.)	Salt and water homeostasis, blood pressure
AT2	Ang II	Fetal tissue, adrenal, ovaries, brain, endothelium	Apoptosis, growth inhibition, vasodilation
Mas	Ang 1-7	Kidneys, heart	Blood flow, renal Na
AT4	Ang III Ang IV	Brain, heart, adrenal, kidneys, blood vessels	Blood flow, renal Na, memory

Ang receptors share several similarities. They are both G-coupled polypeptides that contain approximately 360 amino acids with 7 cell membrane-spanning regions (79; 80). Both genes share sequence homology of approximately 30% (Figure 5) (81). As a result, they are functionally distinct and employ different signal transduction pathways (80; 82). For example, the AT1 receptor mediates the hemodynamic actions,

endocrine functions, and mitogenic effects of Ang II in the kidneys (82; 83), whereas the AT2 receptor regulates fetal organ development and exerts both vasodilatory and antiproliferative effects (84; 85). The AT2 receptor interacts with and modulates the actions perpetuated by the AT1 receptor, likely antagonizing many of them. For instance, AT2 receptor deletion in mice treated with a nitric oxide synthase inhibitor is associated with hypertension as well as reduced sodium and water excretion (86). In contrast, mice with intact AT2 receptors remain normotensive and maintain normal salt and water homeostasis.

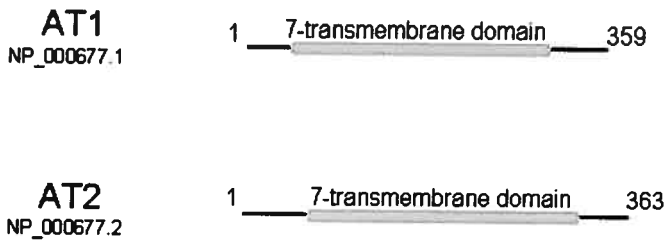


Figure 5. Structure of AT1 and AT2 receptors.

1.3.2 Historical View of the RAS

After 1898, when Tigerstedt and Bergman discovered a substance in both watery and alcoholic extracts of the rabbit renal cortex, renin was found. This is seen as one of the great advances in physiology, although it had little impact at that time (87). Now, more than a century later, the full physiological and pathological significance of renin or even the whole RAS is no longer doubted by medical scientists.

Classically, the RAS has been considered to be a hormonal circulating system. Through vasoconstriction and aldosterone secretion, Ang II plays

an important role in the maintenance of BP and electrolyte balance. Since the identification of the classical enzyme cascade that produces active Ang II in circulating blood, experimental support has accumulated in favour of renin-like activity within many tissues and organs (88). Thus, the notion of the extrarenal RAS was established, leading to the concept of the RAS as a unitary hormonal system, with circulating and tissue components.

During the last decade, it has been established that, apart from its classical actions, Ang II has various other effects induced by direct impact on its receptors or via the local actions of Ang metabolites. Thus, the RAS has been postulated to be a complex humoral system with endocrine, paracrine, autocrine and intracrine properties (89).

1.3.3 General Properties of RAS Members

The contributions of the RAS to the regulation of arterial pressure and the physiopathology of hypertension have long been recognized. It is important to understand that circulating AGT concentrations are generally quite high, being more than 1,000 times greater than plasma Ang I and II concentrations (90-92). Figure 6 depicts the representative plasma AGT concentrations in rats, expressed as pmol/ml, while Ang I and Ang II concentrations are expressed as fmol/ml, indicating that active Ang II concentrations in plasma are a small fraction of Ang II available in the form of AGT (91). Therefore, even small relative changes in the rates of Ang I and Ang II generation may make large absolute differences in circulating concentrations. Nevertheless, changes in AGT synthesis and release occur slowly and, thus, their effects are not as dynamic as the effects of changes in plasma renin concentration (44; 93).

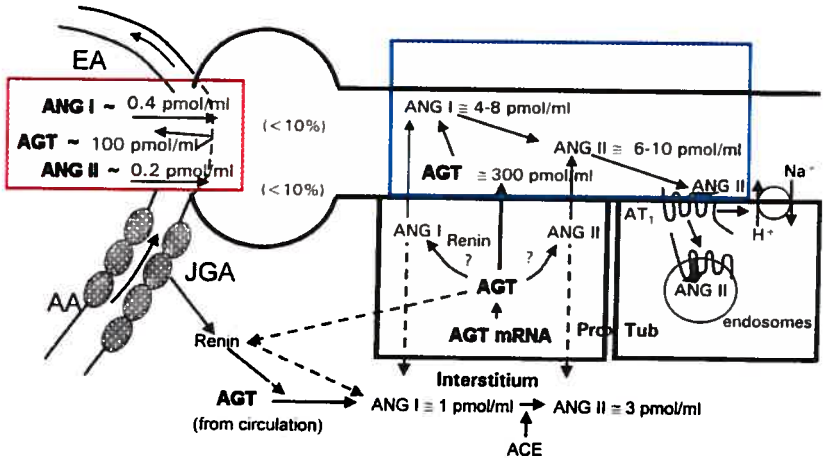


Figure 6. Tubular and interstitial formation, secretion and uptake of angiotensin (Ref. 91).

As discussed above, Ang II exerts its effects via Ang receptors, which trigger intracellular signaling processes. In response to Ang II stimulation, the AT1 receptor interacts with various heterotrimeric G proteins, inducing the phosphorylation of tyrosine kinase (94). The intracellular signaling processes are multiphasic, leading to various biological actions. AT1 receptor activation results in increased protein tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) Activation. These processes are associated with growth factors, cytokines, and inflammatory agents. Thus, in addition to its potent vasoconstrictor actions, Ang II has proliferative and pro-inflammatory properties (95).

One of the earliest detectable events arising from Ang II stimulation is AT1 signaling through phospholipids with the involvement of phospholipase C (PLC), PLD and PLA2. While the stimulation of PLC induces rapid 1,4,5-inositol triphosphate production and diacylglycerol (DAG) release, PLD hydrolyzes phosphatydylcholine to phosphatidic acid and choline. PLA2 is responsible for arachidonic acid release from cell

membrane phospholipids (94). The phospholipid-derived second messengers produced by Ang II-activated PLs induce multiple subsequent events (Ca2+ release from the sarcoplasmic reticulum, PKC activation by DAG, eicosanoid generation, etc.).

The AT2 receptor may counteract some of the actions of the AT1 receptor. Found in large amounts in fetal tissues, the AT2 receptor is considered to be a factor that stimulates growth and development. Preferentially expressed by various secretary cells, it is activated by both circulating and locally-generated Angs (96). As already noted, AT2 has antagonistic properties, being involved in local blood flow control as a vasodilating mechanism (97).

It has been demonstrated that Ang II exerts opposite effects on the growth of certain cells within blood vessels by binding to either the AT1 or AT2 receptor. Ang II induces cell proliferation by activating its AT1 receptor and inhibits cell growth by stimulating the AT2 receptor in different cell types (98). The growth-suppressing effects mediated by the AT2 receptor do not involve repression of fos and c-jun gene expression, but are evoked through the inhibition of cyclin D1 expression and cyclin D1-associated kinase activity (99). Furthermore, AT2 receptor stimulation modulates AT1 receptor-mediated vasoconstriction, and altered AT2 receptor function may contribute to an exaggerated vasoconstrictor action of Ang II (97). Apoptosis is another biological phenomenon which seems to be triggered by the stimulation of AT2 receptors. Elevated AT2 receptor levels have been observed in the brain of rats after global ischemia, eventually leading to delayed neuronal cell death (100).

In addition to its multiple actions, Ang II also functions as a precursor

of its active fragments, represented by Ang III [2-8], Ang IV [3-8], Ang V [3-7] and Ang 1-7 (Figure 4). The heptapeptide Ang III has physio-pharmacological effects similar to those of Ang II (58; 89; 101-104), mediated by their common receptors, AT1 and AT2 (103). Ang IV is involved in blood flow regulation, exploratory behavior, learning, memory, and neuronal development (103). It has been demonstrated that the inhibitory influence of Ang II via AT1, and a facilitory role of Ang IV via its receptor AT4, in the neuronal firing rate, associative and spatial learning (105).

Ang 1-7 produces vasodilation, diuresis and antiproliferative reactions through a specific receptor (Mas). The vasodilating effects of Ang 1-7, more intense in vessels with an intact endothelium than in those with the endothelium removed, are accompanied by the release of nitric oxide (NO), kinins, and prostaglandins (76). Through inhibition of the pressor and proliferative actions of Ang II, Ang 1-7 behaves like a true counteracting factor. In agreement with these data, possible balance has been hypothesized between the vasoconstrictor and hypertensive properties of Ang II and the actions of Ang 1-7 (69). Ang 1-7, one of the active components of the RAS, is involved as a possible counter-regulatory factor in the vasoconstrictor effects of Ang II.

1.4 The Local RAS

Over the last 3 decades, it has become increasingly clear that bioactive Ang peptides can be generated, not only in the systemic circulation, but also as local hormones in several tissues and organs. Since all components of the RAS have been detected in many tissues and organs,

numerous investigations have shifted their focus from its endocrine to its autocrine/paracrine role in specific tissue functions, such as tissue growth and differentiation.

The localization and expression of key RAS components, notably AGT and renin, which are mandatory for the presence of tissue RAS, have been reported in a wide range of tissues (47; 106; 107). The expression of local RAS components in tissues such as the brain, heart, kidneys, adrenals, and gonads has led to the proposition that these components may either potentiate systemic functions, or have entirely separate activities meeting the specific needs of individual tissues (107-109). There is accumulating evidence that changes in tissue/organ-specific RAS may be associated with the pathophysiology of respective tissue/organ functions, giving rise to the possibility that drugs acting on tissue RAS could ameliorate some of these disorders (110).

1.4.1 Discovery of the Local RAS

Evidence accumulated over decades has expanded significantly by more recent findings that have increased the complexity of the RAS. Different Ang receptors and signal transduction pathways have been characterized. Moreover, additional truncated peptides, such as Ang 1-7, have been identified, and alternative Ang II formation by non-renin and non-ACE pathways has been reported. These results have modified our view of the RAS to the concept of "local" or "tissue" RASs. This concept is based on findings of RAS components in "unlikely" places (such as "kidney enzyme" renin in the brain), where endocrine actions of the system cannot

be explained (111-113). This, in turn, has led to new hypotheses and functional concepts of local RAS actions based on the tissue synthesis of Ang II.

The overexpression of RAS genes in Tg mice and rats as well as the knockout of these genes in mice have fostered detailed studies on functions of the local RAS (113). It has also become increasingly clear that these systems are not isolated entities, but can interact with the endocrine RAS as well as other peptide systems (such as the endothelin system) at multiple levels.

Early controversies surrounding the novel concept of tissue RAS arose from the question of local synthesis versus uptake from the circulation. A case in point is the controversy between investigators after the demonstration of renin expression in the heart (75; 114) and those questioning local renin synthesis. This controversy was attributed to the fact that renin mRNA in the heart could only be demonstrated inconsistently, suggesting that studies measuring cardiac renin were biased by artifacts from contamination with plasma renin or active renin uptake from the circulation. The issue should not threaten the concept of the local RAS, since either mechanism could contribute to local AGT synthesis and actions. Modern concepts of the tissue RAS are therefore function-oriented.

1.4.2 The Local RAS in the Kidneys and Its Function

The multiple intrarenal actions of Ang II are based on its predominantly local synthesis and the presence of AT1 receptors within glomerular vessels and various tubular segments. A density gradient of these receptors has been demonstrated by their much higher amounts in superficial than in juxtamedullary glomeruli. Ang receptors can be found mostly in the basal membrane of the tubular epithelium and mesangial cells. This distribution provides the contractile effect of the peptide on mesangial cells and the control of proximal sodium and water reabsorption. Proximal tubular sodium transport is stimulated by enhanced sodium/hydrogen ion antiporter activity on luminal membranes (115). Increased sodium/potassium pumps and sodium/bicarbonate co-transporters on the basolateral membranes of proximal tubular cells (PTCs) also enhance sodium reabsorption (116; 117). Ang II augments sodium reabsorption through actions on sodium/potassium pumps in the medullary thick ascending limb and epithelial sodium channel of cortical collecting tubules (117; 118). At the functional level, Ang II contributes to control of the glomerular filtration rate (GFR).

Aside from its hemodynamic effects, Ang II promotes other processes in the kidneys. It perpetuates the production of nephrotoxic reactive oxygen species (ROS), and stimulates cell proliferation as well as tissue remodeling by enhancing the synthesis of profibrotic cytokines and growth factors. The overexpression of chemokines, chemotactic factors, and cell adhesion molecules contributes to abnormal cellular proliferation and renal fibrosis. Collagen deposition is also enhanced through the inhibition of proteases that normally function to degrade abnormal tissue proteins (119).

Together, these effects increase the development of glomerulosclerosis and tubulointerstitial fibrosis (119; 120).

At the same time, the vasa recta and peritubullary capillaries, being most sensitive to the vasoconstricting action of Ang II, contribute to both tubulo-glomerular feedback and the control of water and ion elimination. Ang II participates in the long-term regulation of arterial BP through such volume regulatory mechanisms.

1.5 Activation of the Local Renal RAS in DN

Sustained interest in dissecting the pathophysiology of DN has led many to focus on renal hemodynamics. One factor that has fueled this interest is the unequivocal benefit derived from ACE inhibition in DN. Baseline plasma renin activity is found to be reduced in type 2 diabetic patients. The renovascular response to an Ang antagonist is substantially greater than normal, indicating intrarenal RAS activation not reflected in plasma levels (121). Thus, investigators are searching for factors that evoke RAS activation in diabetes. Hyperglycemia is a logical candidate (122-124).

1.5.1 Ang in DN

The beneficial effects of ACEi or Ang receptor blockade in the prevention of diabetic renal disease suggest that Ang II is a major mediator of progressive renal injury (25; 125-127). However, measurement of the activity of circulating RAS components largely points to suppression in DN (121). These observations indicate local renal tissue activation of the RAS

or increased intrarenal sensitivity to Ang II, especially at AT1 receptors, in DN.

mRNA and protein levels of renin are elevated at the onset of DN in spontaneously or streptozotocin (STZ)-induced diabetic rats (128). In early DN, proximal tubule renin mRNA is significantly upregulated, with no change in AGT or ACE mRNA (129). This process is reversed by insulin therapy. In addition, an increase in intrarenal AGT mRNA has been reported in diabetic rats, implying heightened AGT synthesis by proximal tubules (128; 130). In our lab, we have discovered that hyperglycemia stimulates AGT synthesis in a concentration-dependent manner in association with augmented AGT mRNA expression (131). A glucose-response element has been located on the AGT gene promoter that might mediate this effect (129). Recently, we also found that hyperglycemia increases AGT gene expression via ROS in rat proximal tubules, providing evidence of intrarenal RAS activation in DN (132).

In diabetic rat and human kidneys, ACE1 is redistributed towards glomeruli and the renal vasculature but away from the proximal tubules (130; 133). These studies suggest that glomerular ACE mediates nephron injury, possibly by increasing local intraglomerular Ang II formation. In cultured rat mesangial cells, high-glucose levels stimulate Ang II production in a concentration-dependent manner in association with augmented TGF-β production (134). Nevertheless, whole-kidney Ang II levels in diabetic animal models have been reported to be elevated, decreased or

unchanged (128; 135-137). However, whole-kidney levels probably do not reflect Ang II at specific nephron sites.

1.5.2 Ang Receptors in DN

Several studies have documented the expression of renal AT1 and AT2 receptors in DN. Reduced renal expression of the AT1 receptor has been reported in rats after 3 weeks of STZ-induced diabetes and also in diabetic patients (138). However, another investigation found no significant change in AT1 receptor expression, but reduced AT2 receptor expression in the kidneys of rats with early (2-week) STZ-induced diabetes (139). Recently, an experiment was performed on spontaneously hypertensive rats (SHR) after 32 weeks of STZ-induced diabetes. In long-term DN in the context of hypertension, increased albuminuria and renal structural injury were accompanied by the decreased expression of genes encoding AT1 and AT2 receptors and respective proteins in the kidneys (140). The downregulation of receptor expression implied reduced receptor synthesis, as opposed to the inability of Ang II to internalize and desensitize the AT2 receptor. The decline in renal AT2 receptor expression in diabetic Wistar-Kyoto (WKY) rats without systemic hypertension and with less prominent renal injury was consistent with a glucose-dependent mode of AT2 receptor regulation.

1.5.3 Ang II-induced DN

Ang II has many actions that might cause or contribute to DN (Table 1). It is produced by mesangial cells, in which its biosynthesis is increased by

glucose (134). Thus, mesangial cells might have their own self-contained RAS, and hyperglycemia could stimulate its activity, leading to cellular contraction with reduced filtration surface area (141). Both glucose and Ang II stimulate ECM formation, and collagen accumulation in mesangial cells is blocked by AT1 receptor inhibition (134; 141). TGF-β is an important mediator of collagen accumulation and fibrosis. Because glomeruli from diabetic animals and humans have increased mRNA encoding TGF-β, TGF-β contributes to the pathogenesis of DN (142). Ang II stimulates mesangial cell matrix biosynthesis via the AT1 receptor, and this is mediated by TGF-β (16). In rat mesangial cells, glucose-induced TGF-β secretion is abrogated by AT1 receptor blockade (134). In humans, the progression of DN is elicited, at least in part, by Ang II action via TGF-β (143). These observations suggest that glucose stimulates Ang II synthesis, leading to increased TGF-β-induced matrix accumulation.

Several studies have reported AT2 receptor downregulation in diabetic animal models (139; 140). If intrarenal Ang II production is augmented in diabetes, AT2 receptor downregulation could create an imbalance in which AT1 receptor stimulation is unopposed. A reduction of renal nitric oxide (NO) in diabetes could occur because of diminished NO synthesis, decreased availability of the substrate L-arginine, or failure of the AT2 receptor to generate NO (25; 139; 144).

In summary, these data and other investigations indicate that intrarenal RAS activation might play an important role in DN.

1.6 Clinical Trials of RAS Blockade in Diabetic Patients

Because intrarenal RAS activity contributes to DN, many clinical trials have explored the effects of RAS blockade and obtained surprising results in hypertensive patients with or without diabetes. Since the mid-1980s, numerous clinical studies have assessed the metabolic effects of ACEi or Ang receptor blockers (ARBs). Besides these inhibitors, a novel direct renin inhibitor, Aliskiren, has just become available in the USA, Switzerland, and Europe. The long-term potential of Aliskiren and direct renin inhibition is being investigated in a clinical program known as ASPIRE HIGHER, focusing on the benefits of the drug in hypertensive patients with heart failure (HF) or kidney failure. Preliminary data from this program indicate that BP is reduced in these patients (145).

1.6.1 Clinical Trails of ACEi in Diabetic Patients

Six clinical studies in which ACEi were given to patients with hypertension or other risk factors for cardiovascular disease (CVD) indeed suggest that they decrease the risk of ESRD (Table 3).

The Heart Outcomes Prevention Evaluation (HOPE) trial found a 34% reduction ($P < 0.001$) in the relative risk of new-onset diabetes in patients treated daily with up to 10 mg of the ACEi ramipril (146). Similarly, the effect of enalapril on the incidence of new-onset diabetes was assessed in a subgroup of patients from studies of left ventricular dysfunction (SOLVD). A significant 78% reduction ($P < 0.0001$) in the incidence of new-onset diabetes was noted in comparison to placebo (147). Together with the small number of patients included in the sub-analysis, this limited wider interpretation of the findings. Nevertheless, the data from both HOPE and

SOLVD suggested that ACEi prevent the development of diabetes in subjects with CVD, meriting further investigation in prospective trials.

Table 3. Clinical evidence for prevention of diabetes with ACE inhibitors

Clinical study	Comparison	Reduction in ESRD risk
HOPE	Ramipril vs. placebo	34 % (15-49%, $P < 0.001$)
SOLVD	Enalapril vs. placebo	78% (54-90%, $P < 0.0001$)
CAPPP	Captopril vs. beta blockers/diuretics	22% (6-33%, $P = 0.039$)
ALLHAT	Lisinopril vs. amlodipine	17 % (8.1% vs. 9.8%, $p < 0.46$)
	Lisinopril vs. diuretics	30% (8.1% vs. 11.6%, $p < 0.001$)
ASCOT–BPLA	Amlodipine ± perindopril vs. atenolol ± bendroflumethiazide	34 % (22-37%, $P < 0.001$)
DREAM	Ramipril vs. placebo	7 % (18.1% vs. 19.5 %)

The Captopril Prevention Project (CAPPP), which compared the effects of captopril and conventional therapy (diuretics or beta-blockers) on cardiovascular morbidity and mortality in more than 10,000 patients with hypertension (diastolic BP > 100 mmHg on 2 occasions), included new-onset diabetes as a secondary endpoint. After a mean follow-up of 6.1 years, the incidence of new-onset diabetes was reduced by 14% overall in the captopril group ($P = 0.039$) and by 22% in the subgroup of patients previously untreated for hypertension ($P = 0.041$) (148).

The Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial (ALLHAT) was a large randomized study designed to determine whether newer classes of anti-hypertensive agents lowered the incidence of coronary heart disease or other CVD events better than the diuretic chlorthalidone (149). After a mean of 4.9 years, no differences were seen between the treatment groups in terms of primary outcomes.

However, analysis of a sub-group of individuals who were non-diabetic at the start of the study found that the relative risk of developing diabetes in patients treated with the ACEi lisinopril was reduced by 30% compared with the diuretic, and by 17% compared with the calcium antagonist amlodipine.

Similarly, in the Blood Pressure-Lowering Arm of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT-BPLA), hypertensive patients assigned to treatment with amlodipine ± perindopril were 34% less likely to develop new-onset diabetes than those randomized to atenolol ± bendroflumethiazide ($P < 0.001$) (150). At the outset, approximately 75% of patients did not have diabetes. During the 5-year study, 8% of patients in the amlodipine group, the majority of whom also received perindopril, developed diabetes, compared with 11.4% of those on atenolol-based therapy. These findings may be the result of the diabetogenic effect of the diuretic-beta-blocker combination and the positive effect of the ACEi (151).

The Diabetes REduction Approaches with ramipril and rosiglitazone Medications (DREAM) Trial was a large-scale study specifically designed to investigate drug therapies for the prevention of diabetes. A total of 5,269 patients without CVD but with impaired fasting glucose or impaired glucose tolerance were recruited. Patients were randomized to receive up to 15 mg ramipril daily or placebo and followed for 3 years. Treatment with ramipril did not significantly reduce the incidence of diabetes or death over this period (ramipril 18.1% versus placebo 19.5%), although the Kaplan-Meier

survival curves tended to diverge by the end of the trial. However, there was a significant benefit versus placebo for the secondary endpoint of regression to normoglycemia (152). Regardless of the reasons for ramipril failure to reduce new-onset diabetes in the DREAM trial population, considerable evidence supports the use of ACEi in patients with hypertension and/or CVD, with the additional purpose of normalizing glucose homeostasis and decreasing the incidence of diabetes, especially when compared to many other antihypertensive drugs.

The results of a new clinical study, Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE), were published in September 2007. ADVANCE is the largest-ever trial to assess the treatment of type 2 diabetes patients with a fixed-dose combination of an ACEi, perindopril, and a thiazide-type diuretic, indapamide, versus placebo in a population of 11,140 patients with a mean age of 66 years, recruited from 22 countries in Asia, Australia, Europe, and North America. Over a mean follow-up duration of 4.3 years, the results showed that a composite of major macrovascular and microvascular events was reduced by 9% with active treatment vs placebo ($P = 0.041$). The relative risk of death from CVD was decreased by 18% ($P = 0.027$) and all-cause mortality by 14% ($P = 0.025$) in the active treatment arm, with significant declines also seen in total coronary events (14%) and total renal events (21%) with active treatment (153; 154). ADVANCE is a very important trial because it provides further confirmatory evidence that reducing BP is important in diabetic patients, and combination treatment with ACEi affords significant kidney protection in type 2 diabetes patients.

1.6.2 Clinical Trails of ARBs in Diabetic Patients

Like ACEi, ARBs are known to exert positive metabolic effects. However, only recent clinical studies of ARBs have been prospectively designed to include new-onset diabetes as an endpoint (Table 4). As with ACEi, initial evidence of the effects of ARBs on new-onset diabetes came from trials in which they were compared with conventional therapies. In the Losartan Intervention For Endpoint reduction in hypertension (LIFE) trial, patients with hypertension were randomized to receive either losartan- or atenolol-based treatment and were followed for at least 4 years. At the end of the study, 25% risk reduction in new-onset diabetes was observed in the ARB group compared to the beta-blocker group (155).

Table 4. Clinical evidence for prevention of diabetes with ARBs

Clinical study	Comparison	Reduction in ESRD risk
LIFE	Losartan vs. atenolol	25 % (6.0% vs. 8.0%, $P < 0.001$)
LAPINE	Candesartan vs. HCTZ	78% (0.5% vs. 4.1%, $P < 0.05$)
VALUE	Valsartan vs. amlodipine	88% (13.1% vs. 16.4%, $P < 0.001$)
CHARM	Candesartan vs. placebo	19 % (6% vs. 7.4%, $P = 0.02$)
SCOPE	Candesartan vs. placebo	18 % (4.9% vs. 6%, $P = 0.09$)

The Antihypertensive Treatment and Lipid Profile In a North of Sweden Evaluation (ALPINE) study investigated metabolic outcomes in 392 patients with hypertension (156). Patients were randomized to either candesartan or hydrochlorothiazide (HCTZ) and were followed for 1 year. Significantly more patients in the HCTZ group were diagnosed with diabetes during follow-up.

Additional evidence of the antidiabetic effects of ARB therapy came from the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) study, which compared valsartan with amlodipine in more than 15,000 patients with hypertension. After a mean follow-up of 4.2 years, new-onset diabetes was diagnosed in significantly fewer patients in the valsartan group (13.1%) than in the amlodipine group (16.4%, $P < 0.001$) (157).

The Candesartan in Heart failure – Assessment of Reduction in Mortality and Morbidity (CHARM) program included the reduction of new-onset diabetes in HF patients treated with candesartan compared to placebo as a pre-specified secondary endpoint (158). The investigators concluded that candesartan decreased the risk of new-onset diabetes in HF patients, and that this benefit was in addition to diminutions in the primary endpoint of mortality and hospitalizations for HF.

The Study on COgnition and Prognosis in the Elderly (SCOPE) also reported an 18% relative risk reduction in new-onset diabetes with candesartan compared to placebo (159). Although this finding did not achieve statistical significance, it does further support that ARBs can help to decrease the risk of diabetes in high-risk patients, such as those with hypertension

1.7 Gene Regulation of AGT

Plasma and tissue AGT concentrations both contribute directly to circulating Ang I and Ang II levels. Thus, changes in these concentrations are important, as they can influence the degree of RAS activation. AGT is

regulated by several hormonal factors, and some of the *cis*- and *trans*-regulatory elements involved in the control of its transcription have been identified.

1.7.1 AGT Regulation by Hormonal Factors

Many *in vivo* and *in vitro* studies have demonstrated that glucocorticoids (i.e. dexamethasone), estrogens, Ang II, and thyroid hormones all stimulate AGT synthesis and release (160; 161). These hormones increase AGT mRNA levels in the rat liver (47; 162; 163). The hormonal regulation of AGT mRNA has also been investigated in hepatocytes and other cell lines, such as PTCs and adipocytes (164). The effects of steroids are neutralized by antiglucocorticoids and antiestrogens. Thyroidectomy decreases plasma AGT concentration, and T_3 restores it. Ang II has been consistently found to stimulate AGT synthesis, and insulin inhibits its expression in PTCs in culture (131; 165). However, it has also been suggested that insulin downregulates AGT expression in adipocytes (166). The fall in plasma AGT that occurs during chronic ACE inhibition is probably due to decreased positive feedback by Ang II, or to increased cleavage by plasma renin and its reduced synthesis (167), although other mechanisms are possible since ACE inhibition does not block only the RAS.

Plasma AGT and estrogen concentrations rise in parallel during pregnancy. Oral contraceptive pills containing synthetic estrogens cause a dose-dependent elevation of plasma AGT (168), but there seems to be no direct relationship between the increase in plasma AGT and BP. The

estrogen-induced increase in AGT is tissue-dependent. The effect of estrogen on the liver is important, but it does not stimulate AGT production in adipose tissue, and contradictory results have been reported in the brain (169). Bilateral nephrectomy leads to heightened hepatic synthesis and plasma concentrations of AGT (170), but the manner in which it does so is thus far unknown. The additive effects of these various stimuli suggest that they trigger AGT synthesis through different transcription pathways.

These observations indicate that a chronic increase in plasma AGT might modulate RAS activity, and may also cause an abnormally short feedback loop between Ang II and renin release. The rise in plasma Ang II after plasma AGT elevation exerts a short negative feedback loop on renin release in the normal physiological state, and this limits the direct effect of changes in plasma AGT on BP.

1.7.2 AGT Expression in Diseases

Several pathological conditions lead to augmented AGT production, which contributes to local Ang II generation. The medial layer of an injured aorta contains increased amounts of AGT mRNA, as might be expected for a member of the serpin family, which suggests that AGT is involved in myointimal proliferation in response to vascular injury (171). Enhanced ACE activity and AGT synthesis also occur after experimental left ventricular hypertrophy (172), indicating, along with other studies, that the cardiac renin system may help modulate heart growth and hypertrophy. The change in renal hemodynamics caused by experimental HF leads to a

specific increase in renal AGT mRNA, suggesting that it contributes to activation of the intrarenal renin system (173).

1.7.3 Transcriptional Regulation of AGT

AGT production is mainly regulated by gene transcription, although there is evidence of post-transcriptional regulation, as when Ang II increases the stability of AGT mRNA (174). Recent studies in mice indicate that the proximal promoter region of the mouse AGT gene (-96 to +22) is sufficient for AGT gene expression in mouse fibroblast cells during their differentiation into adipocytes (175). A liver-specific factor (AGF2) binds to the proximal promoter element (-96 to +52), and a ubiquitous nuclear factor (AGF3) binds to the core promoter element (-6 to +22). These 2 factors seem to act synergistically (175). The core promoter region of the human AGT gene in HepG2 cells has been analyzed (176). Electrophoretic mobility shift assays have demonstrated that a ubiquitously-expressed nuclear factor (AGT core promoter-binding factor 1) binds to a region between positions -25 to -1, denoted as AGCE1 (AGT core promoter element 1), located between the TATA box and the transcription initiation site. AGCE1 appears to play a major role in activating AGT transcription, particularly by downstream core elements. This region is probably more complex with several nuclear factors binding to its 5'- or 3'-side (177), which may be important for the general rate of transcription initiation and also for determining the pattern of AGT gene expression. These studies point to the importance of regulatory regions where several natural variants of the AGT gene have been detected.

1.8 Insulin and AGT

As discussed above, insulin could modulate AGT gene expression in adipocytes and PTCs. However, the results are contradictory in 2 different cell types. In this section, I will discuss in more detail the gene regulation effects of insulin, for a clear hypothesis on how it inhibits AGT expression in PTCs.

Insulin, the most important hormone implicated in blood glucose control and energy metabolism, acts by stimulating glucose influx and metabolism in adipocytes and muscles, and by inhibiting gluconeogenesis in the liver. Since its discovery, insulin has been subjected to extensive research to elucidate its activity in various metabolic processes, including glucose homeostasis, substrate metabolism, gene regulation, protein synthesis, and protein degradation (178). This hormone is secreted by β-cells of the pancreatic islets of Langerhans in response to increased circulating glucose levels after a meal. Impaired insulin actions, either due to insulin hyposecretion or defects in insulin signaling, cause serious problems in glucose homeostasis, and subsequently lead to DM.

1.8.1 Insulin Signaling

Insulin acts through a cell surface receptor that belongs to a subfamily of growth factor receptor tyrosine kinases. This subfamily is comprised of 3 members: insulin receptor (IR), type 1 insulin-like growth factor (IGF) receptor, and IR-related receptor (179). The IR consists of a heterodimeric α_2, β_2 structure. Insulin-binding to the IR α-subunit results in conformational

changes, leading to enhanced intrinsic protein tyrosine kinase activities of the β-subunit by multi-site tyrosine phosphorylation. Phosphorylated IR can phosphorylate several cytosolic IR substrates, including insulin receptor substrates (IRSs), Src homology collagen (Shc), and adaptor protein with pleckstrin homology, Src homology 2 (SH2) domains, and Casitas B-lineage lymphoma. The phosphorylated proteins dock downstream affector molecules that contain the SH2 domain (180), which are then able to activate 2 key signaling pathways. These pathways are the phosphatidylinositol 3-kinase (PI3K)-AKT/PKB pathway (181), which is responsible for most of the metabolic actions of insulin, and the Ras-MAPK pathway (180), which regulates the expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation (Figure 7) (182).

The Ras-MAPK pathway goes through the formation of complexes between the exchange factor mammalian son of 7 less (mSOS) and growth factor receptor binder-2 (Grb2) (183). Grb2 can be activated by IRS or Shc, 2 direct substrates of IR kinase. Activation of the IRS-Grb2-mSOS complex results in subsequent activation of the Ras, Raf, MEK, and extracellular signal-regulated kinase (ERK) pathway. Activated ERK1/2 phosphorylates a downstream ribosomal protein kinase, $p90^{rsk}$. Both ERK1/2 and $p90^{rsk}$ can be translocated to the nucleus where they phosphorylate translocation factors, and contribute to the mitogenic and growth-promoting effects of insulin.

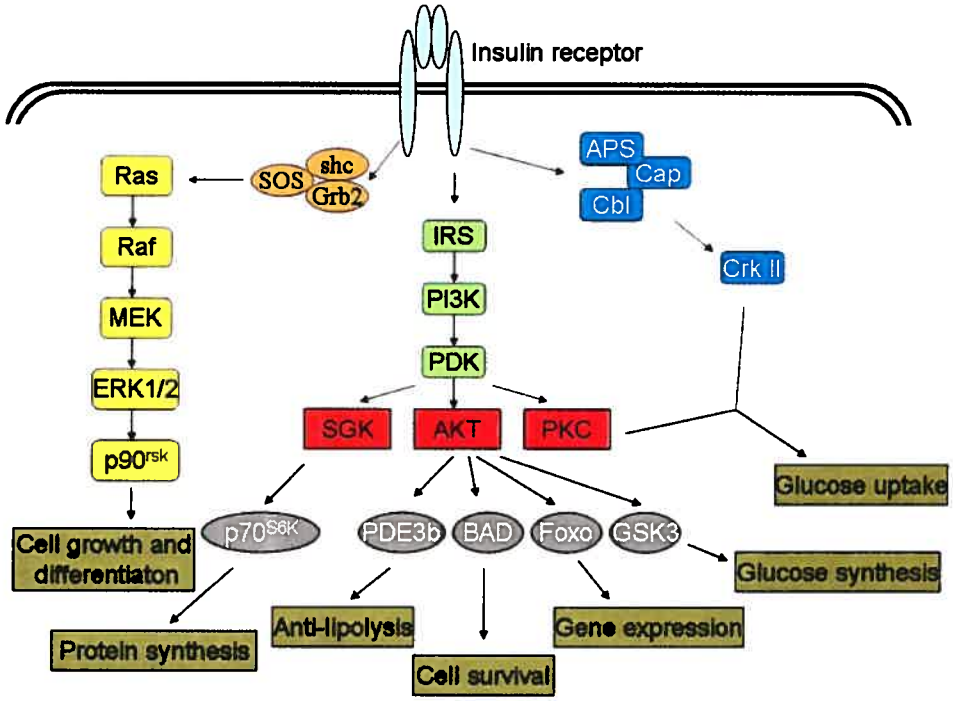


Figure 7. Insulin signaling pathway and its cellular function.

The second pathway that mediates insulin signalling through the IRS complex involves PI3K activation (178; 181). PI3K phosphorylates phosphatidylinositol (PI) lipids at position 3 of the inositol ring, and generates 3 phosphorylated forms of PI, such as phosphatidylinositol 3,4,5 triphosphate (PIP_3) (184), which is implicated in activation of the mammalian target of Rapamycin (mTOR) and phosphoinositide-dependent kinase (PDK). mTOR has been found to phosphorylate eIF4E-binding protein (4EBP1). PDK is related to serine/threonine protein kinases responsible for the phosphorylation and stimulation of several downstream signaling protein kinases, such as PKB/AKT, p70 ribosomal S6 kinase ($p70^{s6k}$) and PKC-zeta. PKB phosphorylates glycogen synthase kinase 3 (GSK3), cGMP-inhibitable phosphodiesterase 3b, BAD, and FOXO transcription factors, leading to the stimulation of glycogen synthesis and

inhibition of lipolysis, apoptosis and gene expression, respectively (185; 186). Activation of these serine/threonine protein kinases has been hypothesized to mediate the metabolic effects of insulin at the level of glucose transport, GLUT4 translocation, and protein synthesis (187). Interest in the PKC family has been rekindled by observations that the addition of inhibitors of its atypical isoforms results in partial suppression of glucose uptake in adipocytes and that heightened PKC activity is associated with increased intramyocellular triglycerides, a hallmark of insulin resistance (188).

1.8.2 Insulin Effect on Gene Regulation

While insulin has long been known to modulate intracellular metabolism by altering the activity or intracellular location of various enzymes, it has also been recognized that regulation of gene transcription is a major action of this hormone. Insulin has been reported to regulate the expression at least 100 genes (189). A recent report revealed changes in the mRNA levels of about 800 genes during 3-hour insulin infusion in the skeletal muscles of healthy individuals (190). Many metabolic effects are mediated by the regulation of gene expression with positive actions on glycolytic and lipogenic enzymes and negative impacts on gluconeogenic enzymes. Furthermore, insulin affects the mRNA stability of specific genes (PEPCK, GLUT4, and CYP450 2B/E), promotes translation by the activation of $p70^{s6k}$, 4EBP1, and eIF4E, stimulates the phosphorylation and dephosphorylation of many metabolic enzymes, and plays a role in hepatic growth and regeneration. These are all mechanisms of protein regulation by insulin.

Table 5. Insulin response sequences/elements.

IRE/IRS type	Genes	IRE/IRS sequence	Effect of insulin
GAPDH-IRE	GAPDH; Apo A-I	CCCGCCTC	Positive
SRE motif	FAS; HK II; SREBP-1c; ACC2	TCACCCC	Positive
AP-1 motif	Collagenase-1	TGA(G/C)TCA	Positive
Ets motif	Prolactin; somatostatin	(C/A)GGA(A/T)	Positive
E-box motif	Pyruvate kinase	CANNTG	Positive
SREBP motif	Glucokinase	ATCACCCCAC	Positive
TTF-2 motif	Thyroglobulin	C(T/A)(A/G)A(A/G)(C/A)AAACA	Positive
C1A/C1B motif	Glucagon	CACGCCTGACTG	Negative
PEPCK-like motif	PEPCK; G6Pase; IGFBP-1; TAT; Apo CIII	T(G/A)TTT(T/G)(G/T)	Negative
NF-1-like motif	GLUT4	TTTGGATTGAAGCCAAT	Negative
GC-box-like motif	ANG	CCTTCCCGCCCTTCA	Negative

Regarding gene regulation, an important question is: how does insulin's signal pass from the IR on the plasma membrane to a specific *trans*-acting element and bind to its *cis*-acting element? The promoter elements that mediate the effect of insulin on gene transcription are referred to as insulin-response elements or insulin-response sequences (IREs/IRSs). Unfortunately, while no single consensus on IRE is apparent in multiple insulin-regulated genes (191), there is some consensus on IREs in a small number of genes, such as TGTTTTT in PEPCK, G6Pase, TAT, Apo CIII, and IGFBP-1, although their mechanisms of regulation by insulin are different (192). Seven distinct IREs that mediate the positive effects of insulin on gene transcription have been characterized. Several reports have identified potentially novel stimulatory IREs in other genes that do not match any of these sequences (192; 193). In contrast, only 4 negative IREs have been identified, and 2 of them have been well-characterized. Table 5 lists 11 distinct classes of IRSs/IREs that have been defined.

Although the *cis*-acting elements that mediate the actions of insulin on gene transcription have been defined for a significant number of genes, the transcription factors responsible for the transactivation of these target sequences remain unknown. Because there is no consensus IRE in many insulin-regulated genes, no common insulin-responsive factor (IRF) could mediate all the insulin effects on gene regulation. It is possible, however, to identify more *trans*-acting elements by DNA-protein interaction technology. Data published during the past 10 years provide evidence that insulin mediates its action on gene transcription through the insulin-signaling pathway that directly modifies the phosphorylation/dephoshorylation of some transcription factors, such as c-Fos/c-Jun, thyroid transcription factor-2, hepatic nuclear factors 1 and 3, and forkhead transcription factor box O1 (FOXO1, previously known as Fkhr). Then, it regulates its targeted gene expression on the promoter region (192-195), or perhaps an intracellular receptor interacts directly with insulin-like steroid hormone receptor, and performs its gene regulation function through the receptor. However, this has not yet been identified.

Two major pathways, PI3K/AKT and ERK, are known to play a major role in transcription regulation. Insulin stimulates sterol response element-binding protein-1 (SREBP-1) gene transcription through sterol-response element (SRE). Studies of the promoter regions of insulin-regulated genes suggest that SRE is also a responsive motif of fatty acid synthesis, acetyl-Co-A Carboxylase-2, and hexokinase II (196; 197). It has been suggested that insulin modulates these genes through phosphorylated GSK3 by PI3K/AKT, which results in reduced GSK3 activity and may thus stabilize SREBP-1c nuclear content by inhibiting its

degradation (198; 199).

Insulin controls glucose production by inhibiting the expression of 2 rate-limiting enzymes in gluconeogenesis and glycogenolysis, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) (195; 200; 201). Interestingly, both have the same IRE, but in different copy numbers. One IRE is located between -425 and -399 in the mouse PEPCK promoter, with 3 IREs between -198 and -159 (region B of its insulin response region) in the mouse G6Pase promoter. The signaling pathways for these effects are not completely understood, but are thought to require activation of the lipid kinase PI3K. Among the PI3Ks, AKT has been implicated as a mediator of insulin's inhibition of a reporter gene driven by a PEPCK promoter spanning the putative IRE. FOXO1 (202) is phosphorylated in an insulin-responsive manner by PIP_3-dependent kinases, such as AKT and Sgk. Phosphorylation leads to nuclear exclusion and inhibition of FOXO1-dependent transcription (203; 204). Studies in hepatoma cells suggest that FOXO1 and its closely-related isoform FOXO3 possess the ability to regulate the transcription of reporter genes containing IRE from G6Pase and PEPCK promoters in an insulin-dependent manner (201). These data indicate that FOXO1 is a positive transcription factor of PEPCK and G6Pase. Insulin inhibits the expression of these genes by phosphorylating FOXO1, leading to its translocation.

Although these experiments support the hypothesis that FOXO1 and its orthologs are the IRFs that bind the PEPCK-like IRE motif and mediate the inhibitory effect of insulin on gene transcription through this element, other results do not concur with this model. Several studies suggest that

PKB is either not required or not sufficient for insulin-regulated PEPCK and G6Pase gene expression (205). In addition, a detailed base-by-base analysis of the PEPCK IRE indicated that the effect of insulin mediated through this element on heterologous gene transcription does not correlate with FOXO1-binding (206). This raises another question: are there other unknown IRFs with different roles in gene regulation?

Because the PEPCK, G6Pase, IGFBP-1, tyrosine aminotransferase (TAT), and apolipoprotein CIII genes share the same IRE (Table 1), it has been assumed that the same *trans*-acting element mediates the effect of insulin on the transcription of all these genes. However, the results suggest that these IREs might function differently. Such findings also raise the question of whether a different mechanism modulates specific gene transcription.

We have previously reported from Dr. John S.D. Chan's lab that insulin inhibits rAng expression in PTCs, and that a putative IRE is located in the rAGT promoter region between -882 and -855 (165; 207). With Southwestern blotting, we found 2 nuclear proteins that bind this element of AGT, and the binding profile was modulated by glucose and insulin stimulation. However, further experiments are required to acquire more detailed information.

1.9 The Heterogenous Nuclear Ribonucleoprotein Family (hnRNP)

The hnRNP family of more than 20 different proteins shares common structural domains, and extensive research has shown that they play central roles not only in mRNA processing, metabolism and transport but also in DNA repair, telomere biogenesis, cell signaling and the regulation of gene expression at both the transcriptional and translational levels (208).

Through these key cellular functions, individual hnRNPs have a variety of potential roles in tumor development and progression, including the inhibition of apoptosis, angiogenesis and cell invasion.

1.9.1 Structure of the hnRNP Family

As RNA polymerase II (RNA poly II) transcripts are synthesized, they associate with proteins to form hnRNP complexes. It is within these hnRNP complexes that pre-mRNAs are processed to form mature mRNA transcripts before being exported from the nucleus (209). Immunopurified hnRNP complexes from human HeLa cells contain more than 20 individual proteins designated as hnRNP Al (34 kD) through hnRNP U (120 kD) (210).

All hnRNPs share some structural motifs (Figure 8). The most common is the RNA-binding domain (RBD), also known as the RNA recognition motif (RRM), which is generally located at the N-terminus. RNA appears to bind to the surface of the RBD and, therefore, remains exposed and accessible for interaction with other factors (211). Binding studies have shown that sequence-specific elements of DNA or RNA are bound by hnRNPs through the RRM (209). In addition to binding to nucleic acids, the RRM of certain hnRNPs, i.e. hnRNP A1, is involved in protein-protein interactions. Indeed, hnRNP A1, through its N-terminal RBD, interacts with IκBα, resulting in nuclear factor kappa-B (NF-κB) activation (212).

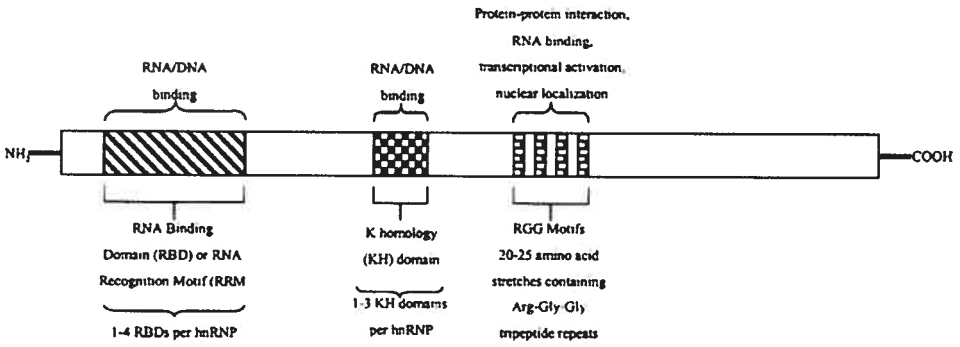


Figure 8. General structural domains of the hnRNP family (Ref. 211).

hnRNPs also have auxiliary regions, for example, RGG and KH (K homology) motifs. However, such regions are found in only some but not all hnRNP proteins. RGG motifs are 25-amino-acid stretches with Arg-Gly-Gly tripepetide repeats. Besides being involved in RNA-binding, they are also implicated in protein-protein interactions, transcriptional activation, and nuclear localization (209). The KH domain, first described in hnRNP K (213), is 50 amino acids long, and contains conserved octapeptide repeats of Ile-Gly-X2-Gly-X2-Ile (X being any amino acid). hnRNP K has 3 KH domains, and mutational analysis studies have confirmed that the octapeptide repeats are essential for RNA-binding.

1.9.2 Transcriptional Function of the hnRNP Family

hnRNPs are involved in different cellular functions, and through such functions, this family of proteins could potentially regulate cell physiology via various mechanisms. In my thesis, I focus on the transcriptional effects of hnRNPs.

The genome is tightly packed into DNA/protein structures, referred to as chromatin. These DNA/protein structures are modified by various

mechanisms such as acetylation or methylation (214). Using formaldehyde cross-linking on HeLa cells, hnRNP U was demonstrated to bind directly to chromosomal DNA (215). Subsequently, yeast 2 hybrid and co-immunoprecipitation assays revealed that hnRNP U interacts with p300, a protein known to acetylate histones. Contact between this protein complex and DNA results in the local hyperacetylation of histones (216), providing a connection between hnRNPs and chromatin remodeling. In addition to hnRNP U, the chromatin-remodeling role of other hnRNP members is beginning to emerge. For example, hnRNP K has been shown to interact with the chromatin-remodeling enzyme histone methyltransferase, but the resultant effect on chromatin remains unknown (217).

Transcription factors can either function as activators or repressors of gene expression, and hnRNP members have been identified as transcription factors. hnRNP K, which will be described later, and hnRNP U have been the most extensively studied. hnRNP U represses transcription by acting as a RNA poly II elongation inhibitor (218), hnRNP U associates with the general transcription factor TFII H *in vivo* and prevents phosphorylation of the C-terminal domain (CTD) of RNA poly II by TFII H, a process required for elongation (218). A recent report described hnRNP U associating directly with RNA poly II, and antibodies against hnRNP U inhibited RNA poly II transcription. Therefore, hnRNP U association with co-factors dictates whether it functions as an inducer or inhibitor of transcription (219). Interestingly, hnRNP P2 also interacts with RNA poly II through its N-terminal domain and associates with SR proteins (which

mediate mRNA-splicing) through the C-terminus (220). These interesting observations suggest that hnRNP P2 may function as an adaptor molecule linking transcription to RNA-splicing.

hnRNP A1 is another family member which has been shown to mediate transcription and DNA-binding in an independent manner. The transcriptional activation and DNA-binding activities of NF-κB protein are inhibited by IκBα-binding. Previously, I reported that hnRNP A1 interacts with and leads to IκBα-degradation. Cells lacking hnRNP A1 expression are defective in NF-κB-dependent transcriptional activation. However, hnRNP A1 overexpression increases IκBα-degradation and NF-κB-dependent transcription (212).

Besides the direct effects of hnRNPs on transcription, the post-translational modification of certain hnRNPs also affects their ability to function as transcription factors. hnRNP D mediates transcription by interacting with TATA-binding protein (TBP) and p300, and is therefore a transcription factor (221). hnRNP D phosphorylation by PKA enhances whereas GSK3β phosphorylation inhibits the transcriptional activity of this family member (221). Stimulation of hnRNP D by the PKA pathway increases its DNA-binding, yet it remains unclear if GSK3β inhibits transcription by decreasing DNA affinity or preventing co-factor interaction (p300 or TBP) of hnRNP D (222). Nevertheless, another post-translational modification process of hnRNP C1 decreases its interaction with single-stranded DNA (223).

1.9.3 hnRNP F

hnRNP F belongs to the hnRNP H family that is involved in the regulation of alternative splicing and polyadenylation, specifically recognizing poly(G) sequences (G-tracts). The hnRNP H family consists of 4 highly homologous proteins: hnRNP H, hnRNP H', hnRNP F, and hnRNP 2H9.

Members of the hnRNP H family specifically recognize G-tracts that are abundant in both DNA and RNA. These G-tracts are known to form a specific structure, the G-quadruplex, that consists of 4 guanine bases arranged in a square planar conformation and stabilized by hydrogen bonds (224; 225). In DNA, these structures are mainly located in telomeres and in some promoter regions. In RNA, G-tracts are frequent splicing recognition elements found in both introns, and exons, and are crucial for 5' splice site recognition (226-228). They are also abundant downstream of mammalian polyadenylation signals (229). hnRNP F and H, through binding to these G-tracts, are responsible for the regulation of polyadenylation (230) and the splicing regulation of numerous pre-mRNAs, such as Bcl-x (231), rat β-tropomyosin (232), Rous sarcoma virus NRS (233), HIV type 1 TAT (234), HIV-1 tev (235), HIV-1 p17gag instability (236), and c-src (237) pre-mRNAs. Furthermore, mutations in G-tract splice sites correlate with many diseases (238). In some cases, such as neurofibromatosis type 1 disease involving the NF1 gene (239), congenital hypothyroidism involving the TSH-β subunit gene (240), or cystic fibrosis through the CFTR gene (241), mutations directly affect (disrupt or enhance) hnRNP H/F-binding to pre-mRNA (241; 242).

1.9.4 hnRNP K

hnRNP K is an evolutionarily-conserved factor (243) in multiple

subcellular compartments, including the nucleus (226), cytoplasm (244) and mitochondria (245). The finding that hnRNP K is involved in gene expression through chromatin-remodeling (243), transcription (246), pre-mRNA-splicing (247), mRNA export (248) and translation (249) has generated great interest in this factor (250). hnRNP K involvement in these many processes probably reflects the interactions of its multiple domains (250) with a diversity of molecular partners, including DNA, RNA, protein kinases, the GTP/GDP exchange factor Vav, and proteins involved in chromatin-remodeling, transcription, mRNA-splicing and translation (251). Many of these interactions have been shown to be regulated by hnRNP K phosphorylation, induced either by changes in the extracellular environment or by the activity of specific ligands. hnRNP K is not only a kinase substrate but it also regulates the activity of kinases (252). These findings are consistent with a model where hnRNP K bridges signal transduction pathways to sites of nucleic acid-directed processes.

hnRNP K has been found to bind DNA in a sequence-specific manner. The protein binds CT elements located in promoters of the human proto-oncogenes c-Src (253) and c-*myc* (246). Moreover, analysis of the immediate early gene egr-1 in serum-induced rat hepatoma cells reported by Ostrowski et al. supports the observation that hnRNP K is a transcription factor. egr-1 expression is associated with transient hnRNP K recruitment to multiple sites within this gene, including promoter and transcribed regions (251). This protein-DNA interaction is specific as constitutively active β-actin loci fail to recruit hnRNP K (251). Their experiments revealed that hnRNP K binds DNA. Subsequently, its transcriptional activity was demonstrated by introducing mutations within the CT element. hnRNP K

was unable to bind this DNA element, resulting in reduced transcriptional activation (251). Furthermore, hnRNP K was able to stimulate transcription *in vitro* and interact with TBP, a protein required for the recruitment of RNA poly II. When TBP and hnRNP K were co-overexpressed in cells, a CT-rich element reporter gene was activated synergistically (246). Interestingly, hnRNP K-mediated gene activation was shown to increase the level of transcription without altering the decay rate of mRNA (254), suggesting that transcriptional activation by hnRNP K is independent of its mRNA-processing role. hnRNP K, therefore, appears to be a bona fide transcription activator, but it has also been documented as a functional repressor. C/EBP-β is a transcription factor that has been demonstrated to regulate genes involved in acute phase responses. In liver nuclear extracts, hnRNP K was reported to be present in a complex containing C/EBP-β, an interaction that repressed C/EBP-β transcriptional activation (255). Surprisingly, hnRNP K was found to elicit repression of C/EBP-β transcriptional activity in a manner that did not require hnRNP K to bind DNA, but rather through protein-protein interaction. The effect of hnRNPs affecting transcription independently of DNA-binding has also been described for additional members, e.g. hnRNP U.

1.10 Mouse Models in Diabetes Research

Several clinical studies have demonstrated significant renoprotection through blockade of the RAS compared with the effects other antihypertensive drugs, suggesting a crucial role of the intrarenal RAS activation in human kidney diseases. Despite the enthusiasm for ACEis

and ARBs in patients with kidney disease, direct evidence of augmentation of the intrarenal RAS in human is relatively sparse. In human subjects, direct measurements of the intrarenal RAS components, microperfusion studies, or micropuncture investigations are not available. However, accumulating evidence including functional investigations and studies of human biopsy samples emphasize augmentation of the intrarenal RAS in human patients. Therefore, mouse models have played a major role in shaping our current understanding of diabetes. Two major purposes of mouse models are to improve understanding the physiology of diabetes and promote development of new therapeutic compounds. Important contributions have come from both classical laboratory animal models and new ones made using advanced methods of genetic manipulation.

1.10.1 Characterization of Diabetic Nephropathy in Humans and Mice

In humans, DN manifests as a clinical syndrome that is composed of albuminuria, progressively declining GFR, and increased risk for cardiovascular disease (256; 257). The occurrence of cardiovascular disease is an integral component of DN and underscores the systemic nature of this disorder, of which nephropathy is only one aspect. Another key aspect of DN in humans is that it is a late complication of diabetes, occurring progressively in susceptible people only after 15 to 25 years of diabetes (258; 259). Because of issues of cost and convenience, most studies of DN in mice have focused on the earlier harbingers of DN, including the development of albuminuria and histopathologic changes, and have not explicitly used renal insufficiency as an end point (260; 261).

Diabetic albuminuria in humans is associated with the development of characteristic histopathologic features, including thickening of the glomerular basement membrane (GBM) and mesangial expansion. As albuminuria progresses and renal insufficiency ensues, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis develop (262). These pathologic features correlate well with GFR in humans with diabetes and kidney disease and would be important features in a robust mouse model of DN (263; 264).

The major deficiency in animal models of DN is the absence of renal failure. Whereas animal models of diabetic kidney disease exhibit albuminuria and some of the characteristic pathologic changes, reports of renal failure resulting from diabetes in mice or rats are lacking. Furthermore, associated increased risk for cardiovascular disease, neuropathy, and retinopathy has been poorly characterized. Whether the inability to detect renal failure in mice is simply a consequence of studying mice with established diabetes for an insufficient time period or the absence of renal failure reflects an intrinsic resistance to nephropathy of the strains studied thus far remains uncertain.

In contrast to the absence of renal failure, several of the more short-term consequences, including the development of glomerular hyperfiltration, increased albuminuria, and some of the characteristic histopathologic changes, can be detected in animal models. Human DN proceeds through several distinct pathophysiologic stages, including an early stage of glomerular hyperfiltration, followed by the so-called silent

phase in which GFR returns to normal (265). This is followed by the sequential development of microalbuminuria, dipstick-positive proteinuria, and then a progressive decline in GFR leading to ESRD. An optimistic view is that the detection of the early functional and histopathologic changes in mouse models of diabetes reflects inadequate duration of hyperglycemia before the study of these models (264).

1.10.2 Advantages and Disadvantages of Mouse Models in Research of Diabetic Nephropathy

The ideal animal model is simple to describe. It should be easy to produce, maintain, and study while accurately reproducing the human phenotype. Breyer et al. have summarized the advantages and disadvantages of different mouse models in diabetes research (Table 6, Ref. 264). The most frequently used β-cell injuring chemicals are streptozotocin (STZ) and alloxan. Alloxan is a pyrimidine that is given as a single dose. It is rapidlly taken up by β-cell, causes the production of hydroxyl radicals, and produces irreversible b-cell damage within minutes to hours. The permanent insulinopenia with hyperglycemia and ketoacidosis typically starts after 12 hours and insulin therapy is required to keep the animals alive. The severity and lack of titratability of alloxan make it less frequently used than STZ.

Table 6. Some mouse models of diabetes studied for DN.

Mouse Model	Diabetic Type	Advantages	Disadvantages
Streptozotocin	Type 1	Well established, reproducible timing; may be established in strains both resistant and susceptible to DN	Potential for nonspecific toxicity; strain-dependent dosing necessary; biohazard: potential mutagen
Ins2 Akita	Type 1	Commercially available (JAX); autosomal dominant mutation	Presently only C57BL/6 commercially available; C57BL/6 relatively resistant to nephropathy; hyperglycemia in females is mild
Db/db	Type 2	Available on multiple strains; commercially available	Infertile; autosomal recessive; mutation in leptin receptor is a very rare cause of obesity and type 2 diabetes in humans
High-fat diet	Type 2	Onset can be determined by the investigator	Only C57BL/6 reported as susceptible; hyperglycemia not prominent

Modified from Ref. 259

STZ is a fungal fermentation product that selectively damages pancreatic β-cell, possibly via oxidant damage (266). In many species, a signal injection of a large dose of STZ (requiring approximately 150 to 200 mg/kg to obtain chronic hyperglycemia) is sufficient to cause insulin-deficient diabetes. A regimen using multiple injections of low dose of STZ has also been devised. This regimen typically calls for daily intraperitoneally injections of 40 to 50 mg/kg STZ for 5 days (267). This model of type 1 diabetes typically develops albuminuria (268), even on the relatively sclerosis-resistant C57BL/6J background (269); however, the potential for collateral tissue toxicity complicates the interpretation of the cause of this albuminuria. The multiple dose technique more consistently produces animals with sufficient beta-cell loss to cause diabetes, while at the same time leaving enough residual insulin secretary capacity to avoid ketoacidosis and the need for treatment with insulin. This is a major practical advantage.

To circumvent the potential nonspecific tissue toxicity that occurs in the

STZ model of type 1 diabetes, one may use the recently described insulin-2 *Akita* ($Ins2^{Akita}$) mouse mutant model of type 1 diabetes (270). Akita mouse is severely deficient in insulin production, but with enough residual insulin to allow the animal to live. The C57BL/6-Ins2Akita/+ mouse has small islet and greatly reduced insulin production. The Akita mutation is a cysteine to yrosine change at position 7 of the A chain of the Ins2 gene. Since mice have 2 insulin loci, the phenotype of heterozygous mice might have been predicted to be an asymptomatic ~25% reduction in insulin levels. However, the Akita insulin molecular has severe folding problems, causing toxicity to b-cells making the protein, a dominant phenotype, and the reduction in β -cell mass (271).

In type 2 diabetes researches, the *db/db* mutation on the C57BLKS background has been investigated intensively and exhibits many features similar to human DN. The diabetic gene (*db*) is transmitted as an autosomal recessive trait. The *db* gene encodes for a G-to-T point mutation of the leptin receptor, leading to abnormal splicing and defective signaling of the adipocyte-derived hormone leptin (272). In the C57BLKS/J *db/db* mouse, hyperinsulinemia is noted by 10 day of age, and blood glucose levels are slightly elevated at 1 mo of age (7.2 ± 2.3 mM) (273). Progressive hyperglycemia is noted with mean levels of glucose of 28.6 ± 13.2 mM at 16 wk of age (273). By 2 to 4 mo of age, diabetic mice have a 20 to 30% increase in glomerular size. Glomerular hypertrophy at the onset of diabetes may be due to alteration of glomerular hemodynamics as there is evidence of glomerular hyperfiltration in *db/db* mice during the early stages of diabetes (274).

The progression of diabetes in *db/db* mice on the C57BL/6 background in the diabetic phenotype is less severe than that in C57BLKS/J, and as these mice age, plasma glucose seems to normalize. More recently, some investigators have reported observing that a subset of approximately 50% of C57BL/6J *db/db* mice develop more persistent hyperglycemia. In these mice, more robust albuminuria and renal histopathologic diabetic changes have been reported. Unfortunately, the factors that cause this subgroup of C57BL/6J *db/db* mice to develop persistent hyperglycemia remain to be elucidated. Nevertheless, although the leptin mutation may not produce as robust a model for DN on the C57BL/6 background as it does on the C57BLKS/J background, it does provide a clear advantage for genetic studies because most transgenic and knockout strains are available on this background and can cleanly introgressed onto this strain (264).

1.11 Hypothesis and Aims of These Studies

Rationale: DN is the major cause of ESRD. Hyperglycemia and RAS activation play major roles in the progression of DN. The existence of a local intrarenal RAS has now been well-accepted. AGT is the sole substrate of the RAS. The regulation of renal AGT gene expression, however, is not well-understood. During the last several years, our lab has focused on the mechanism(s) of regulation of rAGT gene expression in renal proximal tubular cells (RPTCs) under high glucose conditions. In previous studies, we showed that high glucose stimulates and insulin inhibits AGT gene expression and cell hypertrophy in immortalized RPTCs

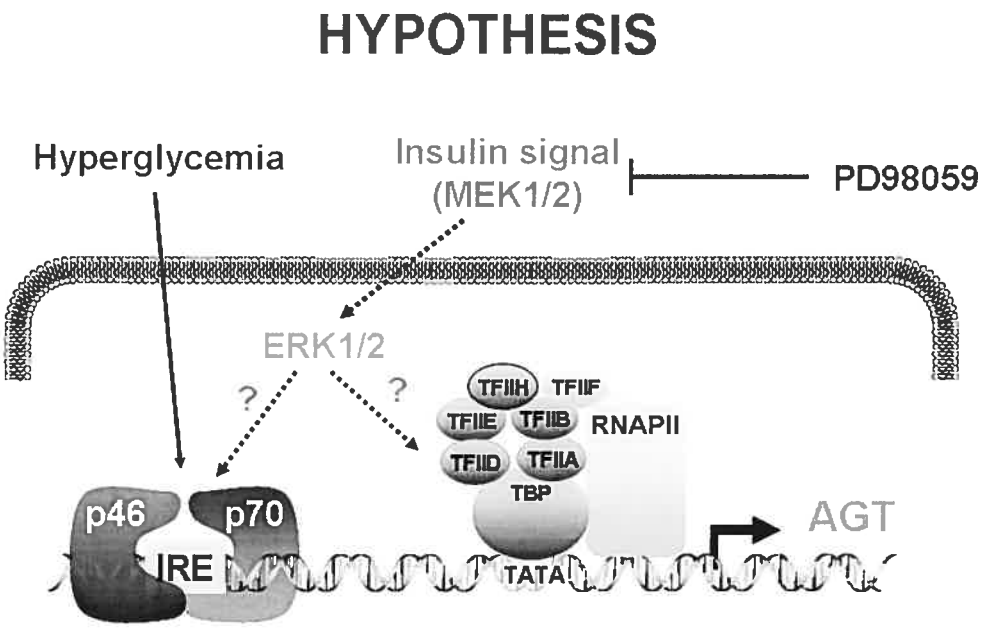
(IRPTCs). Moreover, we identified a novel IRE motif that contains nucleotides N-878 to N-864 (5'-CCT TCC CGC CCT TCA-3') upstream of the transcription start site of the rAGT gene promoter. This AGT-IRE binds to 2 major nuclear proteins with apparent molecular weights (MW) of approximately 48 and 70 kD in IRPTCs, as revealed by Southwestern blotting. It appears that high glucose and insulin enhance and inhibit the expression of 48- and 70-kD nuclear protein expression in IRPTCs, respectively. These data suggest that 48- and 70-kD nuclear proteins might mediate the effect of high glucose and insulin on AGT gene expression in IRPTCs. However, these 2 nuclear proteins have not been identified, and the molecular events that link them to hyperglycemia and the transcriptional regulation of rAGT are largely unknown. The main goal of the present studies is to identify and clone the 2 nuclear proteins and to investigate their actions on AGT gene expression.

Hypothesis: We hypothesize that the 48- and 70-kD nuclear proteins specifically bind to IRE and that both have transcriptional activity on the rAGT promoter. Subsequently, both exert their effects on the regulation of rAGT expression, could be modulated by its IRE, and interact with other cis- and trans-element factors at the gene transcription level.

AIM 1: To clone and identify the 48-kD nuclear protein and investigate its role in AGT gene expression.

AIM 2: To clone and identify the 70-kD nuclear protein and investigate its role in AGT gene expression.

AIM 3: To elucidate the essential roles of these 2 nuclear proteins in AGT regulation and their subsequent influence on RAS activation and the progression of renopathology in hyperglycemia, i.e. RPTC hypertrophy.



Chapter 2 : Article 1

Heterogenous nuclear ribonucleoprotein F modulates angiotensinogen gene expression in rat kidney proximal tubular cells.

HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN F MODULATES ANGIOTENSINOGEN GENE EXPRESSION IN RAT KIDNEY PROXIMAL TUBULAR CELLS

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ABSTRACT

We previously identified an insulin-responsive element (IRE) in the rat angiotensinogen (ANG) gene promoter that binds to 2 nuclear proteins with apparent molecular weights of 48 and 70 kiloDalton (kDa) from rat immortalized renal proximal tubular cells (IRPTCs). The present studies aimed to identify and clone the 48 kDa nuclear protein and to define its action on ANG gene expression. Nuclear proteins were isolated from IRPTCs and subjected to 2-dimensional electrophoresis. The 48 kDa nuclear protein was detected by Southwestern blotting and subsequently identified by mass spectrometry, revealing that it was identical to 46 kDa heterogeneous nuclear ribonucleoprotein F (hnRNP F), a nuclear protein that binds to TATA-binding protein and associates with RNA polymerase II and also interacts with nuclear cap-binding complex. We cloned the hnRNP F cDNA from IRPTCs by reverse transcriptase-polymerase chain reaction. Bacterially-expressed recombinant hnRNP F bound to the rat ANG-IRE, as revealed by gel mobility shift assay. The addition of polyclonal antibodies against hnRNP F yielded a supershift in gel mobility. Transient transfer of sense and antisense hnRNP F cDNA in IRPTCs inhibited and enhanced angiotensinogen gene expression, respectively. High glucose stimulated and insulin inhibited hnRNP F expression in IRPTCs. Expression studies indicated that hnRNP F is present in the kidney, testis, liver, lung and brain but not in the spleen. In conclusion, our studies demonstrate that hnRNP F binds to rANG-IRE and modulates renal ANG gene expression, implicating that dysregulation of hnRNP F might

affect renin-angiotensin system activation and, subsequently, kidney injury in diabetes.

Abstract: 249 words; Text: 5,131

INTRODUCTION

Insulin regulates the expression of over 100 genes by affecting the transcription, mRNA stability or mRNA translation (1, 2). The underlying mechanism(s) of insulin regulation of gene transcription and mRNA stability are poorly understood. One major obstacle in hampering the progress at the transcriptional level is that there is no consensus insulin response element (IRE) that can be account for the regulation of all insulin responsive genes. This is in contrast with consensus-responsive elements that have been described for other hormones, i.e., the steroid hormone receptors. However, some genes whose transcription is inhibited by insulin appears to share a common IRE (T(G/A)TTT(T/G)(G/T)) core sequence including phosphoenolpyruvate carboxykinase (PEPCK), insulin-like growth factor-binding protein-1 (IGFBP-1), tyrosine aminotransferase, glucose-6-phosphatase, apolipoprotein C III, and aspartate aminotransferase (3-8). Transacting factors that interact with the above common IRE have been tentatively identified, but none have been directly shown to mediate an insulin response. Moreover, other genes contain a well-defined IRE but its sequence is substantially different from the common IRE (2), thus it has been postulated that no single common transacting factor could be associated with all IREs of different genes.

Angiotensinongen (ANG) is a glycoprotein consisting of 452 amino acid residues with an apparent molecular weight of 62-65 kiloDalton (kDa) (9, 10). ANG is principally expressed in the liver and is the sole substrate in the renin-angiotensin system (RAS). In addition to the well-characterized systemic RAS, the presence of a local intrarenal RAS has now been generally accepted (11,12). The mRNA and protein for all RAS components, including ANG, renin, angiotensin-converting enzyme (ACE) and angiotensin II (Ang II) receptors (AT_1R and AT_2R subtypes), are expressed in murine and rat immortalized renal proximal tubular cells (IRPTCs) (13-20). Several studies have reported that Ang II levels, ANG and renin mRNA expression are evelvated in early diabetic kidney (16, 21, 22), suggesting that hyperglycemia and/or augmented intrarenal Ang II may be directly or indirectly responsible for renal proximal tubular hypertrophy and tubulointerstitial fibrosis in diabetes.

In previous studies, we showed that high glucose stimulates and insulin inhibits ANG gene expression and cell hypertrophy in IRPTCs (23-31). Moreover, we identified a putative insulin responsive element (IRE) motif containing nucleotides N-878 to N-864 (5' CCT TCC CGC CCT TCA 3') upstream of the transcription start site of the rat ANG gene promoter (32). This ANG-IRE binds to 2 major nuclear proteins with apparent molecular weights of approximately 48 and 70 kiloDaltons (kDa) from IRPTCs, as revealed by Southwestern blotting (31). It appears that high glucose and insulin enhanced and inhibited the expression of 48 and 70 kDa nuclear protein expression in IRPTCs, respectively. These data suggest that 48 and

70 kDa nuclear protein might mediate the effect of high glucose and insulin on ANG gene expression in IRPTCs.

The present studies aimed to identify and clone the 48 kDa nuclear protein and to investigate its action on ANG gene expression. We have identified the 48 kDa nuclear protein as 46 kDa heterogenous nuclear ribonucleoprotein F (hnRNP F) by 2-dimensional (2-D) electrophoresis and mass spectrometry. We demonstrated that hnRNP F binds to ANG-IRE and modulates ANG gene expression in IRPTCs. Finally, high glucose stimulated and insulin inhibited hnRNP F expression in IRPTCs. These studies demonstrate that 46 kDa hnRNP F is one of the IRE-binding proteins (BPs) that binds to the rat ANG gene promoter and modulates ANG gene expression in IRPTCs.

MATERIALS AND METHODS

D(+)-glucose, D-mannitol and insulin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Gamma-[^{32}P-ATP] (3000 Ci/mol) and D-threo-[1,2 ^{14}C]choramphenicol were obtained from Amersham-Pharmacia Biotech (Baie d'Urfé, QC, Canada). Rabbit polyclonal antiserum raised against the full-length human hnRNP F and antiserum predominantly recognizing hnRNP F (CTARRYIGIVKQAGLER corresponding to amino acids 215 to 230 of human hnRNP F (33)) was a gift from Dr. Taka-aki Tamura (Chiba University, Chiba, Japan) and Dr. Christine Milcarek (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA), respectively. The characteristics of these antisera have been reported (34, 35). The bacterial

expression vector pGex 4T-3, and mammalian expression vector pcDNA 3.1, were purchased from Amersham-Pharmacia Biotech and InVitrogen Inc. (Burlington, ON, Canada), respectively. Restriction modified enzymes were acquired from either InVitrogen Inc., Amersham-Pharmacia Biotech or Roche Diagnostics (Laval, QC, Canada).

Oligonucleotides for rat ANG-IRE N-882 to N-855 (5' CCT CCC TTC CCG CCC TTC ACT TTC TAG T 3') (32), mutants of ANG N-882 to N-885 (M1, 5' CCT CCC TTC CAT TAC TTC ACT TTC TAG T 3' , M2, 5' CCT CCC TTA AAT AAG ACC ACT TTC TAG T 3', M3, 5 CCT CCC TTC CCT TCC TTC ACT TTC TAG T 3 , M4, 5 CCT CCC TTC CCT CCC TTC ACT TTC TAG T 3 , rANG-IRE motif N-878 to N-864 (5' CCT TCC CGC CCT TCA 3'), concanmeric rANG-IRE motif (3 X N-878 to N-864, 5 CCT TCC CGC CCT TCA CCT TCC CGC CCT TCA CCT TCC CGC CCT TCA 3), IRE of human glyceraldehyde phosphate dehydrogenase gene (hGAPDH-IRE, N-473 to N-477, 5' CCA ACT TTC CCG CCT CTC AGC CTT TGA A 3) (36), IRE of rat glucagon gene (N-267 to N-242, 5' AGT TTT CAC GCC TGA CTG AGA TTG A 3') (37) were synthesized by InVitrogen Inc. The oligonucleotide containing the consensus SP1-binding site (5 -TCG CCC CGC CCC CGA TCG AAT-3) (38) was purchased from Promega (Fisher-Scientific Inc., Montreal, QC, Canada).

The plasmid containing the concatameric rANG-IRE motif DNA was constructed by inserting the double-stranded concatameric rANG-IRE motif oligonucleotide with the Not-1 enzyme restriction site added on both termini into the polyclonal site of pcDNA 3.1 by conventional methodology. The

double-stranded concatameric rANG-IRE motif DNA fragment was then incised from the plasmid and used for labeling as probe.

The expression vectors, plasmid containing the coding sequence for chloramphenicol acetyltransferase (CAT) without the promoter (pOCAT) or with Rous sarcoma virus enhancer/promoter sequence (pRSV/CAT) fused to the 5 -end of the CAT coding sequence, respectively, were a gift from Dr. Joel F. Habener (Massachusetts General Hospital, Boston, MA). Thin-layer chromatography plates were purchased from Fisher Scientific (Montreal, Quebec, Canada).

Cell Culture

IRPTCs at passages 12 to 16 were used in the present studies. The characteristics of IRPTCs, which express the mRNA and protein of ANG, renin, ACE, and Ang II receptors, have been described previously (39).

Cellular and Tissue Nuclear Extract Preparation

Nuclear extracts from 10 plates (150 x 20 mm) each of confluent IRPTCs previously incubated in DMEM with 5 mM glucose and 20 mM D-mannitol, 25 mM glucose or 25 mM glucose plus insulin (10^{-7} M) for 24 h, and rat tissues (liver, kidney, testis, lung, brain and spleen) were prepared according to the method of Henninghausen and Lubon (40) with slight modifications, as we have described elsewhere (31, 41).

2-D Electrophoresis

2-D electrophoresis was carried out with the IPGphor Isoelectric Focusing Unit (Amersham-Pharmacia Biotech). For isoelectrofocusing (IEF), precast 13 cm IPG strips (pH 3-10, non-linear, Immobiline DryStrips, Amersham-Pharmacia Biotech) were pre-equilibrated with 750 μg of nuclear extracts in 250 μl rehydration buffer (8 M urea, 1 M thiourea, 4% CHAPS, 2% IPG buffer, 1%NP-40, 0.1 M DTT, and 0.0001% of bromophenol blue (BPB)) for more than 12 h according to the supplier's manual. IEF was run for 90 kilovolt-hours at 25°C. After IEF separation, the IPG strips were immediately equilibrated for 15 min with a buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% sodium dedocyl sulfate (SDS), 2% DTT and 0.0001% of BPB. Then, the strips were re-equilibrated with another buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide and 0.0001% BPB for an additional 15 min. For 2-D separation, the IPG strips were placed above 10% polyacrylamide gel (PAGE) containing SDS and electrophoresed. Amersham's rainbow markers served as molecular weight markers. IRPTC nuclear extracts (100 μg) were run on the same 10% PAGE-SDS as controls. Each sample was divided in 2 strips for 2-D electrophoresis. One gel was stained with Coomassie Brilliant Blue R-250 (Amresco Inc., Solon, OH, USA) to visualize proteins. The other was electrotransferred to a Hybond C-extra membrane (Amersham-Pharmacia Biotech) for Southwestern blotting.

Southwestern Blotting

Southwestern blotting was performed according to the procedure of Kwast-Welfeld et al. (42) with slight modifications (31,41). Briefly, IRPTC nuclear proteins (200 μg) were resolved on a 4 to 20% PAGE-SDS gradient or on 10% PAGE-SDS (43), then electrotransferred to a Hybond C-extra membrane, which was incubated with 10% (W/V) non-fat milk proteins in a binding buffer containing 10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.25 mM EDTA and 2.5% glycerol (V/V) for 24 h at 4°C. The membrane was washed at least twice with binding buffer containing 0.25% non-fat milk proteins. Subsequently, it was hybridized overnight with ^{32}P-labelled concanmeric ANG-IRE motif DNA (approx. 1.0 to 2.0 pmol; 10^6 cp/ml) in binding buffer containing 0.25% non-fat milk proteins and 300 μg/ml non-denatured herring sperm DNA at 4°C. The membrane was finally washed, air-dried and exposed for autoradiography.

MALDI-Mass Spectrometry

Spots on the gel corresponding to positive signals of the Southwestern blot membrane were picked up for MALDI-Mass Spectrometry (MALDI-MS). All MALDI-MS analyses were performed at the Quebec Genome Centre (McGill University, Montreal, QC, Canada). Briefly, protein samples were first cleaved by trypsin and then subjected to MALDI-MS. MALDI-MS analysis was conducted at 20 kV accelerating voltage and 23 kV reflecting voltage. For protein identification, peptide mass fingerprints were searched by the Mascot

program developed by Matrix Science Ltd. (freely accessible on http:\\www.matrixscience.com).

Cloning of hnRNP F

hnRNP F was cloned from IRPTCs by conventional RT-PCR. Sense and antisense primers corresponding to nucleotides N+1 to N+19 (5 ATG ATG CTG GGC CCT GAG G 3) and N+1228 to N+1245 (5 TCG TAC CCA CCT ATA CTA ATC 3) of rat hnRNP F cDNA (35) were used in PCR. In addition, the Not 1 enzyme restriction site was added on the 5 and 3 end of sense and antisense primers, respectively. hnRNP F cDNA was then subcloned in sense and antisense orientation at the polyclonal site (Not 1) of the bacterial expression vector pGex 4T-3, or mammalian expression vector pcDNA 3.1, by conventional methodology.

Purification of Recombinant hnRNP F from Bacteria

E.Coli BL-21 cells (Amersham-Pharmacia Biotech) were transformed by pGex 4T-3 containing rat hnRNP F cDNA. Expression of the fusion protein [GST fused with hnRNP F (GST-hnRNP F)] in BL-21 cells was induced by the addition of 0.5 mM isopropylthiogalactoside (IPTG) into the culture medium with incubation for 4 h. The bacteria were then harvested, and GST-hnRNP F fusion proteins were purified from the bacterial extracts by GST affinity column chromatography according to the manufacturer's protocol

(Amersham-Pharmacia Biotech). The purified GST-hnRNP F fusion proteins were used in gel mobility shift assays.

GMSAs

Gel mobility shift assays (GMSAs) were performed according to methodology described elsewhere (31, 41), employing the labeled monomeric ANG-IRE motif DNA as probe. Briefly, the rANG-IRE DNA fragment was 5' end labeled with [γ-^{32}P]-ATP by T_4 polynucleotide kinase. Purifed GST-hnRNP F fusion proteins (1 μg) or GST (5 μg) or IRPTC nuclear proteins (5 μg) in the presence of 0.3 units of poly(dI/dC) in 20 mM Hepes (pH 7.6), 1 mM EDTA, 50 mM KCl, 2 mM spermidine, 1 mM DTT, 0.5 mM PMSF and 10% glycerol (V/V) were incubated for 30 min at room temperature. Then, the 5'-labelled probe (~0.1 pmol) was added and further incubated for 30 min at room temperature. After being chilled on ice, the mixture was run on 5% (W/V) non-denaturing PAGE and exposed for autoradiography.

In competition assays, 100- to 300-fold molar excess of unlabeled DNA fragments was added to the reaction mixture and incubated for 30 min at room temperature before incubation with the labeled probe. In super-shift assays, 0.5 to 2 μl of polyclonal antibodies against hnRNP F was added in the reaction mixture and incubated for 30 min on ice before incubation with the labeled probe.

Mammalian Expression of Recombinant hnRNP F

Mammalian expression vector pcDNA 3.1 containing rat hnRNP F cDNA in sense (pcDNA 3.1/hnRNP F (+)) or antisense (pcDNA 3.1/hnRNP F (-)) orientation was transfected into IRPTCs with Fugene 6 reagent according to the instruction manual provided by the supplier (Roche Diagnostics). We optimized the DNA concentration for gene transfection at 2 μg per 0.5 to 1 x 10^6 cells. Forty-eight h after transfection, the cells were either harvested and assayed for hnRNP F protein and ANG mRNA by Western blotting and RT-PCR, respectively.

Western Blotting for hnRNP F

Briefly, the cellular proteins and nuclear extracts were dissolved in 700 μl lysis buffer [62.5 mM Tris-HCl, pH 6.8, containing 2% (W/V) SDS, 10% glycerol, 50 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1% (W/V) BPB], sonicated for 15 sec, heated at 95°C for 5 min, and centrifuged at 12,000 x g for 5 min. Thirty-five μl of the supernatants were subjected to 10% PAGE-SDS gel and then transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham-Pharmacia Biotech). The membrane was initially blotted for anti-hnRNP F antibody (1 :30,000 dilution) and then reblotted for anti-β-actin antibody (1:10,000 dilutions) using chemiluminescent developing reagent (Roche Diagnostics). The relative densities of the hnRNP F and β-actin bands were quantified by computerized

laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Amersham-Pharmacia Biotech).

RT-PCR for Quantification of ANG mRNA in IRPTCs

Total RNA, isolated with TRIzol reagent (InVitrogen Inc.) according to the supplier's protocol and quantified by its absorbance at 260 nm, was deployed in RT-PCR to quantify the amount of ANG mRNA expressed in IRPTCs as described previously (25, 26). The sense and antisense rat ANG primers were 5'-CCT CGC TCT CTG GAC TTA TC-3' and 5'-CAG ACA CTG AGG TGC TGT TG-3', corresponding to N+729 to N+748 of exon 2 and N+111 to N+130 of exon 3 of rANG gene (9), respectively. The sense and antisense rat β-actin primers were 5'-ATG CCA TCC TGC GTC TGG ACC TGG C-3' and 5'-AGC ATT TGC GGT GCA CGA TGG AGG G-3', corresponding to the nucleotide
sequences of +155 to +179 of exon 3, and nucleotide sequences of +115 to +139 of exon 5 of the rat β-actin gene (44), respectively.

To identify rANG and β-actin cDNA fragments, 10 µl of the PCR product were electrophoresed on 1.2% agarose gels and transferred onto a Hybond XL nylon membrane (Amersham-Pharmacia Biotech). Digoxigenin-labeled oligonucleotide 5'-GAG GGG GTC AGC ACG GAC AGC ACC-3', corresponding to nucleotide +775 to +798 of rANG cDNA (9) (i.e., nucleotide +828 to +851 of exon 2 of rANG gene (44)) prepared with a digoxigenin oligonucleotide 3' end labeling kit (Roche Diagnostics), was used to hybridize

the PCR products on the membrane. After stringent washing, the membrane was detected with a digoxigenin luminescence kit (Roche Diagnostics) and exposed to Kodak BMR film (Eastman Kodak Co., Rochester, NY). After rANG mRNA analysis, the same membrane was stripped and rehybridized with a β-actin oligonucleotide probe (sequence: 5'-TCC TGT GGC ATC CAT GAA ACT ACA TTC-3', corresponding to nucleotides +9 to +35 of exon 4 of the rat β-actin gene (44)). ANG mRNA levels were normalized by corresponding β-actin mRNA levels.

Chloramphenical Acetyl Transferase (CAT) assay

The method of construction of the rANG-CAT fusion gene, pOCAT/rANG N-1498/+18 and mutant pOCAT/rANG N-1498/+18 with mutated IRE has been described previously (31, 32). Control plasmid or ANG-CAT fusion gene were transfected into IRPTCs using lipofectamine (InVitrogen, Inc.). We have optimized the DNA concentration for gene transfection at 2-3 μg per 0.5 to 1 x 10^6 cells. Thus, in the present studies, a total of 2 μg of supercoiled DNA (i.e., 2 μg of pRSV/CAT, 1 μg of pOCAT /rANG N-1498/+18 or mutant pOCAT/rANG N-1498/+18 plus 1 μg of pcDNA 3.1 or pcDNA 3.1/hnRNP F) was used routinely in cell transfection. 24 h after the transfection, the media were replaced with fresh 5 mM D-glucose DMEM and incubated for another 24 h. The cells were then harvested and assayed for CAT activity (30).

To normalize the efficiency of transfection, 0.5 μg of pTK/hGH [a vector with the thymidine kinase (TK) enhancer/promoter fused to the 5-human growth hormone (hGH) gene] was co-transfected with pRSV/CAT or pOCAT/rANG N-1498/+18 as described previously (30). The plasmid pRSV/CAT served as a positive control to monitor the efficiency of transfection of rANG-CAT fusion gene. The level of transfection efficiency for pRSV/CAT in IRPTCs ranged from 60 to 90%, i.e., the percentage of conversion of ^{14}C chloramphenicol to mono- and di-acetyl chloramphenicol. The transfection efficiency of pOCAT/rANG N-1498/+18 in IRPTCs ranged from 25% to 35% compared with pRSV-CAT. The inter- and intra-assay coefficient variation of transfection for pOCAT/rANG N-1498/+18 in IRPTCs are 25% and 12% (N = 10), respectively. The method for chloramphenicol acetyltransferase (CAT) assay has been described previously (30).

Statistical analysis

Three to 5 separate independent experiments were performed per protocol, and each treatment group was run in duplicate. The data were analyzed by one-way ANOVA and the Bonferroni test. A probability level of $p \leq 0.05$ was regarded as statistically significant.

RESULTS

Identification of Rat IRE-BPs in IRPTCs

Figure 1A shows the staining of nuclear proteins after 2-D electrophoresis. Southwestern blotting of IRE-BPs after 2-D electrophoresis is displayed in **Figure 1B**. It is apparent that 2 positive spots with an apparent molecular weight of 46-48 kDa were identified, and these spots were cut out and subjected to MALDI-MS. The MS results are displayed in **Figure 2**. The 2 spots identified a common protein designated as RIKEN (Accession number 4833420I20 (Mus musculus)). Database analysis revealed that RIKEN is identical to the rat hnRNP F cDNA sequence reported by Yoshida et al. (34).

To confirm the authencity of rat hnRNP F revealed by Southwestern blotting, we stripped the radioactivity from the membrane and reblotted with rabbit polyclonal antiserum against hnRNP F, as shown in **Figure 1C**. It is apparent that the proteins interacting with anti-hnRNP F were superimposable with positive signals detected by Southwestern blotting, as seen in **Figure 1B**. These data confirm that the proteins that interact with rat ANG-IRE are identical to hnRNP F.

Gel Mobility Shift Assay

Bacterially expressed recombinant hnRNP F was employed to study the interaction of putative rANG-IRE (N-878 to N-864) with hnRNP F. **Figure 3** illustrates the analysis of GST-hnRNP F fusion proteins by 10% PAGE-SDS gel. Three major bands with apparent molecular weights of 26, 44 and 69-72 kDa were induced by isopropylthiogalactosidase (IPTG) (**Figure 3A**). The 44 and 69-72 kDa species interacted with rabbit antiserum against hnRNP F, but not the 26 kDa species (**Figure 3B**). The 26 kDa molecular species was the

induced GST protein, according to information provided by the supplier (Amersham-Pharmacia Biotech). The 69-72 kDa molecular species had the molecular weight of hnRNP F (46 kDa) fused with GST (26 kDa), whereas the 44 kDa molecular species was likely partially-degraded GST-hnRNP F fusion proteins. Partially-purifed GST-hnRNP F (lane 7 in **Figure 3A** and **3B**) was used in subsequent GMSAs.

When the labeled rANG-IRE DNA was incubated with GST-hnRNP F, 1 major band consisting of 2 species appeared with retarded mobility (**Figure 4A**). No slowly-migrating band was observed when the labeled DNA was incubated with GST (bacterial extract of empty vector pGex 4T-3). The addition of an unlabeled rANG-IRE was effective in competing with the binding of labeled rANG-IRE DNA to the fusion proteins(s) (100- and 300-fold molar excess of unlabeled DNA fragment), but not the unlabeled DNA fragment of hGAPDH-IRE, rat glucagon-IRE, and the SP1 consensus sequence (**Figure 4B**). Unlabeled mutants (M3 and M4) of rANG-IRE (N-878 to N-864) were effective in competing with the binding of labeled rANG-IRE DNA but not the unlabeled mutants (M1 and M2) of rANG N-882 to N-855 in competing with the binding of labeled rANG-IRE DNA (**Figure 5**). These studies showed that the mutation of 4 nucleotides in N-878 to N-864 was sufficient to completely abolish binding with hnRNP F, indicating that the rANG N-874 to N-867 sequence localized within nucleotides N-878 to N-864 is important for binding to hnRNP F.

The addition of rabbit antiserum against hnRNP F induced a supershift of rANG-IRE binding with GST-hnRNP F fusion protein in a dose-dependent

manner (**Figure 6**). These data further confirm that rat ANG-IRE binds to hnRNP F.

Figure 7 reveals that the labeled rANG-IRE binds 3 major IRPTC nuclear proteins (labeled bands A, B and C). Nuclear proteins in all three bands are completely displaced by 100 X fold-excess of unlabeled rANG-IRE. The addition of rabbit anti-hnRNP F induced a supershift of rANG-IRE binding with nuclear proteins in band A but not band B and C. No supershift bands were observed with rabbit non-immune immunoglobulin G (IgG). These results demonstrate that rANG-IRE binds to endogenous nuclear hnRNP F in IRPTCs as well as with two unidentified nuclear proteins.

Effect of hnRNP F on ANG mRNA Expression in IRPTCs

HnRNP F expression (**Figure 8A**) from IRPTCs transiently transfected with pcDNA 3.1/hnRNP F (+) was significantly higher ($p<0.05$) than in IRPTCs transiently transfected with empty vector, pcDNA 3.1 or pcDNA 3.1/hnRNP F (-), as analyzed by Western blotting. It is apparent, however, that basal ANG mRNA levels in IRPTCs transiently transfected with pcDNA 3.1/hnRNP F (+) were significantly lower (50% decrease, $p<0.05$) than those in non-transfected IRPTCs (**Figure 8B**). In contrast, ANG mRNA levels were significantly higher (200% increase, $p<0.01$) in IRPTCs transiently transfected with pcDNA 3.1/hnRNF (-) than those in non-transfected IRPTCs (**Figure 8B**). These results demonstrate that transfected sense and antisense hnRNP F cDNA

respectively suppressed and enhanced the expresssion of endogenous ANG mRNA in IRPTCs.

Effect of hnRNP F on rANG Gene Promoter Activity

Figure 9 indicates that co-transfection with hnRNP F significantly suppresses rANG gene promoter activity. However, co-transfection with hnRNP F had no inhibitory effect on mutant rANG gene promoter activity. These studies support the notion that hnRNP F modulates ANG gene expression at the transcriptional level via binding to the IRE.

Effect of High Glucose and Insulin on hnRNP F Expression in IRPTCs

Figure 10A shows the Southwestern blot analysis of hnRNP F expression in IRPTC nuclear extract by employing the labeled rANG-IRE. After Southwestern blot analysis, the same menbrane was blotted with the polyclonal antibodies against the hnRNP F (**Figure 10B**). Southwestern blotting analysis reveals that high glucose levels (25 mM) enhanced and insulin suppressed the expression of 48- and 70-kDa molecular species from IRPTCs. The polyclonal antibodies against the hnRNP F interacted with a predominant molecular species of 48-kDa and a minor species of 46-kDa. The 48-kDa molecular species detected by Southwestern blotting (**Figure 10A**) is superimposed or corresponding to the 48-kDa species detected by Western blotting (**Figure 10B**). These data demonstrate that the 70 kDa IRE-BP is immunologically different from the hnRNP F. The expression of HnRNP

F and unidentified 70 kDa IRE-BP is regulated by high glucose and insulin in IRPTCs.

Tissue Distribution of hnRNP F in Rat Tissues

Figure 11 illustrates the tissue distribution of the hnRNP F protein in various rat tissues by Western blotting. It is apparent that the hnRNP protein is present in the nuclear extracts of kidney, liver, testis, lung and brain but not in the spleen.

DISCUSSION

The present studies employed a combination of Southwestern blotting and proteomics and identified hnRNP F as one of the nuclear proteins that binds to IRE of the rat ANG gene promoter and inhibits ANG gene expression in IRPTCs.

We have previously demonstrated that high glucose enhances the expression of both 48 and 70 kDa nuclear proteins in IRPTCs, and insulin inhibits this increment, suggesting that 48 and 70 kDa nuclear proteins may mediate the effect of high glucose and insulin on ANG gene expression in IRPTCs. Since the molecular structure of the 48 and 70 kDa IRE-binding proteins is unknown, the present studies aimed to identify their molecular structure. It is apparent that multiple IRPTC proteins are resolved by 2-D electrophoresis. A second gel run simultaneously with the first gel for

Southwestern blotting demonstrated 2 positive spots with an apparent molecular weight of 46-48 kDa, and pI of 5.0 to 6.0 that closely matched the stained protein spots on the first gel. After tryptic digestion and MALDI-MS, these two spots were found to have matching the partial amino acid sequence deduced from RIKEN cDNA (Accession number 4833420I20, gi 19527048 and NM 133834). The cDNA sequence of RIKEN is identical to the hnRNP F sequence as reported by Yoshida et al (34) (Accession number BAA 37095). Using specific rabbit antiserum against hnRNP F, we confirmed the identity of hnRNP F on the membrane from Southwestern blot. Western blotting revealed positive signals superimposed on the positions of the 2 spots detected by Southwestern blotting. The reasons why hnRNP F is present in 2 different forms (same apparent molecular weight but different pI) is presently unclear. One possibility is that these proteins might be variants with different phosphorylated forms.

A broad spot with an apparent molecular weight of 68 to 70 kDa (denoted by broken rectangle in **Figure 1**) was detected by Southwestern blotting. Subsequent MALDI-MS analysis of this spot, however, could not yield a definite peptide with a significant high Masscot score. These data suggest that the positive broad spot detected by Southwestern blotting is a cluster of several different proteins.

To demonstrate that hnRNP F interacts with rANG-IRE, we cloned hnRNP F from IRPTCs by conventional RT-PCR and expressed it in a bacterial system for use in GMSAs. Our GMSAs revealed that labeled rANG-IRE DNA interacted with GST-hnRNP F fusion protein(s), but not GST

proteins. The addition of unlabeled rANG-IRE DNA effectively displaced labeled ANG-IRE at or greater than a 100-fold molar excess of unlabeled DNA, whereas unlabeled hGAPDH-IRE, rat glucagon-IRE, SP1 consensus sequence, and mutants M1 and M2 of rANG-IRE were not effective in displacing labeled rANG-IRE. In contrast, M3 and M4 were effective in competing with labeled ANG-IRE. These studies demonstrate that nucleotides N-878 to N-864 represent the rANG-IRE motif, which is essential for binding to hnRNP F fusion proteins.

Most convincingly, the addition of specific rabbit polyclonal antibodies against hnRNP F yielded a supershift of labeled rANG-IRE binding with hnRNP F fusion proteins in a dose-dependent manner. Moreover, the addition of rabbit polyclonal antibodies against hnRNP F also yielded a supershift of labeled rANG-IRE binding with IRPTC nuclear proteins but not with rabbit immunoglobulin G. Taken together, these data demonstrate unequivocally that hnRNP F binds to rANG-IRE.

There are at least 20-30 independent hnRNP species in mammalian cells (45, 46). Since antibodies against hnRNPs affect splicing (47-49), and hnRNPs are associated with poly (A)-containing RNA, i.e., pre-mRNA and mRNA, and found to be constituents of the splicesome (47), they are believed to be important in splicing events. HnRNP F is a member of a subfamily of hnRNP proteins which includes at least F, H and H□proteins (33, 50). These proteins are highly related; hnRNP H and H□are 96% identical and 78 and 75% identical to hnRNP F, respectively (33, 50). HnRNP F has the unique nucleotide binding property of binding to (G/C)-stretch of double-stranded

DNA and RNA sequences via its GY-rich motif and RNA-binding domains, respectively (34). Studies on hnRNP F and H/H have revealed that these proteins participate at various steps in the processing of cellular mRNA, i.e., in alternating splicing of the c-src gene (51), the β-tropomyosin gene (52), and the thyroid hormone receptor gene (53). Furthermore, Veraldi KL et al. (54) have demonstrated that differential changes in expression levels of hnRNP F and H/H are involved in controlling the synthesis of membrane-bound versus secreted antibodies during the development of memory B cells into plasma cells. Most interestingly, studies by Yoshida et al (55) and Gamberi et al. (56) have demonstrated that hnRNP F could bind to TATA-binding protein, associates with RNA polymerase II and interacts directly with nuclear cap-binding protein (CBP) complex, suggesting that hnRNP F could modulate gene expression at the transcriptional and post-transcriptional levels. Thus, these data strongly indicate that the members of this subfamily are important for the control of gene expression at both the transcriptional and post-transcriptional levels in various cells.

Surprisingly, over-expression and down-expression of hnRNP F inhibits and augments ANG mRNA expression and ANG gene promoter activity in IRPTCs, respectively. To the best of our knowledge, the present report is the first to demonstrate that hnRNP F can modulate ANG gene expression in kidney proximal tubular cells *in vitro*. At present, the molecular mechanism(s) of hnRNP F action on ANG gene expression is not known. One possibility is that hnRNP F behaves like a negative transacting protein and

inhibits the binding of other positive transacting factor(s) (i.e., the p70 kDa IRE-BPs) to TBP and RNA polymerase II, subsequently attenuating ANG gene expression. The second possibility is that hnRNP F overexpression exhausts the availability of nuclear CBPs for capping pre-mRNAs, which would subsequently attenuate the formation of mature ANG mRNA in the cytoplasm. The third possibility is that hnRNP F could bind to unidentified splicing silencer(s) in ANG pre-mRNA and subsequently alter the normal splicing of ANG pre-mRNA in the nucleus. Finally, it is possible that hnRNP F is a negative regulatory protein, but its negative effect is normally neutralized by interaction (heterodimerization) with the unidentified 70 kDa IRE-BP (an hypothetical positive regulatory transacting factor that binds to rANG-IRE) in cells. HnRNP F overexpression will then exceed the neutralization ability of the unidentified 70 kDa IRE-BP and subsequently attenuate ANG mRNA expression. More studies are definitely needed along these lines to elucidate the mechanism(s) of action of hnRNP F on ANG gene expression in IRPTCs.

Finally, our studies reveal that high glucose stimulated and insulin inhibited the expression of hnRNP F in IRPTCs. To the best of our knowledge, this is the first report that hnRNP F could be modulated by high glucose and insulin in kidney proximal tubular cells *in vitro*. Our tissue distribution analysis revealed that the hnRNP F protein is detectable in the nuclear extracts of rat liver, kidney, testis, brain and lung but not in the spleen. Rat liver, kidney, testis (epididymis), brain and lung are known to express ANG mRNA (57, 58). These observations raise the possibility that the expression of hnRNP F might have a role in regulating ANG gene expression in these tissues.

In summary, we have identified a nuclear protein that binds to rANG-IRE by a combination of Southwestern blotting and proteomics. This IRE-binding protein is identified as hnRNP F. hnRNP F modulates ANG gene expression in IRPTCs. Finally, it appears that high glucose and insulin regulate hnRNP F expression in IRPTCs and hnRNP F is detected in various rat tissues that express ANG mRNA. The present studies raise the possibility that the expression of hnRNP and the unidentified 70 kDa IRE-BP(s) may play an important role in regulating local intrarenal RAS activation. Dysregulation of the expression of hnRNP F and unidentified 70 kDa IRE-BPs may contribute to renal injury in diabetes via altering local intrarenal RAS activation.

APPENDIX

Abbreviations used in this article: ACE, angiotensin-converting enzyme; ANG, angiotensinogen; Ang II, angiotensin II; BPB, bromophenol blue; CBP, CAP-binding protein; CREB, cAMP-responsive element-binding protein; DMEM, Dulbecco's modified Eagle medium; DN, diabetic nephropathy; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GMSA, gel mobility shift assay; hGAPDH, human glyceraldehyde phosphate dehydogenase; hnRNP F, heterogenous nuclear ribonucleoprotein F; IPTG, isopropylthiogalactosidase; IRE, insulin-responsive element; IRE-BP, IRE-binding protein; IRPTCs, immortalized renal proximal tubular cells; kDa, kiloDalton; MALDI, Matrix assisted laser desorption/ionization technique; MS, mass spectrometry; PCR, polymerase chain reaction; RAS, renin-angiotensin system; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-D, 2 dimensional

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LEGENDS

Figure 1: Rat ANG IRE-BPs detected by 2-dimensional (2-D) electrophoresis, Southwestern and Western blottings. A, rat IRPTC nuclear extracts were subjected to 2-D electrophoresis and then stained with Coomassie Brilliant Blue R-250. M: Amersham Pharmacia Biotech's rainbow molecular mass markers. N.E.: rat IRPTC nuclear extracts without isoelectrofocusing. B, Southwestern blotting analysis of ANG IRE-BPs from IRPTC nuclear proteins after 2-D electrophoresis. After 2-D electrophoresis, the nuclear proteins were transferred onto a Hybond C-extra membrane, hybridized with radioactively-labeled ANG-IRE (N-878/N-864), washed, and subjected to autoradiography. The arrow heads indicate the proteins that were determined to be hnRNP F by later mass spectrometry. The broken box denotes the proteins that were subjected to mass spectrometry, but their identities could not be determined. C, Western blotting analysis of the 2-D electrophoresis membrane from panel B using anti-hnRNP F antibody (obtained from Dr. Christine Milcarek, Univ. of Pittsburg School of Medicine, Pittsburg, PA, USA).

Figure 2: Mass spectrometry analysis of proteins dectected by Southwestern blotting. Spot 1 was isolated from 2-D gel and then subjected to tryptic digestion and MALDI-MS analysis. A, a

typical MALDI-MS peptide fingerprint of spot 1. B, peptide sequence homology with hnRNP F identified by MALD-MS and database search (BOLD letter). The sequence coverage of hnRNP F reached 32%. Similar results were obtained from spot 2 (not shown).

Figure 3: Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS- PAGE) and Western blotting analysis of glutathione S-transferase (GST)-rat hnRNP F (GST-hnRNP F) fusion proteins from bacterial culture. A, Bacterial extracts were subjected to 10% SDS-PAGE and then stained with Coomassie Brilliant Blue R-250. B, Western blotting analysis of GST-hnRNP F fusion proteins from bacterial culture with rabbit anti-hnRNP F antiserum. Lane 1 (Amersham-Pharmacia Biotech® rainbow markers), lanes 2 and 3 [30 μg, crude bacterial extract (*E. Coli* transformed with control plasmid, pGex 4T-3 without and with 0.5 M isopropylthiogalactoside (IPTG) induction, respectively], lanes 4 and 5 [30 μg, crude bacterial extract (*E. Coli* transformed with the plasmid, pGex 4T-3 containing hnRNP F cDNA) without and with 0.5 M IPTG induction, respectively], lane 6 (10 μg, purified GST protein after the GST affinity column chromatography) and lane 7 (10 μg, purified GST-hnRNP F

fusion protein after the GST affinity column chromatography). Similar results were obtained in 2 additional experiments.

Figure 4: Gel mobility shift assay of the radioactively-labeled rANG-IRE DNA fragment with GST-hnRNP F fusion protein(s). (A) The labeled DNA probe (0.1 pmol) was incubated with GST (5 μg) or GST-hnRNP F fusion protein(s) (0.1 to 2. 0 μg each) in the presence of 0.3 units of poly dI-dC. (B) Competition with 100- and 300-fold molar excess of unlabeled hGAPDH-IRE (N-473/-446), rGlucagon-IRE (N-266/-242), concensus SP1 sequence, and rANG-IRE motif is shown in lanes 4 to 5, lanes 6 to 7, lanes 8 to 9, and lanes 10 to 11, respectively. Similar observations were made in 2 other experiments.

Figure 5: Autoradiography in gel mobility shift assay of the radioactively-labeled rANG-IRE DNA fragment with GST-hnRNP F fusion protein(s). The labeled DNA probe (0.1 pmol) was incubated with GST (5 μg, lane 2) or GST-hnRNP F fusion protein(s) (1 μg each; lanes 3 to 13) in the presence of 0.3 units of poly dI-dC. Competition with 100- and 300-fold excess of unlabeled ANG-IRE motif and mutants of rANG N-882/-854 (M1, M2, M3 and M4) is shown in lanes 4 to 5, lanes 6 to 7, lanes 8 to 9, lanes 10

to 11, and lanes 12 to 13, respectively. Similar observations were made from 2 other experiments.

Figure 6: Super-shift gel mobility shift assay of the radioactively-labeled rANG-IRE DNA fragment with GST-hnRNP F fusion protein(s). The labeled DNA probe (0.1 pmol) was incubated with GST (5 μg) or GST-hnRNP F fusion protein(s) (1 μg each, lanes 3-9) in the presence of 0.3 units of poly dI-dC. Rabbit anti-hnRNP F antiserum (0.5 to 2 μl, lanes 4 to 6) or rabbit IgG (0.5 to 2 μg, lanes 7 and 9) were added the reaction mixture and incubated for 30 min on ice before incubation with the labeled probe. Similar observations were made from 2 other experiments.

Figure 7: Super-shift gel mobility shift assay of the radioactively-labeled rANG-IRE with IRPTC nuclear protein(s). The labeled DNA probe (0.1 pmol) was incubated with BSA (5 μg) or IRPTC nuclear protein(s) (5 μg each, lanes 2-7) in the presence of 0.3 units of poly dI-dC. Excessive rat ANG-IRE (100-fold) was added for competition with the labeled probe (lane 3). Rabbit IgG (10 and 5 μg, lanes 4 and 5, respectively) or rabbit anti-hnRNP F antiserum (obtained from Dr. T. Tamura, Chiba, Japan) (1.0 and 0.5 μl, lanes 6 and 7, respectively) were added the reaction mixture and incubated for 30 min on ice before

incubation with the labeled probe. Similar observations were made from 2 other experiments. (SS, supershift band)

Figure 8: Expression of hnRNP F and ANG mRNA in IRPTCs transiently tranfected with pcDNA 3.1, pcDNA 3.1/hnRNP F (+) or pcDNA 3.1/hnRNP F (-). A, Western blotting analysis of hnRNP F and β-actin expression in IRPTCs. 24 h after gene transfection, the cells were incubated for 24 h in 5 mM DMEM containing 5% FBS. Then, cells were collected, extracted and assayed for hnRNP F and β-actin by Western blotting. The relative densities of the hnRNP F band were compared with the β-actin band. The hnRNP F level in pcDNA 3.1-transfected cells represents the control level (100%). B, Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of endogenous rat ANG and β-actin mRNA expression in IRPTCs. 24 h after gene transfection, the cells were cultured for 24 h in 5 mM DMEM containing 5% FBS. Then, cells were harvested and assayed for rat ANG mRNA by RT-PCR as described in *Materials and Methods*. The relative densities of the PCR band of ANG mRNA were compared with the β-actin band. The ANG mRNA level in pcDNA 3.1-transfected cells represents the control level (100%). Results were expressed as the means ± SD of 3 determinations (*p≤

0.05, **p≤ 0.01). Similar results were obtained in 3 other experiments.

Figure 9: Effect of hnRNP F on rANG gene promoter activity in IRPTCs. 48 h after transfection, cells were harvested and assayed for CAT activity. The relative activity in cells transfected with 1 ug of pOCAT/rANG N-1498/+18 or mutant pOCAT/rANG N-1498/+18 was given relative value of 100% (control). Each point represents the mean ± S.D. of three independent experiments (N.S., not significant; *p≤ 0.05; **p≤ 0.01).

Figure 10: Effect of high glucose and insulin on hnRNP F expression in IRPTCs. (A) Southwestern blot analysis. Cells were incubated in 5 mM plus 20 mM D-Mannitol, 25 mM D-glucose or 25 mM D-glucose plus insulin (10^{-7} M) and 5% FBS for 24 h. Then, cells were harvested and nuclear proteins (100 µg) were subjected to Southwestern blotting as described in *Materials and Methods*. (B) Western blot analysis. After Southwestern blot analysis, the same membrane was blottted with rabbit polyclonal antibodies against hnRNP F (obtained from Dr. Christine Milcarek, Univ. of Pittsburg School of Medicine, Pittsburg, PA, USA) and β-actin control and ECL-chemiluminescent developing reagent. The relative densities of hnRNP F bands were compared with the β-

actin control. The level of hnRNP F expressed in IRPTCs in 5 mM glucose medium was considered to be the control (100%). Each bar represents the mean ± SD of three independent experiments (*$p \leq 0.05$, **$p \leq 0.01$).

Figure 11: Distribution of the hnRNP F protein in various rat tissues as analyzed by Western blot analysis. Nuclear proteins (100 µg) were subjected to Western blotting as described in *Materials and Methods* with rabbit polyclonal antibodies against hnRNP F (obtained from Dr. Christine Milcarek, Univ. of Pittsburg School of Medicine, Pittsburg, PA, USA) and β-actin and ECL-chemiluminescent developing reagent. The relative densities of hnRNP F bands were compared with the β-actin control. The level of hnRNP F expressed in IRPTCs was considered to be the control (100%). Each bar represents the mean ± SD of three independent experiments (**$p \leq 0.01$).

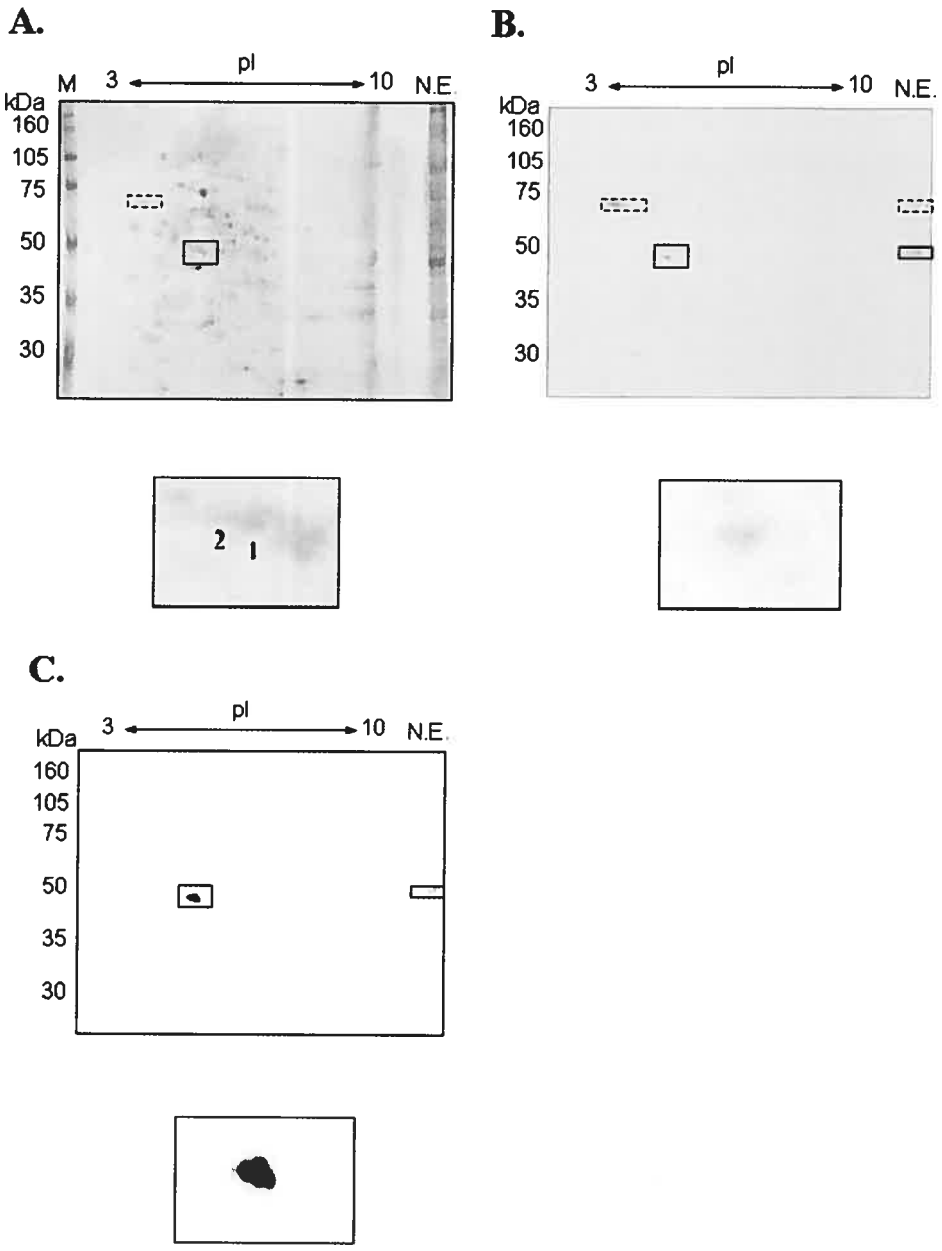
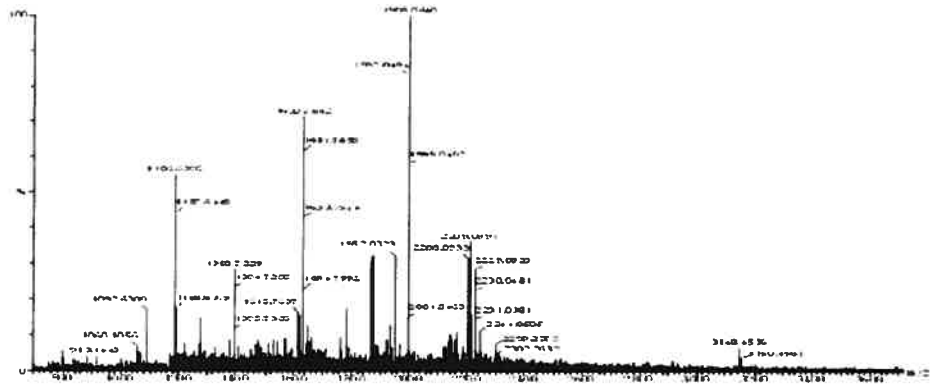

A.
M
3
pI
10
N.E.
kDa
160
105
75
50
35
30
2
1
B.
3
pI
10
N.E.
kDa
160
105
75
50
35
30
C.
3
pI
10
N.E.
kDa
160
105
75
50
35
30

Figure 1

A.



B.

```
  1 MMLGPEGGEG YVVKLRGLPW SCSIEDVQNF LSDCTIHDGV AGVHFIYTRE
 51 GRQSGEAFVE LESEDDVKLA LKKDRESMGH RYIEVFKSHR TEMDWVLKHS
101 GPNSADSAND GFVRLRGLPF GCTKEEIVQF FSGLEIVPNG ITLPVDPEGK
151 ITGEAFVQFA SQELAEKALG KHKERIGHRY IEVFKSSQEE VRSYSDPPLK
201 FMSVQRPGPY DRPGTARRYI GIVKQAGLDR MRSGAYSAGY GGYEEYSGLS
251 DGYGFTTDLF GRDLSYCLSG MYDHRYGDSE FTVQSTTGHC VHMRGLPYKA
301 TENDIYNFFS PLNPVRVHIE IGPDGRVTGE ADVEFATHEE AVAAMSKDRA
351 NMQHRYIELF LNSTTGASNG AYSSQVMQGM GVSAAQATYS GLESQSVSGC
401 YSAGYSGQNS MGGYD
```

Match to Rat hnRNP F

Nominal mass (M_r) 46043; Calculated pI value 5.31

Figure 2

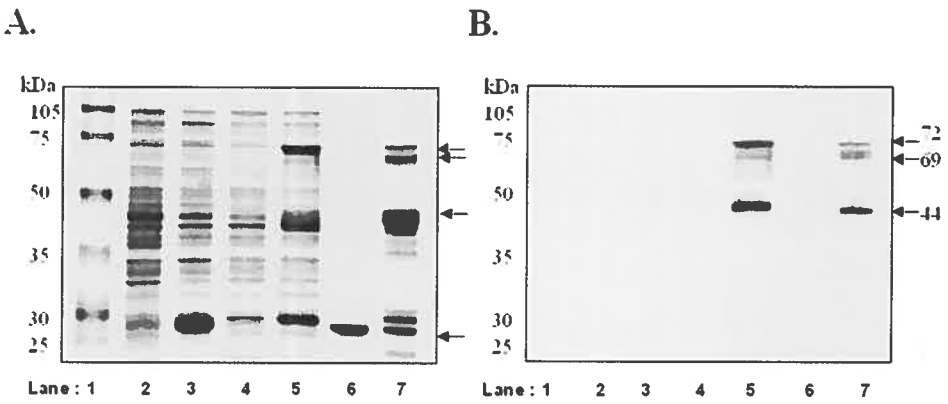

A.
B.
kDa
105
75
50
35
30
25
Lane : 1 2 3 4 5 6 7
kDa
105
75
50
35
30
25
72
69
44
Lane : 1 2 3 4 5 6 7

Figure 3

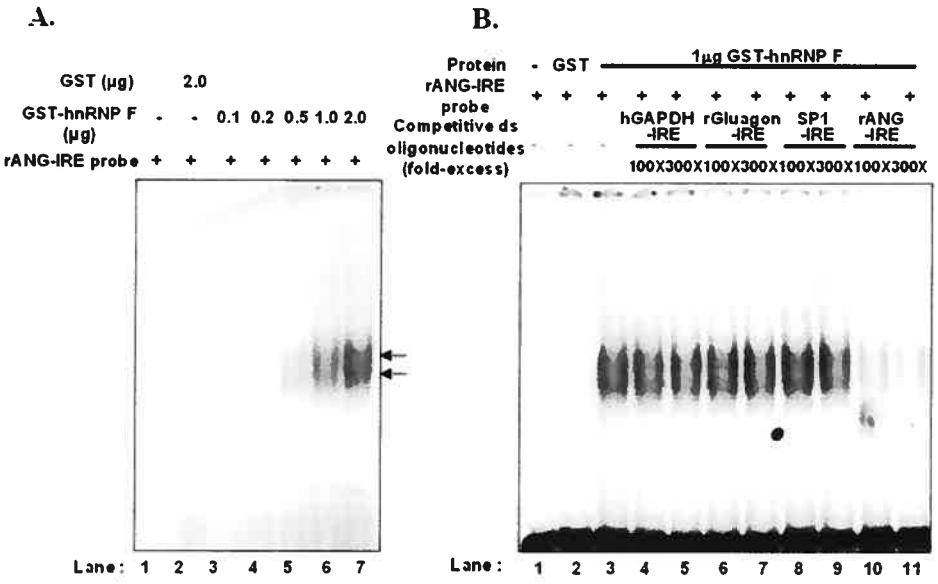

A.
GST (µg) 2.0
GST-hnRNP F (µg) - - 0.1 0.2 0.5 1.0 2.0
rANG-IRE probe + + + + + + +
Lane: 1 2 3 4 5 6 7
B.
Protein - GST 1µg GST-hnRNP F
rANG-IRE probe + + + + + + + + + + +
Competitive ds oligonucleotides (fold-excess)
hGAPDH -IRE
rGluagon -IRE
SP1 -IRE
rANG -IRE
100X 300X 100X 300X 100X 300X 100X 300X
Lane: 1 2 3 4 5 6 7 8 9 10 11

Figure 4

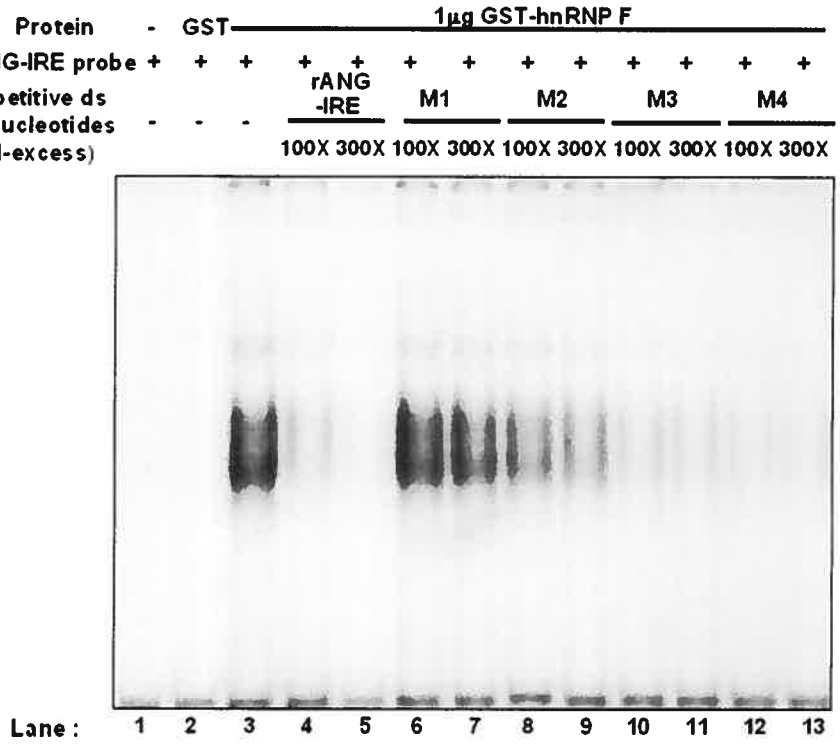

Protein - GST 1µg GST-hnRNP F
rANG-IRE probe + + + + + + + + + + + + +
Competitive ds oligonucleotides (fold-excess) - - - rANG -IRE M1 M2 M3 M4
100X 300X 100X 300X 100X 300X 100X 300X 100X 300X
Lane : 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 5

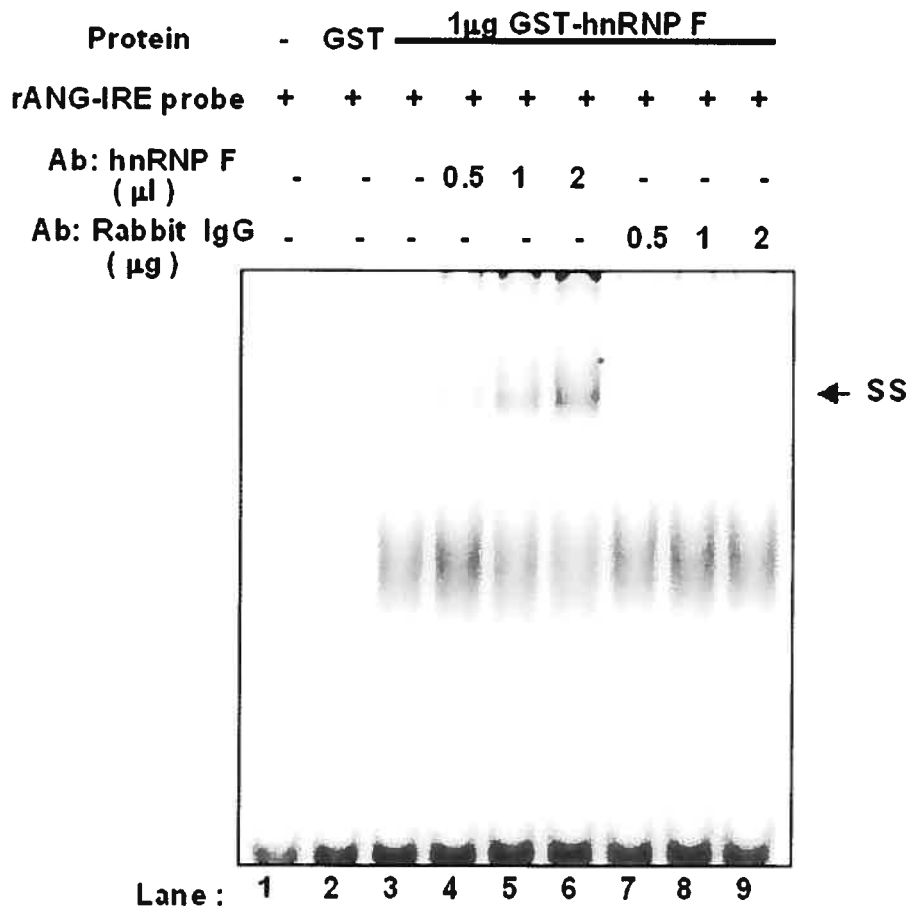

Protein - GST 1µg GST-hnRNP F
rANG-IRE probe + + + + + + + + +
Ab: hnRNP F (µl) - - - 0.5 1 2 - - -
Ab: Rabbit IgG (µg) - - - - - - 0.5 1 2
SS
Lane : 1 2 3 4 5 6 7 8 9

Figure 6

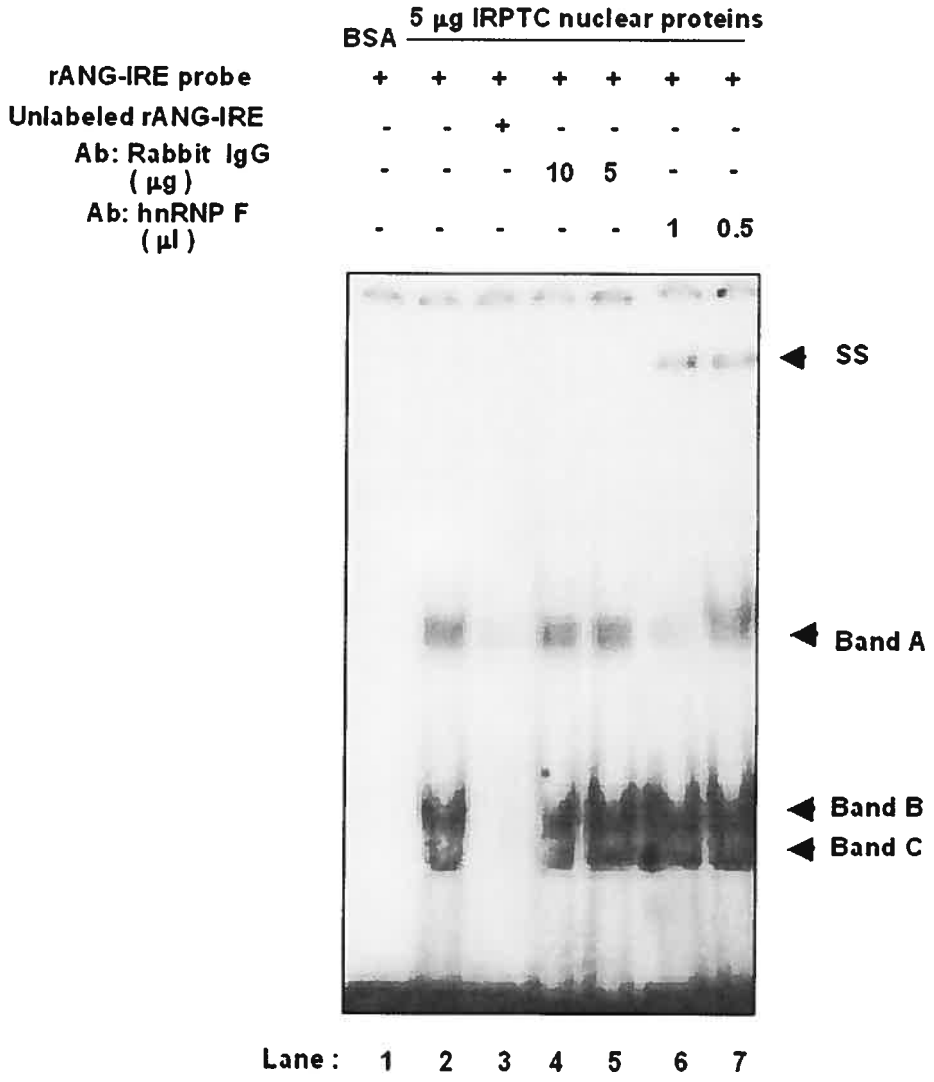

BSA
5 µg IRPTC nuclear proteins
rANG-IRE probe + + + + + + +
Unlabeled rANG-IRE - - + - - - -
Ab: Rabbit IgG (µg) - - - 10 5 - -
Ab: hnRNP F (µl) - - - - - 1 0.5
SS
Band A
Band B
Band C
Lane : 1 2 3 4 5 6 7

Figure 7

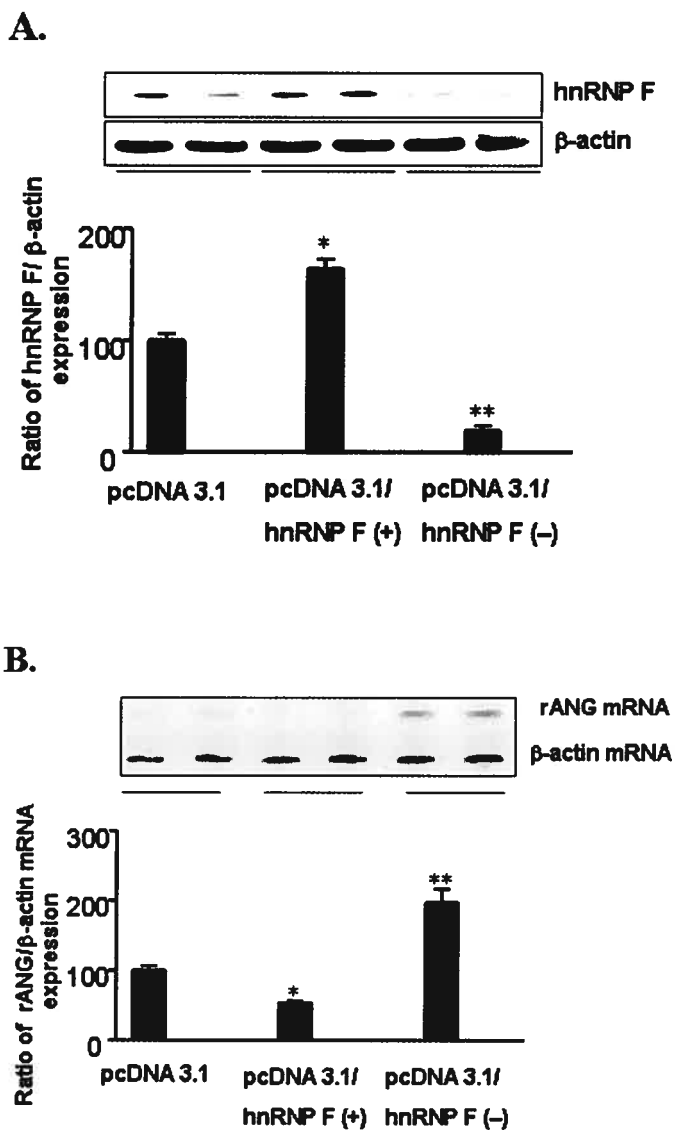

A.
hnRNP F
β-actin
Ratio of hnRNP F/ β-actin expression
200
100
0
*
**
pcDNA 3.1
pcDNA 3.1/ hnRNP F (+)
pcDNA 3.1/ hnRNP F (–)
B.
rANG mRNA
β-actin mRNA
Ratio of rANG/β-actin mRNA expression
300
200
100
0
*
**
pcDNA 3.1
pcDNA 3.1/ hnRNP F (+)
pcDNA 3.1/ hnRNP F (–)

Figure 8

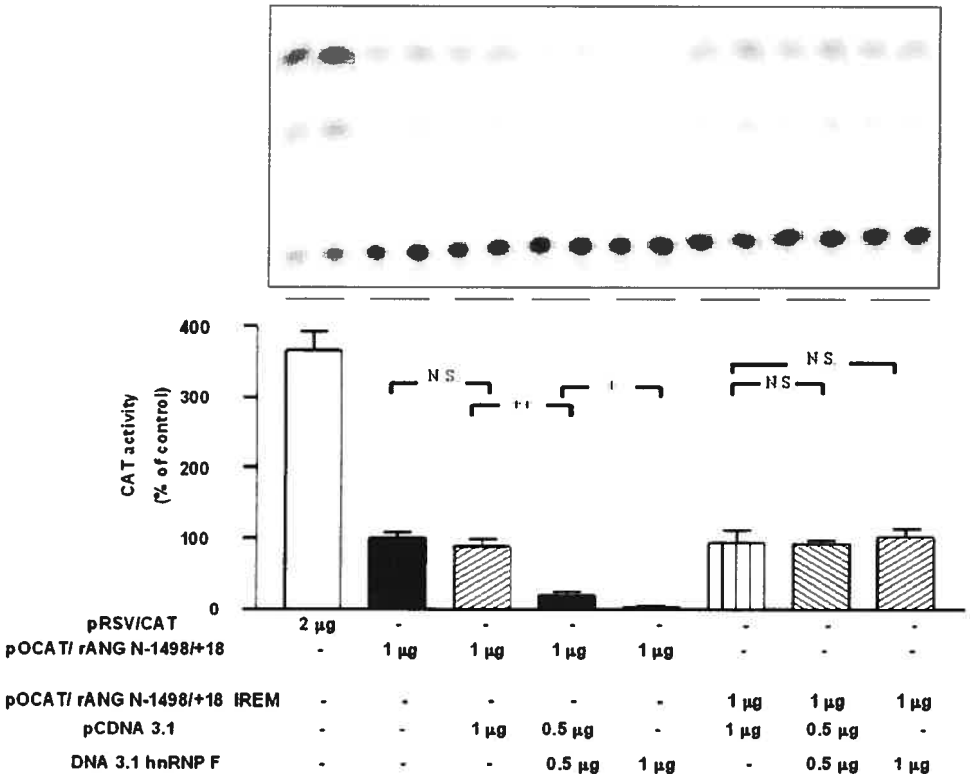

CAT activity
(% of control)
400
300
200
100
0
NS
NS
NS
pRSV/CAT 2 µg - - - - - - -
pOCAT/ rANG N-1498/+18 - 1 µg 1 µg 1 µg 1 µg - - -
pOCAT/ rANG N-1498/+18 IREM - - - - - 1 µg 1 µg 1 µg
pCDNA 3.1 - - 1 µg 0.5 µg - 1 µg 0.5 µg -
DNA 3.1 hnRNP F - - - 0.5 µg 1 µg - 0.5 µg 1 µg

Figure 9

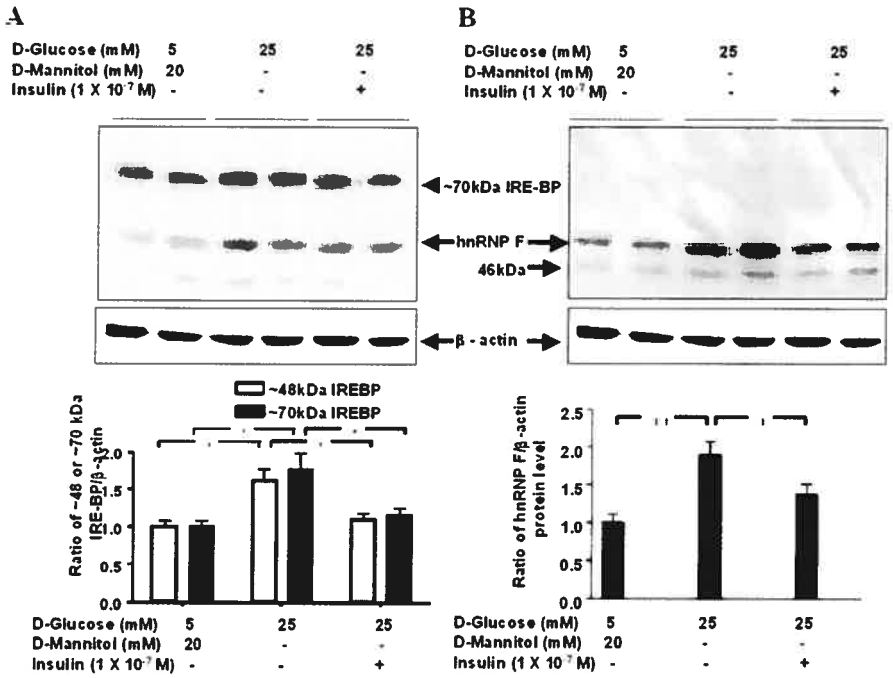

A
B
D-Glucose (mM) 5 25 25
D-Mannitol (mM) 20 - -
Insulin (1 X 10⁻⁷ M) - - +
~70kDa IRE-BP
hnRNP F
46kDa
β - actin
~48kDa IREBP
~70kDa IREBP
Ratio of ~48 or ~70 kDa IRE-BP/β-actin
Ratio of hnRNP F/β-actin protein level
0.0
0.5
1.0
1.5
2.0
2.5

Figure 10

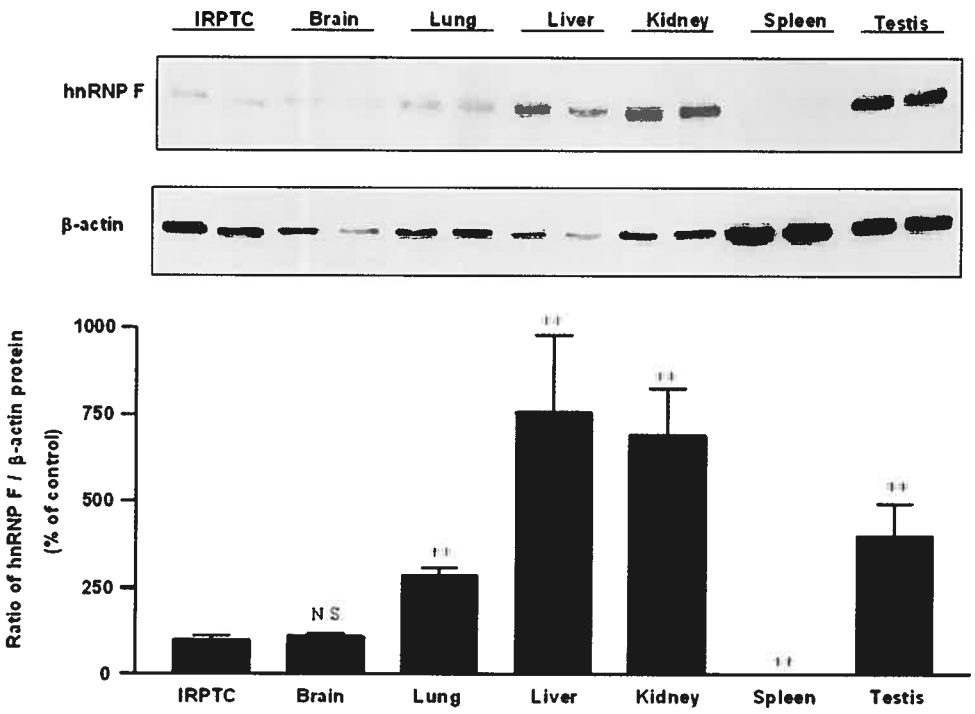

IRPTC
Brain
Lung
Liver
Kidney
Spleen
Testis
hnRNP F
β-actin
Ratio of hnRNP F / β-actin protein (% of control)
1000
750
500
250
0
N S
IRPTC
Brain
Lung
Liver
Kidney
Spleen
Testis

Figure 11

Chapter 3 : Article 2

Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells.

HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN K MODULATES ANGIOTENSINOGEN GENE EXPRESSION IN KIDNEY CELLS

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Keywords: Heterogeneous nuclear ribonucleoprotein K, angiotensinogen, kidney

Abstract: 173 words; Text: 4,839

ABSTRACT

The present studies aimed to identify the 70-kiloDalton nuclear protein that binds to an insulin-responsive element in the rat angiotensinogen gene promoter and to define its action on angiotensinogen gene expression. Nuclear proteins were isolated from rat kidney proximal tubular cells and subjected to 2-dimensional electrophoresis. The 70-kiloDalton nuclear protein was detected by Southwestern blotting and subsequently identified by mass spectrometry, which revealed that it was identical to 65-kDa heterogenous nuclear ribonucleoprotein K (hnRNP K). HnRNP K bound to insulin-responsive element of rat angiotensinogen gene was revealed by gel mobility shift assay and chromatin immunoprecipitation assay. HnRNP K inhibited angiotensinogen mRNA expression and promoter activity. In contrast, hnRNP K down-expression by small interference RNA enhanced angiotensinogen mRNA expression. Finally, *in vitro* and *in vivo* studies demonstrated that high glucose increases and insulin inhibits hnRNP K expression in rat kidney proximal tubular cells. In conclusion, our experiments revealed that hnRNP K is a nuclear protein that binds to the insulin-responsive element of the rat angiotensinogen gene promoter and modulates angiotensinogen gene transcription in the kidney.

INTRODUCTION

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD), accounting for 30-50 % of all new ESRD cases in North America (1-3). Both clinical and animal studies indicate that intensive insulin therapy and prolonged treatment with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (Ang II)-AT_1 receptor blockers delay the progression of nephropathy in diabetes, but neither strategy cures nephropathy (4-12). While such results support the concept that hyperglycemia and the renin-angiotensin system (RAS) are involved in the development and progression of DN, the molecular mechanism(s) linking hyperglycemia to RAS activation remain largely undefined.

Angiotensinogen (ANG), is a glycoprotein consisting of 452 amino acid residues with an apparent molecular weight (MW) of 62-65 kiloDalton (kDa), is the sole substrate in the RAS cascade (13, 14). ANG is principally produced by the liver and cleaved by renin from the kidney to form angiotensin I (Ang I), which is then further processed by ACE to form Ang II. However, physiological, biochemical and molecular studies have provided convincing evidence for other pathways of Ang II production. Among these, the existence of an intrarenal RAS is of special interest and is now generally accepted (15, 16). Renal proximal tubules (RPTs) contain all components of the RAS, including messenger RNAs and proteins, such as ANG, renin, ACEs, and Ang II receptors (AT_1 and AT_2 subtypes) (17-23). We have reported that ANG protein is synthesized and secreted from rat immortalized renal proximal tubular cells (IRPTCs) (24), providing evidence that intrarenal Ang II is

probably derived from ANG synthesized within renal proximal tubular cells (RPTCs) *in vivo*. Thus, the local formation of Ang II may play an important role in the development of nephropathy in diabetes.

We have established that high glucose (i.e., 25 mM) stimulates ANG gene expression via reactive oxygen species generation, activation of protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK) and hexosamine biosynthesis pathway (HBP) signalling in IRPTCs (25-28). RAS blockers and stable transfer of antisense rat ANG cDNA into IRPTCs inhibit transforming growth factor-beta 1 (TGF-β1) gene expression and cellular hypertrophy in high glucose (29, 30). These investigations strongly indicate that ANG and TGF-β1 gene expression are essential for the high-glucose effect on RPTC hypertrophy and kidney injury. We have also established that insulin inhibits the stimulatory effect of high glucose levels on ANG gene expression and the induction of hypertrophy in IRPTCs (31-33). Moreover, a putative insulin-responsive element (IRE) containing nucleotides N-878 to N-864 (5' CCT TCC CGC CCT TCA 3') upstream of the transcription start site of the rat ANG gene promoter has been identified, and it binds to 2 major nuclear proteins with apparent MW of approximately 48 and 70 kDa from IRPTCs, as revealed by Southwestern blotting (33). We recently reported that the 48-kDa nuclear protein is identical to 46-kDa heterogenous nuclear ribonucleoprotein F (hnRNP F) (34). Furthermore, transient transfer of sense and antisense hnRNP F cDNA respectively inhibits and enhances ANG gene expression in IRPTCs (34).

The present studies aimed to identify the 70-kDa nuclear protein and to investigate its action on ANG gene expression. We identified the 70-kDa nuclear protein as 65-kDa heterogenous nuclear ribonucleoprotein K (hnRNP K) by 2-dimensional (2-D) electrophoresis and mass spectrometry (MS). Recombinant hnRNP K bound to ANG-IRE, as shown by gel mobility shift assay (GMSA) and chromatin immunoprecipitation assays. Over-expression and down-expression of hnRNP K inhibited and enhanced ANG gene expression in IRPTCs, respectively. Finally, *in vitro* and *in vivo* studies revealed that high glucose or hyperglycemia increased and insulin inhibited hnRNP K expression in rat kidney proximal tubular cells. These experiments demonstrated that 65-kDa hnRNP K is a nuclear protein that binds to the rat ANG gene promoter and modulates ANG gene expression in the kidneys.

MATERIALS AND METHODS

D(+)-glucose, D-mannitol and insulin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Insulin implant ((Linplant) and gamma-[^{32}P-ATP] (3,000 Ci/mol) were obtained from Linshin Ltd. (Scarborough, ON, Canada) and Amersham-Pharmacia Biotech (Baie d'Urfé, QC, Canada), respectively. Plasmid containing full-length hnRNP K cDNA (pcDNA 3/hnRNP K) and rabbit polyclonal antiserum (#54) recognizing

hnRNP K (QNSVKQYADVEGF corresponding to amino acids 452 to 464 of human hnRNP K) were generated in Karol Bomsztyk's laboratory as described previously (35). Mouse monoclonal antibody against human hnRNP K/L (clone 3C2), a gift from Dr. Gideon Dreyfuss (Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadephia, PA, USA), has been reported elsewhere (36). The bacterial expression vector pGex 4T-3 and mammalian expression vectors, pcDNA 3.1 and pRC/RSV were purchased from Amersham-Pharmacia Biotech and InVitrogen Inc. (Burlington, ON, Canada), respectively. Restriction modified enzymes were acquired from either InVitrogen Inc., Amersham-Pharmacia Biotech or Roche Diagnostics (Laval, QC, Canada).

Oligonucleotides for rat ANG-IRE N-882 to N-855 (5' CCT CCC TTC CCG CCC TTC ACT TTC TAG T 3') (33), mutants of ANG N-882 to N-885 (M1, 5' CCT CCC TTC CAT TAC TTC ACT TTC TAG T 3'; M2, 5 CCT CCC TTA AAT AAG ACC ACT TTC TAG T 3 ; M3, 5 CCT CCC TTC CCT TCC TTC ACT TTC TAG T 3 ; M4, 5 CCT CCC TTC CCT CCC TTC ACT TTC TAG T 3), IRE of human glyceraldehyde phosphate dehydrogenase gene (hGAPDH-IRE, N-473 to N-477, 5' CCA ACT TTC CCG CCT CTC AGC CTT TGA A 3) (37), and IRE of rat glucagon gene (N-267 to N-242, 5' AGT TTT CAC GCC TGA CTG AGA TTG A 3') (38) were synthesized by InVitrogen Inc. The oligonucleotide containing the consensus Sp1-binding site (5 -TCG CCC CGC CCC CGA TCG AAT-3) (39) was purchased from Promega (Fisher-Scientific Inc., Montreal, QC, Canada).

Cellular Nuclear Extract Preparation

IRPTCs from passages 12 to 18 were tested. The characteristics of IRPTCs, which express the mRNA and protein of ANG, renin, ACE, and Ang II receptors, have been described previously (40). IRPTC nuclear extracts were prepared from 20 plates (150 x 20 mm), each containing confluent IRPTCs previously incubated in Dulbecco's modified Eagle medium (DMEM) with 5 mM glucose plus 20 mM D-mannitol, 25 mM glucose, or 25 mM glucose plus insulin (10^{-7} M) for 24 h according to the method of Henninghausen and Lubon (41) with slight modifications (33, 34).

2-D Electrophoresis

2-D electrophoresis was carried out with the IPGphor Isoelectric Focusing Unit (Amersham-Pharmacia Biotech) (34). For isoelectrofocusing (IEF), precast 13-cm IPG strips (pH 3-10, non-linear, Immobiline DryStrips, Amersham-Pharmacia Biotech) were pre-equilibrated with 750 μg of nuclear extracts in 250 μl rehydration buffer (8 M urea, 1 M thiourea, 4% CHAPS, 2% IPG buffer, 1% NP-40, 0.1 M DTT, and 0.0001% bromophenol blue (BPB)) for more than 12 h according to the supplier's manual. IEF was run for 90 kilovolt-h at 25°C. After IEF separation, the IPG strips were immediately equilibrated for 15 min with a buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 2% DTT and 0.0001% BPB. Then, the strips were re-equilibrated with another buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide and 0.0001% BPB for an

additional 15 min. For 2-D separation, the IPG strips were placed above 10% polyacrylamide gel containing SDS and electrophoresed (SDS-PAGE). Amersham's rainbow markers served as MW markers. IRPTC nuclear extracts (100 μg) were run on the same 10% SDS-PAGE as the controls. Each sample was divided into 2 strips for 2-D electrophoresis. One gel was stained with Coomassie Brilliant Blue R-250 (Amresco Inc., Solon, OH, USA) to visualize proteins. The other was electrotransferred to a Hybond C-extra membrane (Amersham-Pharmacia Biotech) for Southwestern blotting.

Southwestern Blotting

Southwestern blotting was performed according to the procedure of Kwast-Welfeld et al. (42) with slight modifications (33, 34). Briefly, IRPTC nuclear proteins (200 μg) were resolved on a 4 to 20% SDS-PAGE gradient or on 10% SDS-PAGE (43), then electrotransferred to a Hybond C-extra membrane, which was incubated with 10% (W/V) non-fat milk proteins in a binding buffer containing 10 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl, 0.25 mM EDTA and 2.5% glycerol (V/V) for 24 h at 4°C. The membrane was washed at least twice with binding buffer containing 0.25% non-fat milk proteins. Subsequently, it was hybridized overnight with ^{32}P-labelled ANG-IRE motif DNA (approximately 1.0 to 2.0 pmol; 10^6 cpm/ml) in binding buffer containing 0.25% non-fat milk proteins and 300 μg/ml non-denatured herring sperm DNA at 4°C. The membrane was finally washed, air-dried and exposed for autoradiography.

Matrix-assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS)

Spots on the gel corresponding to positive signals of the Southwestern blot membrane were picked up for MALDI-MS. All MALDI-MS analyses were performed at the Quebec Genome Centre (McGill University, Montreal, QC, Canada). Briefly, protein samples were first cleaved by trypsin and then subjected to MALDI-MS. MALDI-MS analysis was conducted at 20-kV accelerating voltage and 23-kV reflecting voltage. For protein identification, peptide mass fingerprints were searched by the Mascot program developed by Matrix Science Ltd. (freely accessible on http:\\www.matrixscience.com).

Expression of Recombinant hnRNP K

Rat hnRNP K cDNA (35), with the Not 1 enzyme restriction site added on the 5 and 3 ends of sense and antisense primers, respectively, was subcloned at the polyclonal site (Not 1) of the bacterial expression vector pGex 4T-3 by conventional methodology. *E. Coli* BL-21 cells (Amersham-Pharmacia Biotech) were transformed by pGex 4T-3 containing rat hnRNP K cDNA. Expression of the fusion protein [GST fused with hnRNP K (GST-hnRNP K)] in BL-21 cells was induced by the addition of 1 mM isopropylthiogalactoside (IPTG) in the culture medium with incubation for 4 h at 37°C. The bacteria were then harvested, and GST-hnRNP K fusion proteins were purified from the bacterial extracts by GST affinity column chromatography according to the manufacturer's protocol (Amersham-

Pharmacia Biotech). The purified GST-hnRNP K fusion proteins were tested in GMSAs.

GMSAs

These assays were performed according to the methodology described elsewhere (33, 34), employing labeled monomeric ANG-IRE motif DNA as probe. Briefly, the ANG-IRE DNA fragment was 5' end labeled with [γ-^{32}P]-ATP by T_4 polynucleotide kinase. Purifed GST-hnRNP K fusion proteins (0.1 μg) or GST (5 μg) in the presence of 1 μg of poly(dI/dC) in 20 mM Hepes (pH 7.6), 1 mM EDTA, 50 mM KCl, 2 mM spermidine, 1 mM DTT, 0.5 mM PMSF and 10% glycerol (V/V) were sonicated for 30 min and then incubated for 30 min on ice. Subsequently, the 5'-labelled probe (~0.1 pmol) was added and further incubated for 30 min at room temperature. After being chilled on ice, the mixture was run on 5% (W/V) non-denaturing PAGE and exposed for autoradiography.

In competition assays, 100-fold molar excess of unlabeled DNA fragments was added to the reaction mixture and incubated for 30 min at room temperature before incubation with the labeled probe.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to the methodology of Kuo and Allis (44) with slight modifications (45). Briefly, 0.4 ml of 37% formaldehyde was added to 10 ml of overlaying media of IRPTC culture for 15

min at 4°C. After cross-linking, the cells were harvested, washed twice with 1 ml of PBS in Eppendorf tubes, and then lysed with 0.5 ml of immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT) containing the following inhibitors: 10 µg/ml leupeptin, 0.5 mM PMSF, 30 mM p-nitrophenol phosphate, 10 mM NaF, 0.1 mM Na_3VO_4 and 10 mM β-glycerophosphate. After 1 wash with IP buffer, the pellet was suspended in 1 ml IP buffer and sheared in a Bronson sonicator with two 10-s cycles, 1 pulsed and 1 continuous, at an output 3 and 80% duty cycle. Pull-downs were done using anti-K protein antibody with or without blocking peptide (100 µM) and protein A beads (Amersham-Pharmacia Biotech). The beads were washed 5 times with 1 ml of IP buffer without inhibitors. DNA was eluted twice from the beads with 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min with periodic vortexing (at room temperature). Cross-linking was reversed by adding 20 µl of 5 M NaCl and incubating the eluate overnight at 65°C. After adding 5 µg of linear acrylamide, DNA was precipitated with 1.0 ml of 100% ethanol. The pellet was precipitated with 1.0 ml of 70% ethanol, and dissolved in 100 µl of Tris-EDTA (TE) buffer, pH 8.0. Then, 11 µl of 10X protein K buffer (0.1 M Tris, pH 7.8, 50 mM EDTA, 5% SDS) and 1 µl of 20 µg/µl proteinase K were added and incubated at 50°C for 30 min. DNA was extracted with phenol/chloroform, precipitated with ethanol, and the final DNA pellet was dissolved in 20 µl of TE buffer.

Polymerase chain reaction (PCR) amplications were done in 50 µl of 1X PCR buffer containing DNA, 0.5 µM primers (forward primer: 5 CCT TGA

TGC CTC CAA CAA CT 3 and backward primer: 5 GGT GGG AGC TGA GAA GAC AG 3 corresponding to nucleotides N-1043 to N-1026 and N-718 to N-698 of the rat ANG gene promoter (46), respectively), 40 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 1 unit of Tag DNA polymerase (In Vitrogen Inc.). The PCR products were resolved on 2% agarose gel and transferred onto a Hybond XL nylon membrane (Amersham-Pharmacia Biotech). Digoxigenin-labeled oligonucleotide 5' CCT CCC TTC CCG CCC TTC ACT TTC TAG T 3'), corresponding to nucleotide N-882 to N+855 of the rANG gene promoter (46) prepared with a digoxigenin oligonucleotide 3' end labeling kit (Roche Diagnostics), served to hybridize the PCR products on the membrane. After stringent washing, the membrane was detected with a digoxigenin luminescence kit (Roche Diagnostics) and exposed to Kodak BMR film (Eastman Kodak Co., Rochester, NY, USA).

Mammalian Expression of Recombinant hnRNP K

Rat hnRNP K cDNA with flag-tag at the N-terminal in mammalian expression vector pcRC/RSV (35) was transfected into IRPTCs with Lipofectamine according to the instruction manual provided by the supplier (InVitrogen Inc.). We optimized the DNA concentration for gene transfection at 2 μg per 0.5 to 1 x 10^6 cells. Forty-eight h after transfection, total RNAs and nuclear proteins were isolated from IRPTCs and assayed for ANG mRNA by RT-PCR (33, 34) or for flag-hnRNP K protein by Western blotting, respectively.

Small Interfering RNA (siRNA) of hnRNP K

IRPTCs were transfected with 40 nM scrambled Silencer® Negative Control # 1 siRNA (Ambion Inc., Austin, TX, USA) or 40 nM siRNA for hnRNP K (Sense 5 CCA GAU GUA AUG UUU UAG Utt 3 and antisense 5 ACU AAA ACA UUA CAU CUG Gtg 3 . Hnrpk siRNA ID 195920, Ambion Inc.) or 40 nM siRNA for hnRNP F (sense 5 GCA UGG GAC ACC GGU AUA Utt 3 and antisense 5 AUA UAC CGG UGU CCC AUG Ctt 3 . HnRNP F siRNA ID 192101, Ambion Inc.). Transfections were accomplished by using siPORT Amine (Ambion Inc.) according to the manufacturer's instructions. Total cellular RNA and protein were harvested at 48 h post-transfection and then analyzed for ANG and β-actin mRNA, and hnRNP K protein expression by RT-PCR (34) and Western blotting, respectively.

Western Blotting for hnRNP K

Briefly, the cell pellets were lysed in 100 μl RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (W/V) SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin], then incubated on ice for 30 min. The cell lysates were centrifuged at 12,000 x g for 15 min and the supernatants transferred to new tubes. Thirty μg of the supernatants were subjected to 10% SDS-PAGE, then transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham-Pharmacia Biotech). The membrane was initially blotted for anti-flag or anti-hnRNP K antibody (1:4,000 dilutions) and then re-blotted for anti-β-actin antibody

(1:10,000 dilutions) and chemiluminescent developing reagent (Roche Diagnostics). The relative densities of the hnRNP K and β-actin bands were quantified by computerized laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Amersham-Pharmacia Biotech).

Chloramphenicol Acetyl Transferase (CAT) Assay

The method of construction of the rANG-CAT fusion gene, pOCAT/rANG N-1498/+18 (fusion gene containing 1,498 nucleotides upstream of the transcription start site and 18 nucleotides of exon I fused with CAT reporter gene) and mutant pOCAT/rANG N-1498/+18 with mutated IRE has been described previously (32, 34). Control plasmid or fusion gene was transfected into IRPTCs using lipofectamine (InVitrogen Inc.) according to methods described previously (32, 34). 48 h after transfection, the cells were harvested and assayed for CAT activity (32, 34).

To normalize the efficiency of transfection, 0.5 μg of pRSV/β-Gal [a vector with the Rous Sarcoma Viral enhancer/promoter fused to the bacterial β-galactosidase gene] was co-transfected with pOCAT/rANG N-1498/+18 (32, 34). The plasmid pRSV/CAT [a vector with the Rous Sarcoma Viral enhancer/promoter fused to the CAT reporter gene] served as a positive control to monitor the efficiency of transfection of rANG-CAT fusion gene. The level of transfection efficiency for pRSV/CAT in IRPTCs ranged from 60 to 90%, i.e., the percentage of conversion of ^{14}C chloramphenicol to mono- and di-acetyl chloramphenicol. The transfection efficiency of pOCAT/rANG N-

1498/+18 in IRPTCs ranged from 25% to 35% compared with pRSV-CAT. The inter- and intra-assay coefficient variations of transfection for pOCAT/rANG N-1498/+18 in IRPTCs were 25% and 12% (N = 10), respectively.

Animals

The streptozotocin (STZ)-induced diabetic Wistar rat model has been described previously (47). Briefly, adult male Wistar rats (200-250 g) obtained from Charles River Inc. (St-Constant, QC, Canada) were divided into 3 groups: 1. Vehicle-injected controls (10 mM sodium citrate buffer, pH 4.0); 2. Untreated STZ-induced diabetics (65 mg/kg of STZ dissolved in 10 mM sodium citrate buffer administered i.p. after overnight fasting); and 3. Treated STZ-induced diabetics (subcutaneous insulin implant 48 hours after STZ induction). Untreated and treated diabetic rats with blood glucose >25 mM and <7 mM, respectively, were studied. Blood glucose was monitored with a glucose analyzer (Accu-Check Compact, Roche Diagnostics, Laval, QC, Canada).

All animals were allowed free access to rat chow and water. All methods of animal care and sacrifice were approved by the Animal Care Committee of the CHUM.

Isolation of Rat RPTs

Two weeks after the induction of diabetes, the rats were anesthetized and euthanized [Control and treated rats were euthanized at the same time point]. Kidneys were removed immediately for proximal tubule isolation. The renal cortex was separated from the medulla and minced under sterile conditions. Proximal tubules were isolated by Percoll gradient (48) with slight modifications (47). Proximal tubular cells were characterized by their histological appearance (48). A highly-purified preparation of proximal tubules (>97% by microscopy) with >95% viability (determined by trypan blue exclusion) was obtained. Aliquots of freshly-isolated proximal tubules from individual rats were immediately used for total RNA and protein isolation.

Statistical analysis

Three to 5 separate independent experiments were performed per protocol, and each treatment group was run in duplicate. The data were analyzed by 1-way ANOVA and the Bonferroni test. A probability level of $p \leq 0.05$ was regarded as statistically significant.

RESULTS

Identification of 70-kDa IRE-binding proteins (BPs) in IRPTCs

Figure 1A shows the staining of nuclear proteins after 2-D electrophoresis. Southwestern blotting of IRE-BPs after 2-D electrophoresis is

displayed in **Figure 1B**. Positive spots with apparent MW of 70 and 48 kDa were cut out and subjected to MALDI-MS. The MS results of 70-kDa proteins are displayed in **Figure 2**. The 2 spots with an apparent MW of 70 kDa were identified as a common protein (Accession number NM_057141). Database analysis revealed that they are identical to the rat hnRNP K cDNA sequence reported by Ito et al. (49). The 2 spots with an apparent MW of 48 kDa were identified as 46 kDa hnRNP F (34).

To confirm the authencity of rat hnRNP K revealed by Southwestern blotting, we stripped the radioactivity from the membrane and re-blotted it with rabbit polyclonal antiserum against hnRNP K, as shown in **Figure 1C**. It is apparent that the proteins interacting with anti-hnRNP K were superimposable, with positive signals detected by Southwestern blotting, as seen in Figure 1B. These data confirm that the 70-kDa proteins that interact with ANG-IRE are identical to hnRNP K.

GMSA

Bacterially-expressed recombinant hnRNP K proteins were employed to study the interaction of rANG-IRE with hnRNP K. One major band appeared with retarded mobility with labelled rANG-IRE by employing GST-hnRNP K fusion protein (**Figure 3**). No slowly-migrating band was observed when the labeled DNA was incubated with GST (bacterial extract of empty vector pGex 4T-3). The addition of an unlabeled rANG-IRE was effective in competing with the binding of labeled rANG-IRE DNA to the fusion proteins(s) (100-fold molar excess of unlabeled DNA fragment), but not the mutant of

rANG-IRE and the unlabeled DNA fragment of hGAPDH-IRE, rat glucagon-IRE, and the Sp1 consensus sequence (data not shown).

ChIP Analysis of HnRNP K Interactions with Gene Loci

ChIP assays were used to test if hnRNP K interacts with the IRE of the ANG gene promoter *in vivo*. **Figure 4** displays the PCR product of pulled-down DNA with primers specific to the rANG gene promoter. A ~300-bp DNA fragment was apparent in cells that were treated with the cross-linking agent and without blocking peptide (lane 4). In contrast, no PCR product was generated in cells without treatment with hnRNP K antibody (lane 3) or in the presence of blocking peptide (lane 5). These results validate the interaction of hnRPN K with rANG gene loci *in vivo*.

Effect of hnRNP K on ANG mRNA expression in IRPTCs

It is apparent that hnRNP K protein levels in IRPTCs transiently transfected with pRSR/hnRNP K were significantly higher ($p<0.01$) than those in control pRC/RSV-transfected IRPTCs (**Figure 5A**) by Western blotting. In contrast, ANG mRNA expression was significantly lower in pRSV/hnRNP K-transfected cells than those in pRC/RSV-transfected cells analyzed by RT-PCR (**Figure 5B**). These results demonstrated that hnRNP K inhibits ANG mRNA expression in IRPTCs. Most interestingly, hnRNP K overexpression prevented the stimulatory effect of high glucose on ANG mRNA expression in

IRPTCs (**Figure 6**). These data suggest that hnRNP K modulates high glucose stimulation of ANG gene expression in IRPTCs.

Effect of siRNA of hnRNP K on ANG mRNA expression in IRPTCs

Transient transfer of siRNA of hnRNPK and hnRNP F suppressed respective hnRNP K and hnRNP F expression but enhanced ANG mRNA expression in IRPTCs (**Figure 7**). In contrast, transient transfer of negative scrambled silencer had no effect on hnRNP K or hnRNP F protein and ANG mRNA expression in IRPTCs. These data further demonstrate that, like hnNRP F, hnRNP K modulates ANG mRNA expression in IRPTCs.

Effect of hnRNP K on rANG Gene Promoter Activity

Like hnRNP F, co-transfection with hnRNP K significantly suppressed pOCAT-rANG fusion gene promoter activity (**Figure 8**). It appears that hnRNP K is more effective in inhibiting ANG gene promoter activity than hnRNP F. These studies confirm the notion that hnRNP K modulates ANG gene expression at the transcriptional level via binding to IRE.

Effect of High Glucose and Insulin on hnRNP K Expression *In Vitro* and *In Vivo*

Figures 9A and **9B** show the results of respective Southwestern and Western blot analysis of IRPTC nuclear extracts for hnRNP K incubated in normal glucose (5 mM D-glucose plus 20 mM D-Mannitol) or high glucose (25 mM D-glucose) medium in the absence or presence of insulin (10^{-7} M). It is

apparent that high glucose levels enhanced and insulin suppressed hnRNP K expression in IRPTCs *in vitro*. Similarly, studies *in vivo* revealed that hyperglycemia upregulated hnRNP K expression in diabetic rat RPTs, and insulin treatment normalized hnRNP K to non-diabetic levels (**Figure 10**). These data demonstrate that hyperglycemia and insulin regulate hnRNP K expression in diabetic rat RPTs.

DISCUSSION

The present studies identified hnRNP K as one of the nuclear proteins that binds to IRE of the rat ANG gene promoter and inhibits ANG gene expression in IRPTCs.

We reported recently that by employing a combination of proteomics and Southwestern blotting, 46-kDa hnRNP F was identified as the 48-kDa nuclear protein that binds to IRE of the rat ANG gene promoter and inhibits ANG gene expression in IRPTCs (34).

The present studies aimed to identify the molecular structure of the 70-kDa nuclear protein with the same approach as with 46 kDa hnRNP F. It is apparent that multiple IRPTC proteins are resolved by 2-D electrophoresis. A second gel run simultaneously with the first gel for Southwestern blotting demonstrated 2 positive spots with an apparent MW of 70 kDa, and pI of 5.0 to 6.0 closely matched the stained protein spots on the first gel. After tryptic digestion and MALDI-MS, these 2 spots were found to match the partial

amino acid sequence deduced from the hnRNP K sequence as reported by Ito et al. (49) (Accession number NM_057141). Using specific rabbit antiserum against hnRNP K, we confirmed the identity of hnRNP K on the membrane by Southwestern blotting. Western blotting revealed positive signals superimposed on the positions of the 2 spots detected by Southwestern blotting. The reason why hnRNP K is present in 2 different forms (same apparent MW but different pI) is presently unclear. One possibility is that these proteins might be isoforms or variants with different phosphorylated forms. We have further confirmed that the 48-kDa species by MALDI-MS analysis is 46-kDa hnRNP F as we have reported previously (34, data not shown).

HnRNP K was identified as one of these hnRNPs that binds cytidine-rich elements (50-52). HnRNP K is encoded by 1 gene and can be alternatively spliced to at least 4 isoforms with deduced MW in the range of 50-51 kDa but in SDS-PAGE, hnRNP K has an apparent MW of 65 kDa (53). HnRNP K has been localized in the nucleus, cytoplasm and mitochondria, and implicated in chromatin remodeling, transcription, splicing and translation processes (see review by Bomsztyk et al. (54, 55)). HnRNP K binds single-stranded (ss) and double-stranded (ds) DNA motifs (CT element, 5 d-TCCC) within the promoter of c-myc, c-src and c-fos gene, and complexes with Sp1 and TATA-binding protein (TBP) to stimulate gene transcription (56-59). HnRNP K also represses the transcription of thymidine kinase (60), the neuronal nicotinic acetylcholine receptor β4 subunit (61), and osteocalcin (62). Thus, hnRNP K is a multifunctional protein that interacts with DNA, RNA,

transcriptional and translational molecules to alter the *in vivo* rate of gene transcription and translation (either stimulation or repression).

To demonstrate that hnRNP K interacts with rANG-IRE, we expressed hnRNP K in a bacterial system. Our GMSAs revealed that labeled rANG-IRE binds to GST-hnRNP K fusion protein and IRPTC nuclear proteins. The addition of unlabeled rANG-IRE DNA effectively displaced labeled ANG-IRE at or greater than a 100-fold molar excess of unlabeled DNA, whereas mutant rANG-IRE was not effective. Similarly, unlabeled hGAPDH-IRE, rat glucagon-IRE, and the Sp1 consensus sequence were not effective in displacing labeled rANG-IRE (data not shown). These studies indicate that rANG-IRE binds to hnRNP K. Most convincingly, anti-hnRNP K antibodies yielded a supershift of labeled rANG-IRE binding with hnRNP K fusion protein. Most convincingly, our ChIP assays revealed that hnRNP K interacts with rANG gene promoter loci. Taken together, these data unequivocally demonstrate that hnRNP K binds to rANG-IRE.

Interestingly, over-expression and down-expression of hnRNP K inhibits and enhances ANG gene expression in IRPTCs, respectively. To the best of our knowledge, this is the first report that hnRNP K could modulate ANG gene expression in kidney proximal tubular cells *in vitro*. The negative effect of hnRNP K is similar to that of hnRNP F on ANG gene expression (34). At present, the molecular mechanism(s) of hnRNP K action on ANG mRNA expression is not known. One possibility is that hnRNP K behaves like a negative transacting protein and inhibits the binding of other positive transacting factor(s) to TBP and RNA polymerase II, subsequently

attenuating ANG gene expression. This possibility is supported by the findings that the hnRNP K molecule binds directly to TBP and, therefore, may inhibit the basal transcription machinery (36, 51). The second possibility is that hnRNP K over-expression inhibits the formation of an activating transcriptional complex on the promoter and subsequently represses ANG gene expression. This possibility is supported by the studies of Du et al. (61) and Da Silva et al. (63), showing that hnRNP K inhibits Sp1 binding to the promoter of the gene encoding the β_4 subunit of the nicotinic acetylcholine receptor and CD 43 gene promoter, respectively. The third possibility is that hnRNP K could recruit unidentified repressor molecules and subsequently repress ANG gene expression. This possibility is supported by previous studies demonstrating that hnRNP K could bind the murine repressor Zik1 (64). Finally, it is also possible that hnRNP K could form an heterodimer with hnRNP F (a hypothetical stable complex) and then bind to rANG-IRE and subsequently attenuate ANG gene expression. This possibility is supported by the findings that hnRNP K could form an heterodimer or trimer with hnRNP A1/or hnRNP C to inhibit human thymidine kinase promoter (60). Clearly, more work is needed along these lines to elucidate the mechanism(s) of action of hnRNP K on ANG gene expression in IRPTCs.

Finally, our studies revealed that high glucose stimulated and insulin inhibited hnRNP K expression in IRPTCs *in vitro* and in diabetic rat kidneys *in vivo*. To the best of our knowledge, this is the first report that hnRNP K could be modulated by high glucose and insulin in kidney proximal tubular cells *in vitro* and *in vivo*. The exact physiological role(s) of hnRNP K on renal ANG

gene expression is unknown. Studies are ongoing in our laboratory along this line.

In summary, we have established, by a combination of Southwestern blotting and proteomics, that hnRNP K is a nuclear protein that binds to rANG-IRE and inhibits ANG gene expression in IRPTCs. It appears that high glucose and insulin regulate hnRNP K expression in kidney proximal tubular cells. Our studies raise the possibility that hnRNP K expression may play an important role in counter-balancing high glucose stimulation of ANG gene expression and in modulating local intrarenal RAS activation. Dysregulation of hnRNP K expression may contribute to renal injury in diabetes via altered local intrarenal RAS activation.

APPENDIX

Abbreviations used in this article: ACE, angiotensin-converting enzyme; ANG, angiotensinogen; Ang II, angiotensin II; CAT, chloramphenicol acetyl transferase; ChIP, chromatin immunoprecipitation; DN, diabetic nephropathy; ESRD, end-stage renal disease; GMSA, gel mobility shift assay; hnRNP F, heterogenous nuclear ribonucleoprotein F; hnRNP K, heterogenous nuclear ribonucleoprotein K; IP, immunoprecipitation; IEF, isoelectrofocusing; IRE, insulin-responsive element; IRE-BP, IRE-binding protein; IRPTCs, immortalized renal proximal tubular cells; kDa, kiloDalton; MALDI, matrix-assisted laser desorption/ionization technique; MS, mass spectrometry; MW, molecular weight; PCR, polymerase chain reaction; RAS, renin-angiotensin system; RPTCs, renal proximal tubular cells; RT-PCR, reverse transcriptase-polymerase chain reaction; RPTs, renal proximal tubules; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ss, single-stranded; siRNA, small interfering RNA; STZ, streptozotocin; 2-D, 2 dimensional; TBP, TATA-binding protein; TGF-β1, transforming growth factor-beta 1.

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LEGENDS

Figure 1: Rat ANG IRE-BPs detected by 2-D electrophoresis, Southwestern and Western blottings. (A) Rat IRPTC nuclear extracts were subjected to 2-D electrophoresis and then stained

with Coomassie Brilliant Blue R-250. M: Amersham-Pharmacia Biotech's rainbow molecular mass markers. N.E.: Rat IRPTC nuclear extracts without IEF. (B) Southwestern blotting analysis of ANG IRE-BPs from IRPTC nuclear proteins after 2-D electrophoresis. After 2-D electrophoresis, the nuclear proteins were transferred onto a Hybond C-extra membrane, hybridized with radioactively-labeled concanmeric ANG-IRE (N-878/N-864), washed, and subjected to autoradiography. The arrow heads indicate the proteins that were determined to be hnRNP K by later MS. The rectangle denotes the 70-kDa proteins that were subjected to MS, their identities were determined as 65-kDa hnRNP K. (C) Western blotting of the 2-D electrophoresis membrane from panel B using anti-hnRNP K antibody.

Figure 2: MS analysis of proteins detected by Southwestern blotting. Spot 1 was isolated from 2-D gel and then subjected to trypsin digestion and MALDI-MS analysis. Figure 2A shows a typical MALDI-MS peptide fingerprint of spot 1. Figure 2B shows the peptide sequence homology with hnRNP K identified by MALDI-MS and database search (BOLD letters). The sequence coverage of hnRNP K reached 32%. Similar results were obtained from spot 2 (not shown).

Figure 3: GMSA of radioactively-labeled rANG-IRE DNA fragment with GST-hnRNP K fusion protein(s). The labeled DNA probe (0.1 pmol) was incubated with GST (5 μg) or GST-hnRNP K fusion protein(s) (0.1 μg) in the presence of 1 μg of poly dI-dC. Competition with 100-fold molar excess of unlabeled rANG-IRE is shown in lane 4. Anti-hnRNPK antibody yielded a supershift band shown in lane 5.

Figure 4. ChIP analysis with anti-hnRNP K antibody. The cells were lysed, and nuclei were isolated and then sonicated. HnRNP K was immunoprecipitated with (+, lane 4) or without (-, lane 3) anti-hnRNP K antibody or in the presence of blocking peptide (lane 5). Complexes were eluted, cross-linking was reversed, and purified DNA was used as a template in PCR with primers specific to the rat ANG gene promoter. DNA was separated by agarose gel electrophoresis and visualized. Then, the DNA was transferred onto a Hybond XL nylon membrane and hybridized with a DIG-labeled internal DNA probe.

Figure 5: Effect of hnRNP K on ANG mRNA expression in IRPTCs. A. Western blotting of hnRNP K protein in IRPTCs transiently transfected with pRC/RSV or pRSV/hnRNP K. B. RT-PCR analysis of endogenous rANG and β-actin mRNA expression in

IRPTCs transiently tranfected with pRC/RSV or pRSV/hnRNP K. Twenty-four h after gene transfection, the cells were cultured for 24 h in 5 mM DMEM containing 5% FBS. Then, the cells were harvested and assayed for hnRNP K and rANG mRNA by Western blotting and RT-PCR assay, respectively. The relative levels of hnRNP K protein and ANG mRNA were compared with β-actin protein and β-actin mRNA, respectively. The hnRNP K and ANG mRNA level in pRC/RSV-transfected cells represents the control level (100%). The results were expressed as means ± SD of 3 determinations (**$p \leq 0.01$). Similar results were obtained in 3 other experiments.

Figure 6: Over-expression of hnRNP K prevented the high glucose effect on ANG mRNA expression in IRPTCs. RT-PCR analysis of endogenous rANG mRNA expression in IRPTCs stably tranfected with control plasmid pRC/RSV (A) or pRSV/hnRNP K (B). Twenty-four h after synchronization, the cells were cultured for 24 h in 5 mM DMEM plus 20 mM D-glucose, 25 mM D-glucose in the absence or presence or insulin (10^{-7} M) containing 1% depleted FBS. Then, the cells were harvested and assayed for rANG mRNA by RT-PCR. The relative levels of ANG mRNA were compared with β-actin mRNA. The ANG mRNA level in 5 mM D-glucose medium represents the control

level (100%). The results were expressed as means ± SD of 3 determinations (**p≤ 0.01, NS, not significant). Similar results were obtained in 3 other experiments.

Figure 7: Effect of siRNA of hnRNP F and hnRNP K on rANG mRNA expression in IRPTCs. 48 h after transfection, the cells were harvested and assayed for hnRNP F or hnRNP K protein and ANG mRNA expression by Western blotting and RT-PCR, respectively. The level of hnRNP F or hnRNP K and ANG mRNA expression was compared with β-actin protein and mRNA, respectively.

Figure 8: Effect of hnRNP K on rANG gene promoter activity in IRPTCs. 48 h after transfection, the cells were harvested and assayed for CAT activity. Relative activity in cells transfected with 1 μg of rANG N-1498/+18 was given a relative value of 100% (control). Each point represents the mean ± S.D. of 3 independent experiments (N.S., not significant; *p≤ 0.05; **p≤ 0.01).

Figure 9: Effect of high glucose and insulin on hnRNP K expression in IRPTCs analyzed by Southwestern and Western blotting. The cells were incubated in 5 mM plus 20 mM D-mannitol, 25 mM D-glucose or 25 mM D-glucose plus insulin (10^{-7} M) and 5% FBS

for 24 h. Then, the cells were harvested and nuclear proteins (100 µg) were electrophoresed on SDS-PAGE [(4-20% (W/V) gradient gel], transferred onto a polyvinylidene difluoride membrane, analyzed by Southwestern blotting with radioactive rANG-IRE probe (A) and then by Western blotting with rabbit polyclonal antibodies against hnRNP K (B) and ECL-chemiluminescent developing reagent. The same membrane was also hybridized with antibodies against β-actin control. The relative densities of the hnRNP K bands were compared with the β-actin control. The hnRNP K level expressed in IRPTCs in 5 mM glucose medium was considered to be the control (100%). Each bar represents the mean ± SD of 3 independent experiments (*$p \leq 0.05$, **$p \leq 0.01$).

Figure 10: HnRNP K expression in non-diabetic and diabetic rat RPTs. After 2 weeks of STZ-induced diabetes with or without insulin supplementation, RPTs were collected, extracted for proteins and assayed for hnRNP K and β-actin levels by Western blotting. The relative levels of hnRNP K were normalized with β-actin. HnRNP K levels in non-diabetic rats were considered as 100%. Each point represents the mean ± SD of 5 rat RPTs. ***, $p \leq 0.001$.

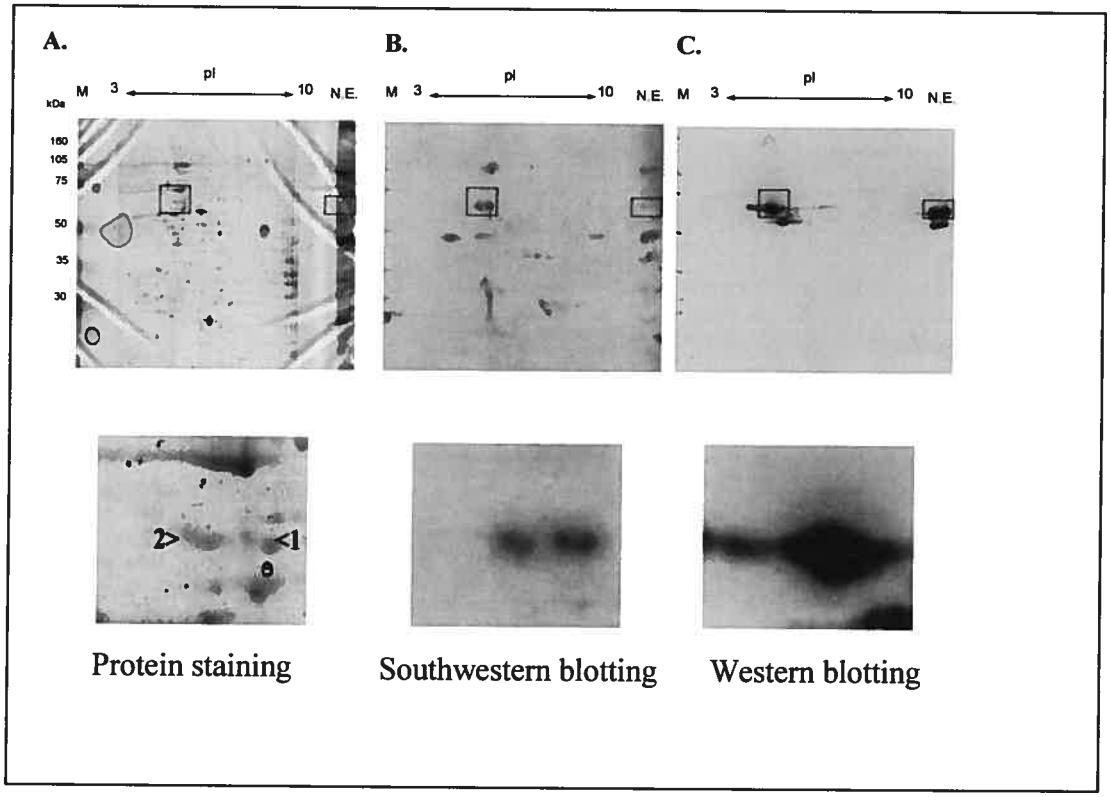
A.
B.
C.
M 3 pI 10 N.E.
kDa
180
105
75
50
35
30
2
1
Protein staining
Southwestern blotting
Western blotting

Figure 1

Matched peptides shown in **Bold Red**

```
  1 METEQPEETF PNTETNGEFG KRPAEDMEEE QAFKRSRNTD EMVELRILLQ
 51 SKNAGAVIGK GGKNIKALRT DYNASVSVPD SSGPERILSI SADIETIGEI
101 LKKIIPTLEE GLQLPSPTAT SQLPLESDAV ECLNYQHYKG SDFDCELRLL
151 IHQSLAGGII GVKGAKIKEL RENTQTTIKL FQECCPHSTD RVVLIGGKPD
201 RVVECIKIIL DLISESPIKG RAQPYDPNFY DETYDYGGFT MMFDDRRGRP
251 VGFPMRGRGG FDRMPPGRGG RPMPPSRRDY DDMSPRRGPP PPPPGRGGRG
301 GSRARNLPLP PPPPPRGGDL MAYDRRGRPG DRYDGMVGFS ADETWDSAID
351 TWSPSEWQMA YEPQGGSGYD YSYAGGRGSY GDLGGPIITT QVTIPKDLAG
401 SIIGKGGQRI KQIRHESGAS IKIDEPLEGS EDRIITITGT QDQIQNAQYL
451 LQNSVKQYSG KFF
```

Match to : Rat hnRNP K (NM_057141)

Nominal mass (Mr): 51230; Calculated pI value: 5.39

Sequence Coverage: 14%

Figure 2

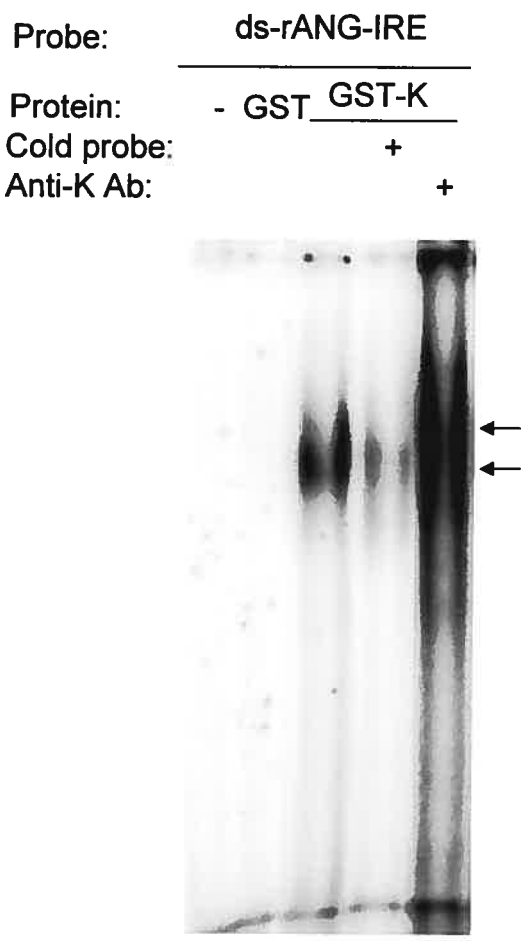

Figure 3

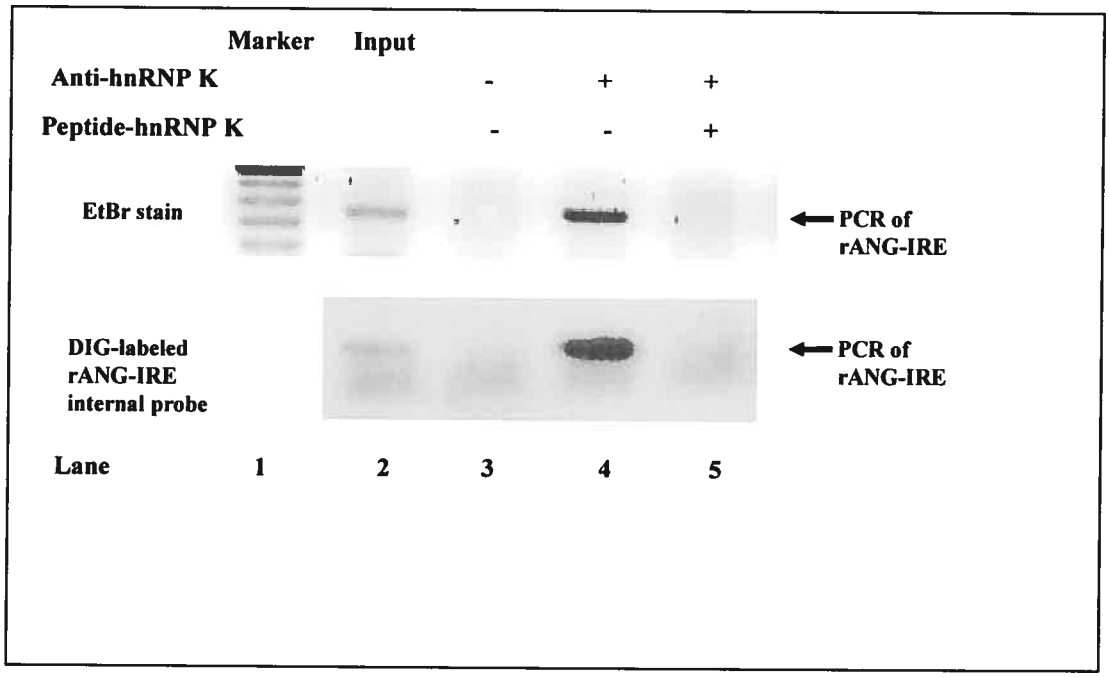
Marker
Input
Anti-hnRNP K
- + +
Peptide-hnRNP K
- - +
EtBr stain
PCR of rANG-IRE
DIG-labeled rANG-IRE internal probe
PCR of rANG-IRE
Lane
1 2 3 4 5

Figure 4

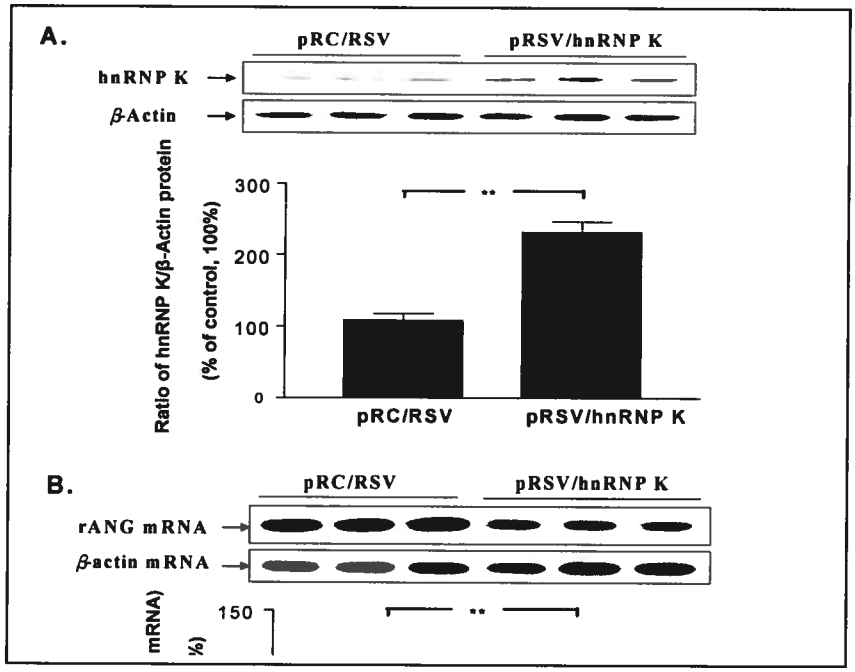
A.
pRC/RSV
pRSV/hnRNP K
hnRNP K
β-Actin
Ratio of hnRNP K/β-Actin protein
(% of control, 100%)
300
200
100
0
**
pRC/RSV
pRSV/hnRNP K
B.
pRC/RSV
pRSV/hnRNP K
rANG mRNA
β-actin mRNA
mRNA)
%)
150
**

Figure 5

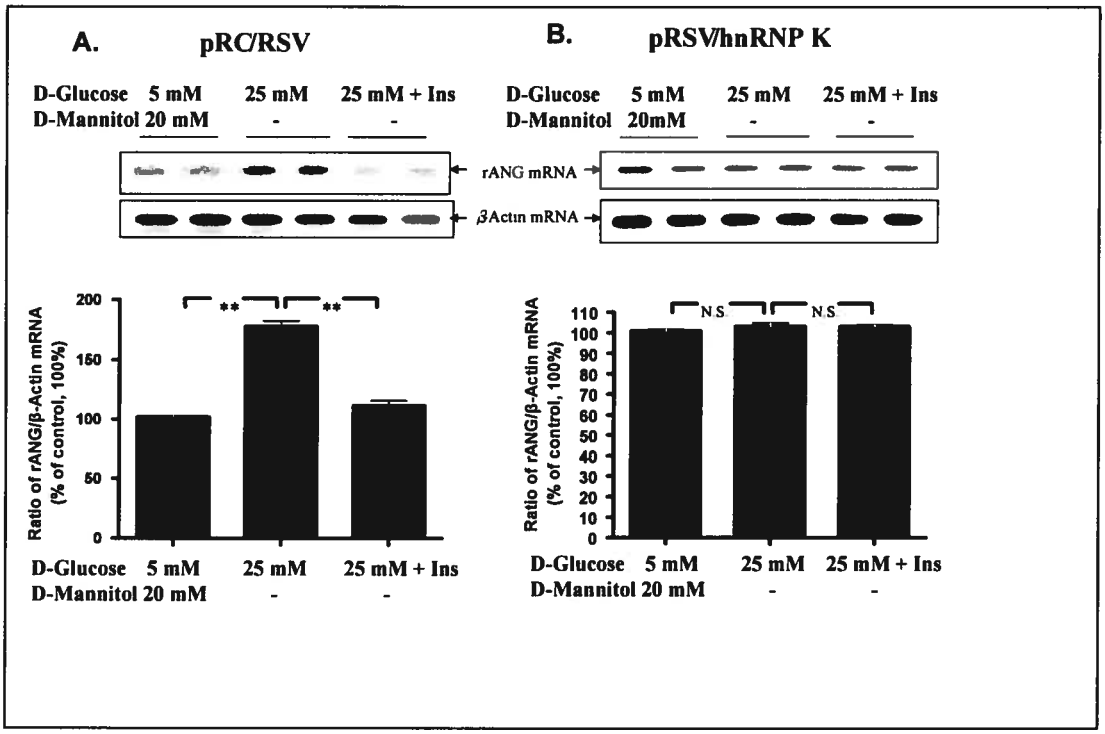
A.
pRC/RSV
D-Glucose 5 mM 25 mM 25 mM + Ins
D-Mannitol 20 mM - -
rANG mRNA
βActin mRNA
Ratio of rANG/β-Actin mRNA (% of control, 100%)
200
150
100
50
0
**
**
D-Glucose 5 mM 25 mM 25 mM + Ins
D-Mannitol 20 mM - -
B.
pRSV/hnRNP K
D-Glucose 5 mM 25 mM 25 mM + Ins
D-Mannitol 20mM - -
Ratio of rANG/β-Actin mRNA (% of control, 100%)
110
100
90
80
70
60
50
40
30
20
10
0
N.S.
N.S.
D-Glucose 5 mM 25 mM 25 mM + Ins
D-Mannitol 20 mM - -

Figure 6

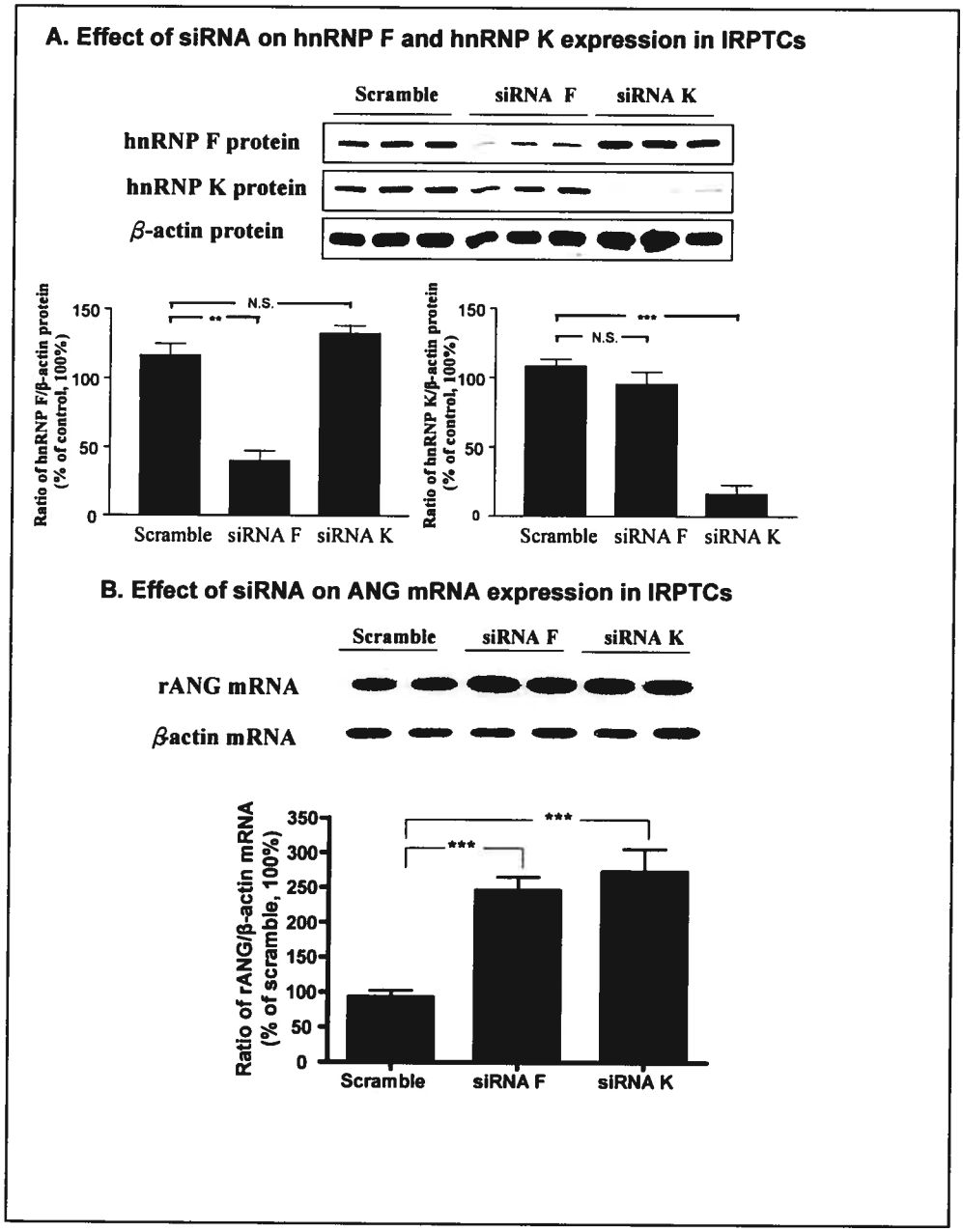
A. Effect of siRNA on hnRNP F and hnRNP K expression in IRPTCs
Scramble
siRNA F
siRNA K
hnRNP F protein
hnRNP K protein
β-actin protein
Ratio of hnRNP F/β-actin protein (% of control, 100%)
150
100
50
0
N.S.
**
Scramble
siRNA F
siRNA K
Ratio of hnRNP K/β-actin protein (% of control, 100%)
150
100
50
0
***
N.S.
Scramble
siRNA F
siRNA K
B. Effect of siRNA on ANG mRNA expression in IRPTCs
Scramble
siRNA F
siRNA K
rANG mRNA
βactin mRNA
Ratio of rANG/β-actin mRNA (% of scramble, 100%)
350
300
250
200
150
100
50
0
***
***
Scramble
siRNA F
siRNA K

Figure 7

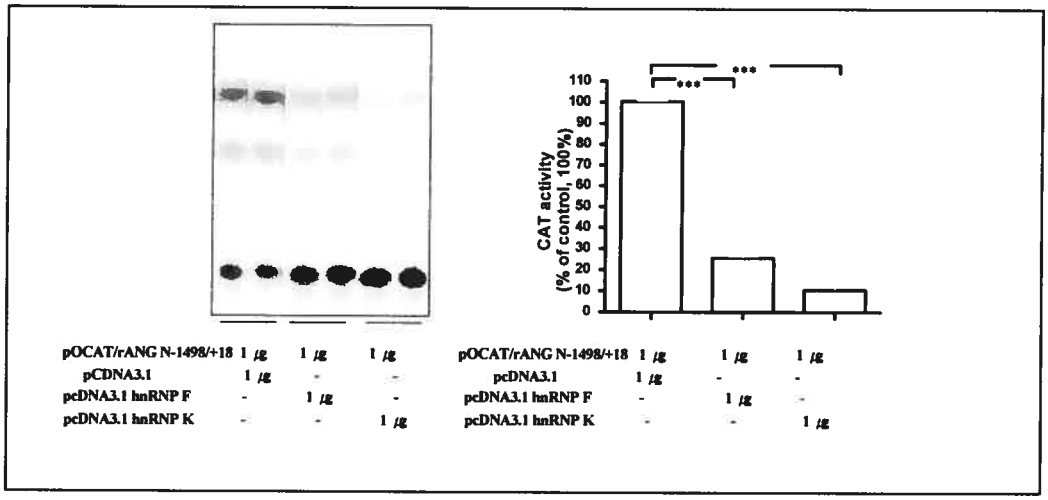
pOCAT/rANG N-1498/+18 1 µg 1 µg 1 µg
pCDNA3.1 1 µg - -
pcDNA3.1 hnRNP F - 1 µg -
pcDNA3.1 hnRNP K - - 1 µg
CAT activity (% of control, 100%)
110
100
90
80
70
60
50
40
30
20
10
0
***
***
pOCAT/rANG N-1498/+18 1 µg 1 µg 1 µg
pcDNA3.1 1 µg - -
pcDNA3.1 hnRNP F - 1 µg -
pcDNA3.1 hnRNP K - - 1 µg

Figure 8

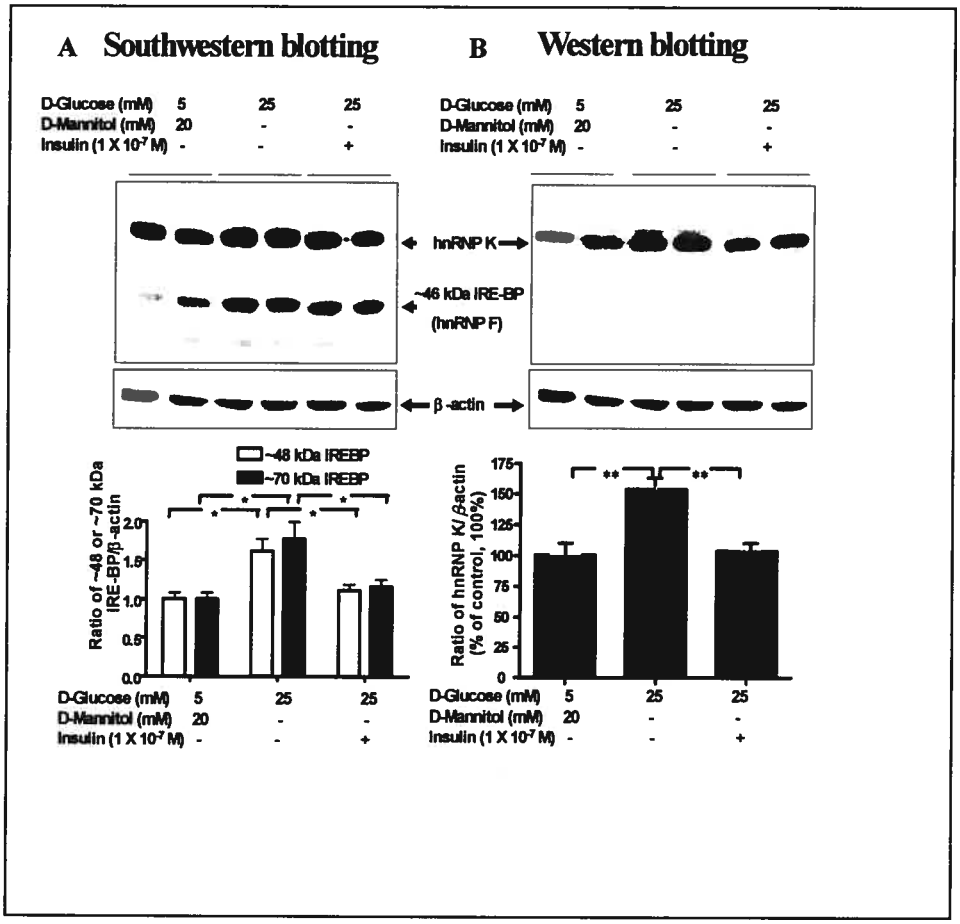
A Southwestern blotting
B Western blotting
D-Glucose (mM) 5 25 25
D-Mannitol (mM) 20 - -
Insulin (1 X 10-7 M) - - +
hnRNP K
~46 kDa IRE-BP
(hnRNP F)
β-actin
~48 kDa IREBP
~70 kDa IREBP
Ratio of ~48 or ~70 kDa IRE-BP/β-actin
Ratio of hnRNP K/ βactin (% of control, 100%)

Figure 9

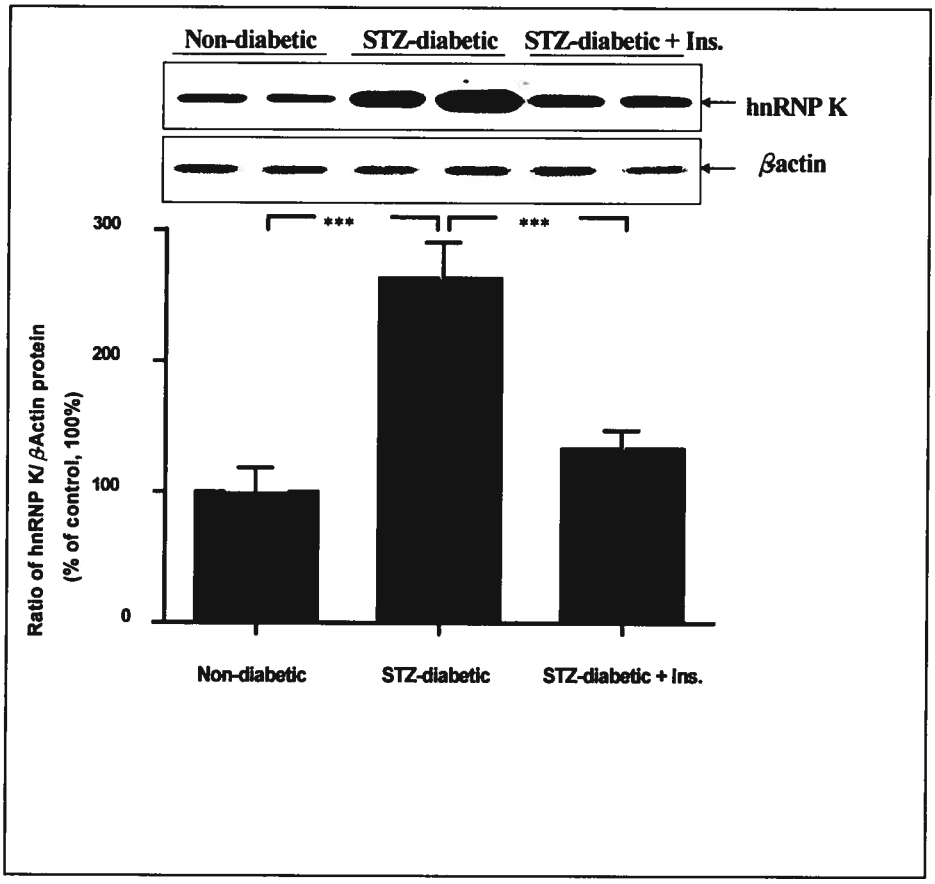
Non-diabetic
STZ-diabetic
STZ-diabetic + Ins.
hnRNP K
βactin
***
***
300
200
100
0
Ratio of hnRNP K/βActin protein
(% of control, 100%)
Non-diabetic
STZ-diabetic
STZ-diabetic + Ins.

Figure 10

Chapter 4 : Article 3

HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN F OVEREXPRESSION ATTENUATES ANGIOTENSINOGEN EXPRESSION, TUBULAR HYPERTROPHY AND TUBULOINTERSTITIAL FIBROSIS IN TRANSGENIC MICE

HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN F OVEREXPRESSION ATTENUATES ANGIOTENSINOGEN EXPRESSION, TUBULAR HYPERTROPHY AND TUBULOINTERSTITIAL FIBROSIS IN TRANSGENIC MICE

Manuscript in preparation

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(Relative contribution: Wei, 75%; Liu, 10%; Chenier, 4%; Guo, 2%; Zhang, 2%; Ingelfinger, 2%; Chan, 5%)

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Abstract

To investigate whether heterogenous nuclear ribonucleoprotein F (hnRNP F) could suppress angiotensinogen (AGT) gene expression and subsequently attenuate renal proximal tubular cell (RPTC) hypertrophy in a high-glucose milieu, rat immortalized RPTCs were stably transfected with control plasmid or plasmid containing hnRNP F cDNA and cultured in normal-glucose (5 mM D-glucose plus 20 mM D-mannitol) or high glucose (25 mM D-glucose) medium. HnRNP F overexpression prevented the high-glucose stimulation of AGT, transforming growth factor-beta1 (TGF-β1), and TGF-β1 receptor type II (TGF-β1 RII) mRNA expression in RPTCs as well as cellular hypertrophy (i.e. total cellular protein content, [^{3}H]-leucine incorporation and $p27^{kip1}$ protein expression) *in vitro*. Transgenic mice specifically overexpressing hnRNP F in their RPTCs displayed attenuation of AGT, TGF-β1 RII and collagen 1α (type IV) mRNA and protein expression with decreased protein/DNA ratio, cellular and nuclear volume, $p27^{kip1}$ protein expression after streptozotocin-induced diabetes. Our observations demonstrate that hnRNP F plays a negative modulatory role in preventing RPTC hypertrophy in diabetes, and its underlying mechanism is mediated, at least in part, via attenuation of intrarenal AGT and TGF-β1 RII expression *in vitro* and *in vivo.*

INTRODUCTION

Diabetic nephropathy (DN), a major cause of end-stage renal disease (ESRD), presently accounts for 30-50% of all new ESRD cases in North America (1-3). While intensive insulin therapy and chronic treatment with renin-angiotensin system (RAS) blockers retard the progression of DN, neither

of them cures or prevents it (4-8). However, the partial success of such therapies suggests that hyperglycemia and RAS activation are major risk factors for DN initiation and progression.

In addition to the circulating RAS, the existence of a local intrarenal RAS has been well-established (9). High glucose and angiotensin II (Ang II) may directly or indirectly induce renal proximal tubular cell (RPTC) hypertrophy and tubulointerstitial fibrosis (10-12). Our laboratory has established that high glucose (25 mM) stimulates rat angiotensinogen (rAGT, the sole substrate in the RAS) gene expression in rat immortalized RPTCs (13,14). RAS blockers and stable transfer of antisense rAGT cDNA prevent high-glucose stimulation of transforming growth factor-beta1 (TGF-β1) and fibrotic gene expression as well as RPTC hypertrophy (10,15). We recently reported that RAS blockers attenuate hypertension, proteinuria, and renal injury in transgenic (Tg) mice overexpressing rAGT in their RPTCs (4,8). Taken together, these data support a crucial role for intrarenal RAS activation in the progression of DN.

We have also demonstrated that insulin inhibits both high-glucose stimulation of rAGT gene expression and RPTC hypertrophy (16,17). A putative insulin-responsive element (IRE), identified in the rAGT gene promoter, modulates rAGT gene transcription through binding with 2 nuclear proteins, heterogenous nuclear ribonucleoprotein F (hnRNP F) and hnRNP K (18-20). The physiological roles of hnRNP F and K in RPTCs *in vivo*, however, remain undefined.

The hnRNPs are a family of more than 20 different proteins that share common structural domains, and extensive research has shown that they play

central roles not only in mRNA processing, metabolism and transport but also in DNA repair, telomere biogenesis, cell signaling and the regulation of gene expression at both the transcriptional and translational levels (21). Through these key cellular functions, individual hnRNPs have a variety of potential roles in disease development and progression.

In the present studies, we investigated whether hnRNP F overexpression could inhibit AGT gene expression and attenuate RPTC hypertrophy in high glucose both *in vitro* and *in vivo*. Our studies revealed that hnRNP F overexpression indeed attenuates AGT and fibrotic gene expression, RPTC hypertrophy and tubulointerstitial fibrosis under these conditions.

MATERIALS AND METHODS

Reagents

D(+)-glucose, D-mannitol, and monoclonal antibodies against β-actin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Full-length hnRNP F cDNA was cloned in our (JSDC's) laboratory (19) and inserted into pEGFP C1 plasmid (Invitrogen, Inc., Burlington, ON, Canada). Rabbit polyclonal antibodies specific for hnRNP F (CTARRYIGIVKQAGLER corresponding to amino acids 215 to 230 of human hnRNP F) (19) and polyclonal antibodies against rat AGT were generated in our laboratory (22). Anti-$p27^{kip1}$ antibody and anti-green fluorescence protein (GFP) antibody were obtained from BD Biosciences (Mississauga, ON, Canada) and Zymed (South San Francisco, CA, USA), respectively. Polyclonal anti-TGF-β1 receptor II (TGF-β1 RII) antibody and monoclonal anti-collagen 1α (type IV) antibody

were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Chemicon International, Inc. (Temecula, CA, USA), respectively. The plasmid pKAP2 containing the kidney-specific androgen-regulated protein (KAP) promoter has been described elsewhere (23). It was a gift from Dr. Curt D. Sigmund (University of Iowa, Iowa, IA, USA). Placebo pellets or pellets containing 5 mg testosterone with a 21-day release schedule (Cat. #A-121) were purchased from Innovative Research of America (Sarasota, FL, USA). Oligonucleotides were synthesized by Invitrogen, Inc. Restriction and modifying enzymes were from either Invitrogen, Inc., La Roche Biochemicals (Laval, QC, Canada), or Amersham-Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Cell Culture and Gene Transfection

Rat immortalized RPTCs were cultured as described previously (19,20). RPTCs from passage 11 were used to establish stable transfectants. The plasmids pEGFP C1 and pEGFP C1/hnRNP F were stably transfected into RPTCs with Lipofectamine reagents, selected in the presence of geneticin (G418, 500 μg/ml), (Invitrogen, Inc.) as described previously (10), and observed under an ECLIPSE TE2000 fluorescence microscope (Nikon, Melville, NY, USA).

Western Blotting

Western blotting was performed as described previously (19,20). The membrane was first blotted with rabbit anti-GFP (1:1,000) or $p27^{kip1}$ (1:2,000),

then re-blotted with anti-β-actin antibodies and exposed to HRP color or chemiluminescent developing reagent (La Roche Biochemicals).

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR assays were performed as described previously (24). Briefly, cDNAs were synthesized from 2 μg of total RNA isolated from mouse RPTs following the manufacturer's protocol (Invitrogen, Inc.). Aliquots of cDNA (1/50 of reverse transcription reactions) were amplified in a MiniOpticon^{TM} MJ mini RT-PCR Detector System with iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in triplicate in 20-μl reaction volumes. The primers used in the RT-qPCR assays are described in Table 1.

Cellular Hypertrophy

RPTC hypertrophy was quantified by total cellular protein content, [^{3}H]-leucine incorporation, and cellular p27^{kip1} expression as described previously (17).

Generation of hnRNP F-Tg Mice

Tg mice overexpressing hnRNP F in their RPTCs were generated as described previously for Tg mice overexpressing rAGT or catalase in RPTCs (23,24). Briefly, full-length hnRNP F cDNA fused with HA-tag (which encodes amino acid residues 98-106 (YPYDVPDYA) of human influenza virus hemagglutinin) at the carboxyl terminal and *NotI* restriction enzyme site at both 5- and 3-termini was inserted into pKAP2 plasmid at the *NotI* site. The

isolated 17-kb KAP2-hnRNP F transgene (digested with *Nde*I and *Spe*I) was then microinjected into 1-cell fertilized mouse embryos obtained from superovulated C57Bl6 × C3H mice (performed at the Clinical Research Institutes of Montreal, Montreal, QC, Canada). Positive Tg founders (identified by mouse tail genomic DNA) were then crossed with wild-type (WT) C57Bl6 mice (Charles River, St-Constant, QC, Canada) to obtain the F_1 generation and bred until homozygous F_4 mice were produced.

The mice were 17-19 weeks of age at the time of data collection. Non-Tg and sex-matched littermates (WT) served as controls. All animals received standard mouse chow and water *ad libitum*. Their care met the standards of the Canadian Council on Animal Care and the Animal Care Committee of the CHUM.

Induction of Transgene Expression

Placebo pellets or pellets containing 5 mg testosterone with a 21-day release schedule were implanted in male and female homozygous Tg mice which were euthanized 2 weeks later. Tissues were harvested and snap-frozen on dry ice. Total RNA was isolated with TRIzol (Invitrogen, Inc.), followed by DNase-I treatment (Invitrogen, Inc.) prior to conventional RT-PCR (23,24). Table 1, lists the primers used.

Induction of Diabetes

Male WT and Tg mice (11-13 weeks old) were divided into 2 groups (6 mice per group): 1. Vehicle-injected controls; and 2. Streptozotocin

(STZ)-induced diabetic animals injected i.p. after 6 hours of fasting with 50 mg/kg of STZ dissolved in 10 mM sodium citrate buffer (pH 4.0). STZ injections were repeated once daily for 5 consecutive days. Forty-eight h after the last injection, blood glucose levels were determined with a Side-Kick Glucose Analyzer (Model 1500, Interscience, Markham, ON, Canada). Only mice with blood glucose levels >20 mM were studied. After 4 weeks of diabetes, the animals were euthanized. Twenty-four hours prior to euthanasia with CO_2 at 4 weeks post-STZ administration, they were housed individually in metabolic cages to collect urine samples later assayed for glucose and ketone levels (Keto-Diastix, Bayer Inc., Healthcare Division, Toronto, ON, Canada), and albuminuria (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA) (23,25). Body weight and blood glucose were measured. Serum creatinine was determined by Beckman Creatinine Analyzer II as described by Ichimura et al. (26). Left kidneys were processed for histology, and the right kidneys were harvested for isolation of renal proximal tubules (RPTs) by Percoll gradient (23,24,27). Aliquots of freshly-isolated RPTs from individual animals were used immediately for quantitation of the protein/DNA ratio, Western blotting and total RNA isolation.

Protein/DNA Ratio in mRPTs

DNA was quantified in quadruplicate (28,29). Briefly, mRPTs were homogenized in 3 ml cold PBS, and aliquots (100 µl) were distributed in 96-well plates at 37°C. Then, 10 µl of 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (La Roche Biochemicals) was added to each well. After incubation at

room temperature for 30 min, DNA concentration was read at λ360 nm excitation and λ460 nm emission (CytoFluor II spectrofluorometer, PerSeptive Biosystems, Cambridge, MA, USA. Protein concentration was measured in quadruplicate in the same samples by *DC* protein assay (Bio-Rad Laboratories). Once protein (μg)/DNA ratios were calculated, the data were presented as percentages of increase in comparison to WT controls (100%).

RPTC and Nuclear Volumes

Kidneys were collected in Tissue-Tek cassettes (VWR Canlab, Montreal, QC, Canada), dipped immediately in ice-cold 4% paraformaldehyde and fixed for 24 h at 4°C. The cassettes were processed by the CHUM Pathology Department. Tissue sections were counterstained with hematoxylin and eosin (H/E). Four to 5 tissue sections per kidney from 6 animals per group were analyzed by light microscopy by 2 investigators blinded to treatment. RPTC and nuclear volumes were measured by image analysis software (Motic Image Plus 2.0, Motic Instruments Inc., Richmond, BC, Canada). Outer cortical RPTs with similar cross-sectional views (equal diameter and clear nuclear structure) were selected.

Mean cell volume was estimated by the Nucleator method (30,31), an unbiased stereological technique estimating the volume of 3-dimensional structures on 2-dimensional sections. Briefly, a cell profile was sampled if its nucleus was visible and if typical nuclear size and the cell centre were 'hit'. The length of a random cell (l^+ and l^-) through the hit point was ascertained by image analysis software. The measurement was repeated 3 times in different

directions (l_1^+, l_1^-, l_2^+, l_2^-, l_3^+ and l_3^-) per cell. The mean intercept of each direction was assessed by the equation $l = ½ (l^+ + l^-)$. Mean intercept length in the third power per cell (l_O^3) was then determined by the equation $l_O^3 = 1/3 (l_1^3 + l_2^3 + l_3^3)$. Mean cell volume ($V_O$) (100 cells were counted per animal) was quantified by the equation $V_O = 4/3 \pi l_O^3$.

Mean nuclear volume (V_N) was estimated (200 nuclei were counted per animal) by the Selector method (28, 29) according to the equation $V_N = \pi/3 \times l_O^3$.

Immunohistochemistry

Immunostaining was performed on 3-µm renal sections by the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnology) (23,24) with either polyclonal anti-hnRNP F (C1004, 1:100 dilution) (19), rAGT (R-16, 1:100 dilution) (23), TGF-β1 RII (1:100), or control rabbit pre-immune serum (1:100 dilution). All sections were counterstained with hematoxylin, dehydrated, and covered with glass slips.

Statistical Analysis

Statistical significance between the experimental groups was analyzed initially by Student's t-test or 1-way ANOVA (analysis of variance) and the Bonferroni test as appropriate. The data are expressed as means ± SD. $p<0.05$ values were considered to be statistically significant.

RESULTS

HnRNP F Overexpression Prevents High Glucose-induced AGT, TGF-β1 and TGF-β1 RII mRNA Expression in RPTCs *in vitro*

To study the effect of hnRNP F on AGT mRNA expression in RPTCs, we stably transfected immortalized rat RPTCs, which express all RAS components (32), with rat hnRNP F cDNA fused to GFP as a tag. GFP-hnRNP F stable clones are shown in **Figure 1A**. In pEGFP C1 empty vector (GFP-EV) stable transfectants, fluorescence was predominantly cytoplasmic, but was exclusively nuclear in GFP-hnRNP F transfectants. **Figure 1B** displays the Western blotting of GFP-EV and GFP-hnRNP F stable transfectants. GFP-hnRNP F fusion protein was expressed as predicted at 72 kDa (the apparent molecular weights of GFP and hnRNP F were 26 and 46 kDa, respectively). Thus, hnRNP F is a nuclear protein. Stable GFP-EV and GFP-hnRNP F transfectants were used in subsequent studies.

To examine the effect of hnRNP F on AGT, TGF-β1 and TGF-β1 RII gene expression, we quantified AGT, TGF-β1 and TGF-β1 RII mRNA expression in GFP-EV and GFP-hnRNP F stable transfectants in normal- and high-glucose medium. After 24 h of high-glucose stimulation, AGT mRNA (**Figure 2A**), TGF-β1 mRNA (**Figure 2B**) and TGF-β1 RII mRNA (**Figure 2C**) were significantly increased (>2-fold) in GFP-EV stable transfectants as compared to normal glucose. HnRNP F overexpression abolished the high-glucose effect on AGT, TGF-β1 and TGF-β1 RII mRNA expression in GFP-hnRNP F stable transfectants.

HnRNP F Overexpression Prevents High Glucose-induced RPTC

Hypertrophy

To demonstrate whether hnRNP F overexpression could prevent RPTC hypertrophy in high glucose via suppression of AGT expression, we determined the cellular hypertrophy index (i.e., total protein content, [^{3}H]-leucine incorporation, and $p27^{kip1}$ protein expression) in stable transfectants cultured in normal- or high-glucose medium. High glucose induced a significant increase in total cellular protein content (**Figure 3A**), [^{3}H]-leucine incorporation (**Figure 3B**) and cellular $p27^{kip1}$ expression (**Figure 3C**) in GFP-EV stable transfectans. HnRNP F overexpression in GFP-hnRNP F stable transfectants prevented the stimulatory effect of high glucose.

Generation of Kidney-specific hnRNP F-Tg Mice

To confirm our *in vitro* data, hnRNP F-Tg mice were generated to produce specific and inducible hnRNP F expression in RPTCs by inserting hnRNP F cDNA into a construct containing the KAP promoter and non-coding DNA, including exons 3 to 5 of human AGT gene (**Figure 4A**). Founders were screened by Southern blotting, whereas F_1, F_2, and F_3 mice were screened by PCR for the hnRNP F-HA transgene (**Figure 4B**). Tissue-specific expression of the hnRNP F-HA transgene was studied under 2-week testosterone implantation in Tg mice. Testosterone pellets (but not placebo) induced hnRNP F-HA transgene expression in the kidneys of female Tg mice (Line 937) (**Figure 4C**). The hnRNP F-HA transgene was expressed in the kidneys of male Tg mice, and exogenous testosterone administration further enhanced its expression in the kidneys but not in other tissues (**Figure 4C**). Renal sections

from male WT and Tg mice were immunostained with anti-hnRNP F antibody. HnRNP F protein expression was significantly higher in RPTs from Tg male mice compared to male WT control littermates (**Figure 4D**).

Taken together, these data demonstrate that the KAP2 promoter directs rat hnRNP F transgene expression in RPTCs and is testosterone-inducible. In subsequent experiments, we used male Tg mice without exogenous testosterone induction since endogenous testosterone was efficient in stimulating transgene expression in RPTCs.

HnRNP F Overexpression Attenuates Protein/DNA Ratios and $p27^{Kip1}$ Expression in RPTs of Diabetic Tg mRPTs

To ascertain the functional role of hnRNP F *in vivo*, diabetes was induced as described in Materials and Methods. Four groups of mice were compared: non-diabetic WT, STZ-induced diabetic WT, non-diabetic Tg, and STZ-induced diabetic Tg. The physical parameters are shown in Table II. Both diabetic WT and hnRNP F-Tg mice exhibited marked hyperglycemia on the day they were sacrificed as well as higher kidney weight/body weight ratio as compared to their non-diabetic counterparts. There were no significant differences in blood glucose, kidney weight/body weight ratio or serum creatinine between non-diabetic WT and non-diabetic hnRNP F-Tg mice as well as between diabetic WT and diabetic hnRNP F-Tg mice. An increase in the albumin/creatinine ratio (ACR) was observed in non-diabetic hnRNP F-Tg mice as compared to non-diabetic WT mice (Table II), but did not reach statistical significance.

Tubular hypertrophy is a well-known characteristic in diabetes induced by STZ (30,33). We quantified biochemical markers of tubular hypertrophy: protein/DNA ratio, cellular and nuclear volume, and $p27^{Kip1}$ expression, in RPTCs of WT and hnRNP F-Tg mice (31). The protein/DNA ratio (**Figure 5A**), cellular volume (**Figure 5B**) and nuclear volume (**Figure 5C**) were significantly higher in RPTCs of diabetic WT and diabetic hnRNP F-Tg mice compared to non-diabetic mice. Similarly, $p27^{Kip1}$ protein expression was significantly stimulated in RPTs of diabetic WT mice compared to non-diabetic WT mice (**Figure 5D**). However, the increased protein/DNA ratio (**Figure 5A**), cellular volume (**Figure 5B**), nuclear volume (**Figure 5C**), and heightened $p27^{Kip1}$ expression (**Figure 5D**) were significantly attenuated in RPTs of diabetic hnRNP F-Tg mice as compared to non-diabetic hnRNP F-Tg mice. Most importantly, the percent increase of these parameters in RPTCs of diabetic hnRNP F-Tg mice was significantly lower in comparison to diabetic WT mice.

HnRNP F Overexpression Attenuates AGT, TGF-β1 RII and Collagen 1α (type IV) Protein Expression in RPTs of Diabetic Tg mice

Since we observed that hnRNP F overexpression inhibits rat AGT mRNA expression in GFP-hnRNP F stable transfectants *in vitro*, we investigated whether hnRNP F overexpression downregulates AGT expression *in vivo*. STZ-induced diabetes increased AGT expression in RPTs of both WT (**Figure 6A, b**) and diabetic hnRNP F-Tg mice (**Figure 6A, d**) as compared to non-diabetic animals (**Figure 6A, a and c**). The increment of AGT expression in RPTs of diabetic hnRNP-Tg mice (**Figure 6A, d**), however, was significantly

attenuated in comparison to diabetic non-Tg mice (**Figure 6A, b**). Semi-quantitative analysis revealed significantly less AGT-positive, stained tubules in diabetic hnRNP F-Tg mice than in diabetic WT mice (**Figure 6B).**

It was apparent that STZ-induced diabetes increased TGF-β1 RII expression in RPTs of both diabetic WT (**Figure 7A**, **b**) and hnRNP F-Tg mice (**Figure 7A, d**) compared to their non-diabetic counterparts (**Figure 7A, a and c**). Again, the increment of TGF-β1 RII expression in RPTs of diabetic hnRNP-Tg mice (**Figure 7A, d**) was significantly attenuated in comparison to diabetic non-Tg mice (**Figure 7A, b**). Semi-quantitative analysis after 4 weeks of diabetes also revealed significantly less TGF-β1 RII-positive, stained tubules in diabetic hnRNP F-Tg mice than in diabetic WT mice (**Figure 7B).** The experiments confirmed that hnRNP F overexpression attenuatesdrenal AGT and TGF-β1 RII expression in diabetes.

HnRNP F Overexpression Attenuates Fibrotic Gene Expression in RPTs of Diabetic Tg mice

AGT mRNA (**Figure 8A**), TGF-β1 RII mRNA (**Figure 8B**), fibronectin mRNA (**Figure 8C**) and collagen α1 (type IV) mRNA (**Figure 8D**) were significantly elevated (2- to 6-fold, $p<0.005$) in RPTs of diabetic WT compared to non-diabetic WT mice. However, the heightened expression of these fibrotic genes was significantly attenuated in RPTs of diabetic hnRNP F-Tg mice.

DISCUSSION

This report demonstrates that hnRNP F overexpression in RPTCs

attenuates the high-glucose stimulation of AGT and TGF-β1 RII expression, cellular hypertrophy and fibrosis, both *in vitro* and *in vivo*, suggesting that hnRNP F expression suppresses RPTC hypertrophy and tubulointerstitial fibrosis in diabetes by dampening AGT gene expression and intrarenal RAS activation.

HnRNPs are pre-mRNA-binding proteins involved in mRNA processing, and approximately 20 of them have been identified (34,35). Accumulating evidence indicates that hnRNP F may regulate gene expression at both the transcriptional and post-transcriptional levels. Indeed, reports suggest that hnRNP F engages in alternative splicing of c-src (36), β-tropomyosin gene (37), thyroid hormone receptor gene (38), and Bcl-x gene (39), as well as in the 3 -end processing of pre-mRNA in B-cell differentiation (40). HnRNP F associates with TATA-binding protein (TBP), RNA polymerase II (Poly II), and even with nuclear cap-binding protein complex (41,42). The molecular mechanism of hnRNP F action on gene transcription, however, is not well-defined.

We previously reported that hnRNP F binds to the IRE of rat AGT gene promoter and inhibits AGT promoter transcriptional activity (19). We also demonstrated that hnRNP F interacts with hnRNP K (another nuclear protein that binds to the same IRE in rat AGT gene promoter) and further inhibits AGT expression in RPTCs in vitro (20). More recently, Chen et al. (43) observed that hnRNP F interacts with the osmoregulatory transcription factor, TonEBP/OREBP, and modulates Hsp90 and PARP-1 gene expression. These findings indicate that hnRNP F may act alone or interact with other

transcriptional factors to modulate specific gene transcription.

The present evidence suggests that the intrarenal RAS plays a key role in hyperglycemia-induced cell hypertrophy via the activation of TGF-β1 signaling (10,44). Our studies in stable transfectants overexpressing hnRNP F indicate that hnRNP F is localized predominantly in the nucleus. HnRNP F overexpression in stable transfectants prevents the high-glucose stimulation of AGT, TGF-β1 and TGF-β1 RII mRNA expression, and circumvents high glucose-induced cellular hypertrophy, as determined by total protein content, [^{3}H]-leucine incorporation, and $p27^{kip1}$ protein expression in RPTCs. These data are consistent with our previous observations that hnRNP F suppresses AGT gene transcription in RPTCs (19) and decreases AGT gene expression preventing cellular hypertrophy in high glucose (10).

In Tg mice overexpressing hnRNP F in their RPTCs, we showed that hnRNP F overexpression fails to prevent the increase in blood glucose and kidney/body weight ratio in diabetic Tg mice (N.B.: The kidney/body weight ratio in hnRNP F-Tg mice tends to decrease as compared to non-Tg controls but the change is not statistically significant). However, hnRNP F overexpression clearly prevented attenuation of the protein/DNA ratio, cellular and nuclear volume, and $p27^{kip1}$ protein expression in RPTCs of diabetic hnRNP F-Tg mice as compared to diabetic non-Tg controls. Moreover, the expression of AGT, TGF-β1 RII, fibronectin and collagen α1 (type IV) mRNA was also significantly reduced in RPTs of diabetic Tg-hnRNP F mice as compared to diabetic non-Tg controls.

The exact reason why hnRNP F-Tg mice had a higher ACR (although not

statistically significant) as compared to WT was not clear in the present studies. Urinary albumin is considered to be the unbalance between glomerular filtration rate and tubular reabsorption (45). It raises the possibility the hnRNP F overexpression may affect the expression or function of some genes associated with tubular reabsorption of albumin, such as megalin and cubilin (46,47). Clearly, more studies along these lines are required.

The present report provides novel observations that hnRNP F overexpression can prevent high glucose-induced RPTC hypertrophy both *in vitro* and *in vivo*. The molecular mechanism(s) of hnRNP F action on AGT gene expression remain undefined. One possibility is that hnRNP F behaves as a negative transacting factor and competes with other positive transacting factor(s) (i.e., cAMP-response element-binding protein (CREB) and activating transcription factor-1 (ATF-1)) for binding to TBP and RNA Poly II, subsequently attenuating AGT gene expression. This possibility is supported by our previous report that CREB and ATF-1 expression is enhanced in RPTCs in high glucose (13), and CREB overexpression augments AGT gene transcription (48). A second possibility is that hnRNP F overexpression may exhaust the availability of nuclear CAP-binding proteins for capping pre-mRNAs, which might then attenuate the formation of mature AGT mRNA in the cytoplasm. Finally, it is also possible that hnRNP F could form a heterodimer with hnRNP K that effectively binds with other positive transcriptional factors to prevent their interaction with the initiation complex.

The physiological importance of attenuation of RPTC hypertrophy in diabetes by hnRNP F overexpression remains to be elucidated. Since studies

have reported that targeting disruption of the $p27^{Kip1}$ gene attenuates kidney hypertrophy and progression of nephropathy in STZ-induced diabetic mice (49,50), we propose that RPTC hypertrophy may be an initial mechanism leading to nephropathy in diabetes.

In summary, we have demonstrated that hnRNP F overexpression suppresses AGT gene expression and subsequently attenuates high glucose-induced cellular hypertrophy in vitro and in vivo, implying that dysregulation of hnRNP F expression may contribute to renal injury in diabetes via altered local intrarenal RAS activation.

APPENDIX

Abbreviations used in this article: ACR, albumin-creatinine ratio; AGT, angiotensinogen; ATF-1, activating transcription factor-1; CREB, cAMP-response element-binding protein; DAPI, 4 ,6-diamidino-2-phenylindole; DN, diabetic nephropathy; ESRD, end-stage renal disease; GFP, green fluorescent protein; GFP-EV, pEGFP C1 empty vector; hnRNP F, heterogenous nuclear ribonucleoprotein F; IRE, insulin-responsive element; KAP, kidney-specific androgen-regulated protein promoter; Poly II, RNA polymerase II; RAS, renin-angiotensin system; RPTCs, renal proximal tubular cells; RPTs, renal proximal tubules; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, real time-quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STZ, streptozotocin; TBP, TATA-binding protein; Tg, transgenic mice; TGF-β1, transforming growth factor-beta1; TGF-β1 RII, TGF-β1 receptor II; WT, wild

type.

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Table I. Primer sequences

Table II. Physical parameters of diabetic mice

LEGENDS

Figure 1. Generation of rat hnRNP F stable transfectants. RPTCs were stably transfected with plasmid DNA, pEGFP C1 empty vector (GFP-EV) or pEGFP-hnRNP F (GFP-hnRNP F). Nuclei were shown by DAPI staining. GFP fusion protein expressions of G418-resistant transfectants were identified by fluorescence microscopy, magnification x 600. (A) and Western blotting (B) as described in Materials and Methods.

Figure 2. Expression of AGT, TGF-β1 and TGF-β1 RII mRNA in stable transfectants. Real time-quantitative PCR (RT-qPCR) analysis of rAGT mRNA (A), TGF-β1 mRNA (B) and TGF-β1 RII mRNA (C) expression in stable transfectants. Cells were incubated with 5 mM D-glucose plus 20 mM D-mannitol or 25 mM D-glucose for 24 h. Then, they were harvested and assayed for gene expression by RT-qPCR as described in Materials and Methods. (*$p<0.05$; **$p<0.01$; ***$p<0.005$; n.s., non-significant).

Figure 3. Cellular hypertrophy in stable transfectants. (A) Total cellular protein content per million cells, (B) [^{3}H]-leucine incorporation, and (C) $p27^{kip1}$ protein expression in RPTCs stably transfected with pEGFP-EV or pEGFP-hnRNP F. The cells were incubated with 5 mM D-glucose plus 20 mM D-mannitol or 25 mM D-glucose for 24 h. (**$p<0.01$; ***$p<0.005$; n.s.., non-significant).

Figure 4. Generation of Tg mice. (A) Schematic map of the kidney androgen-regulated promoter (KAP2)-rat hnRNP F construct. (B) Southern blotting of genomic DNA for founders with radioactive hnRNP F probe. Heterozygous and homozygous F_1, F_2 and F_3 were screened by PCR with specific primers (Table I). (C) RT-PCR product showing tissue expression of hnRNP F-HA mRNA in female and male Tg mice un-induced or induced with testosterone. β-actin and hnRNP F-HA fragments are indicated. Transgenic (line #937) mice were induced with placebo or testosterone for 2 weeks prior to RNA isolation. Br, brain; Hr, heart; Lu, lung; Li, liver; Ki, kidney; Sp, spleen; Ts, testis. (D) Immunohistochemistry of hnRNP F in kidneys of male WT and Tg mice at 20 weeks (magnification, x 200 and 600).

Figure 5. RPTC protein/DNA ratio, cellular and nuclear volume, and $p27^{kip1}$ protein expression in mouse kidneys. RPTs were isolated, extracted and assayed as described in Materials and Methods. RPT protein/DNA ratio (A), cellular volume (B), nuclear volume (C) and $p27^{kip1}$ protein expression (D) are shown in RPTs of diabetic and non-diabetes WT and hnRNP F-Tg mice. The relative densities of the $p27^{Kip1}$ band (D) were normalized with the β-actin band. The protein/DNA ratio, cellular and nuclear volume, or $p27^{Kip1}$ level in non-diabetic WT mice RPTs represents the control level (100%) (*p<0.05; **p<0.01; ***p<0.005; n.s., non-significant).

Figure 6. AGT expression in mice kidneys. (A) Immunohistochemistry of AGT protein expression in kidneys from non-diabetic WT mice (a), STZ-induced diabetic WT mice (b), non-diabetic Tg mice (c), and STZ-induced diabetic Tg mice (d). Magnification x 600. (B) Semi-quantitative analysis of AGT-positive tubules from kidneys of non-diabetic and diabetic WT and hnRNP F-Tg mice (N=6, **p<0.01; ***p<0.005; n.s., non-significant). .

Figure 7. TGF-β1 RII expression in mice kidneys. (A) Immunohistochemistry of TGF-β1 RII protein expression in kidneys from non-diabetic WT mice (a), STZ-induced diabetic WT mice (b), non-diabetic Tg mice (c), and STZ-induced diabetic Tg mice (d). Magnification x 600. (B) Semi-quantitative analysis of TGF-β1 RII-positive tubules from kidneys of non-diabetic and diabetic WT and hnRNP F-Tg mice (N=6, ***p<0.005; n.s., non-significant).

Figure 8. RT-qPCR of AGT, TGF-β1 RII, fibronectin and collagen α1 (type IV) mRNA expression in RPTs. AGT, TGF-β1 RII, fibronectin and collagen IV mRNA levels in RPTs of non-diabetic and diabetic WT and Tg mice are shown in (A), (B), (C) and (D), respectively. Values are expressed as means ± SD, N=6 (*p<0.05; **p<0.01, ***p<0.005; n.s., non-significant).

Table 1. Primers used for conventional RT-PCR or RT-qPCR

Gene	Primer sequences
hnRNP F-HA (BC097275)	S: 5'-AGA GTG ACC GGA GAA GCT GA-3' AS: 5'-GGC GTA GTC AGG CAC GTC GT-3'
Angiotensinogen (rat) (BC087679)	S: 5'-CCT CGC TCT CTG GAC TTA TC-3' AS: 5'-CAG ACA CTG AGG TGC TGT TG-3'
Angiotensinogen (mouse) (NM_007428)	S: 5'-CCA CGC TCT CTG GAT TTA TC-3' AS: 5'-ACA GAC ACC GAG ATG CTG TT-3'
TGF-β 1(rat and mouse) (BC076380)	S: 5'-CCA AAC TAA GGC TCG CCA GTC-3' AS: 5'-GGC ACT GCT TCC CGA ATG TC-3'
TGF-β 1 RII (rat and mouse) (NM_031132)	S: 5'-TCC TTC AAG CAG ACG GAT GT-3' AS: 5'-ATC TTC TCC TGG GAG CAG CT-3'
β -actin (rat and mouse) (NM_031144)	S: 5'-CGT GCG TGA CAT CAA AGA GAA-3' AS: 5'-GCT CGT TGC CAA TAG TGA TGA-3'
Fibronectin (mouse) (NM_010233)	S: 5'-TAG CAG GCT ACC GAC TGA CCG-3' AS: 5'-CAC CCA GCT TGA AGC CAA TCC-3'
Collagen IV A (mouse) (NM_009931)	S: 5'-TGC CGG GTC CAC AAG GTT CAC-3' AS: 5'-GTC CAG GAG GGC CGA TGT CT-3'

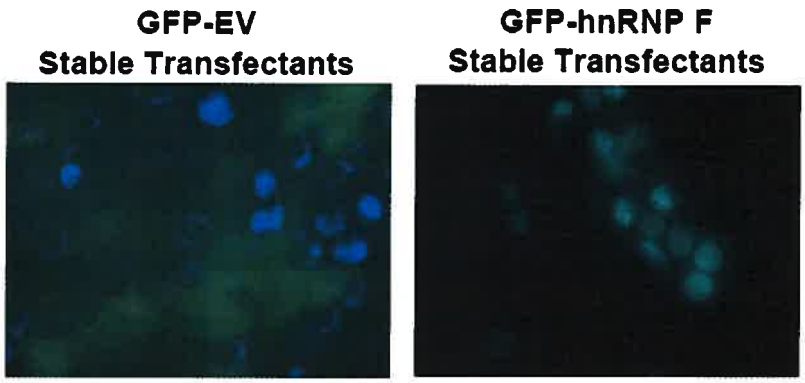
S, sense; AS, antisense

Table 2. The physical parameters of the animals at euthanization.

	WT		hnRNP F-Tg	
	Control	STZ	Control	STZ
Body weight (g)	29.52±2.1	22.83±1.8[a]	31.32±2.6	25.41±1.6[a]
Blood glucose (mM)	7.7±1.7	31.6±4.2[b]	8.2±1.2	30.9±3.6[b]
Kidney weight (g)	0.36±0.08	0.42±0.11	0.34±0.10	0.40±0.16
Ratio of Kidney wt. / body wt.	0.012 ± 0.002	0.018±0.003[c]	0.011± 0.002	0.016±0.003[d]
Urine albumine (µg/ml)	13.6±4.1	18.3±5.9	33.7±10.6	21.4±8.2
Urine creatinine (mg/dl)	44.9±8.7	1.2±0.5	46.4±6.1	1.5±0.7
Urine ACR	29.8±4.5	152.6±28.7[e]	73.9±23.4[f]	141.9±18.5[g]
Serum creatinine (mg)	0.18±0.02	0.19±0.01	0.2±0.02	0.18±0.02

ACR = Albumin/Creatinine Ratio
a. $p<0.001$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.
b. $p<0.001$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.
c. $p<0.05$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.
d. $p<0.01$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.
e. $p<0.05$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.
f. non-significant when hnRNP F-Tg control compared with WT Control : n=6 for each group.
g. $p<0.01$ when STZ (Diabetes) compared with Control (Non-diabetes) : n=6 for each group.

A.


GFP-EV
Stable Transfectants
GFP-hnRNP F
Stable Transfectants

B.

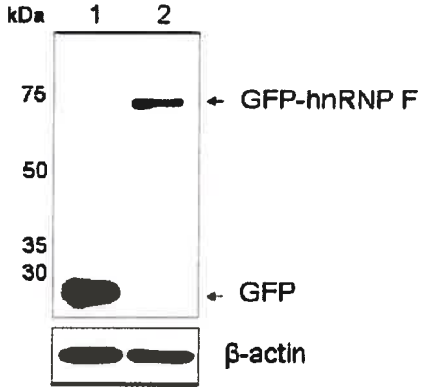
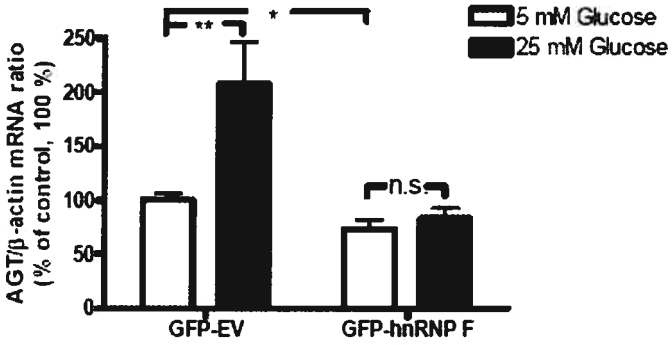
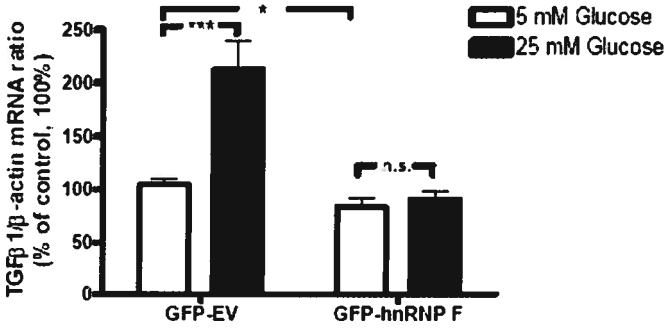

kDa
1
2
75
50
35
30
GFP-hnRNP F
GFP
β-actin

Figure 1

A.


5 mM Glucose
25 mM Glucose
AGT/β-actin mRNA ratio
(% of control, 100 %)
250
200
150
100
50
0
**
*
n.s
GFP-EV
GFP-hnRNP F

B.


5 mM Glucose
25 mM Glucose
TGFβ 1/β-actin mRNA ratio
(% of control, 100%)
250
200
150
100
50
0
***
*
n.s.
GFP-EV
GFP-hnRNP F

C.

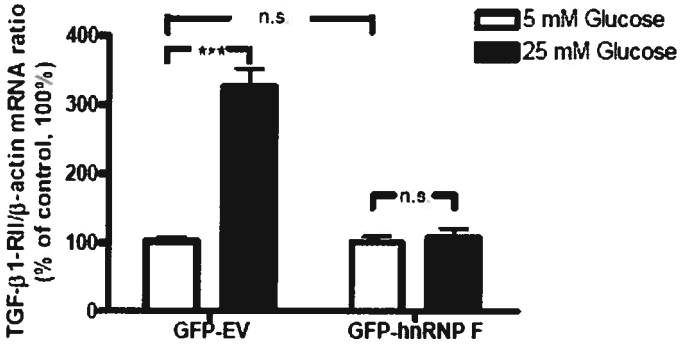
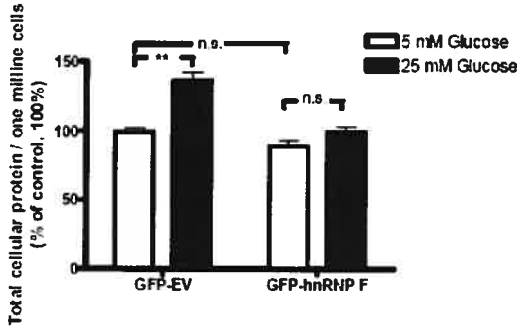
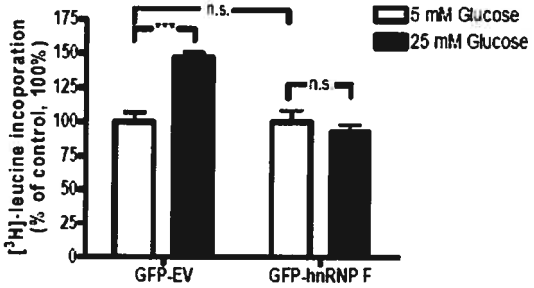

5 mM Glucose
25 mM Glucose
TGF-β1-RII/β-actin mRNA ratio
(% of control, 100%)
400
300
200
100
0
n.s
***
n.s
GFP-EV
GFP-hnRNP F

Figure 2

A.


Total cellular protein / one milline cells
(% of control, 100%)
150
100
50
0
n.s.
**
n.s
5 mM Glucose
25 mM Glucose
GFP-EV
GFP-hnRNP F

B.


[3H]-leucine incoporation
(% of control, 100%)
175
150
125
100
75
50
25
0
n.s.
***
n.s
5 mM Glucose
25 mM Glucose
GFP-EV
GFP-hnRNP F

C.

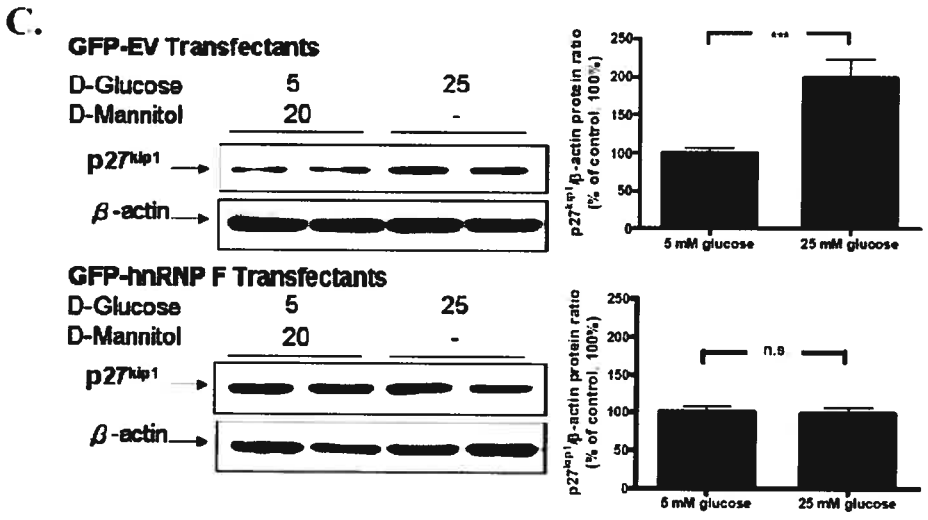

GFP-EV Transfectants
D-Glucose
5
25
D-Mannitol
20
-
p27kip1
β-actin
p27kip1/β-actin protein ratio
(% of control, 100%)
250
200
150
100
50
0
***
5 mM glucose
25 mM glucose
GFP-hnRNP F Transfectants
D-Glucose
5
25
D-Mannitol
20
-
p27kip1
β-actin
p27kip1/β-actin protein ratio
(% of control, 100%)
n.s
5 mM glucose
25 mM glucose

Figure 3

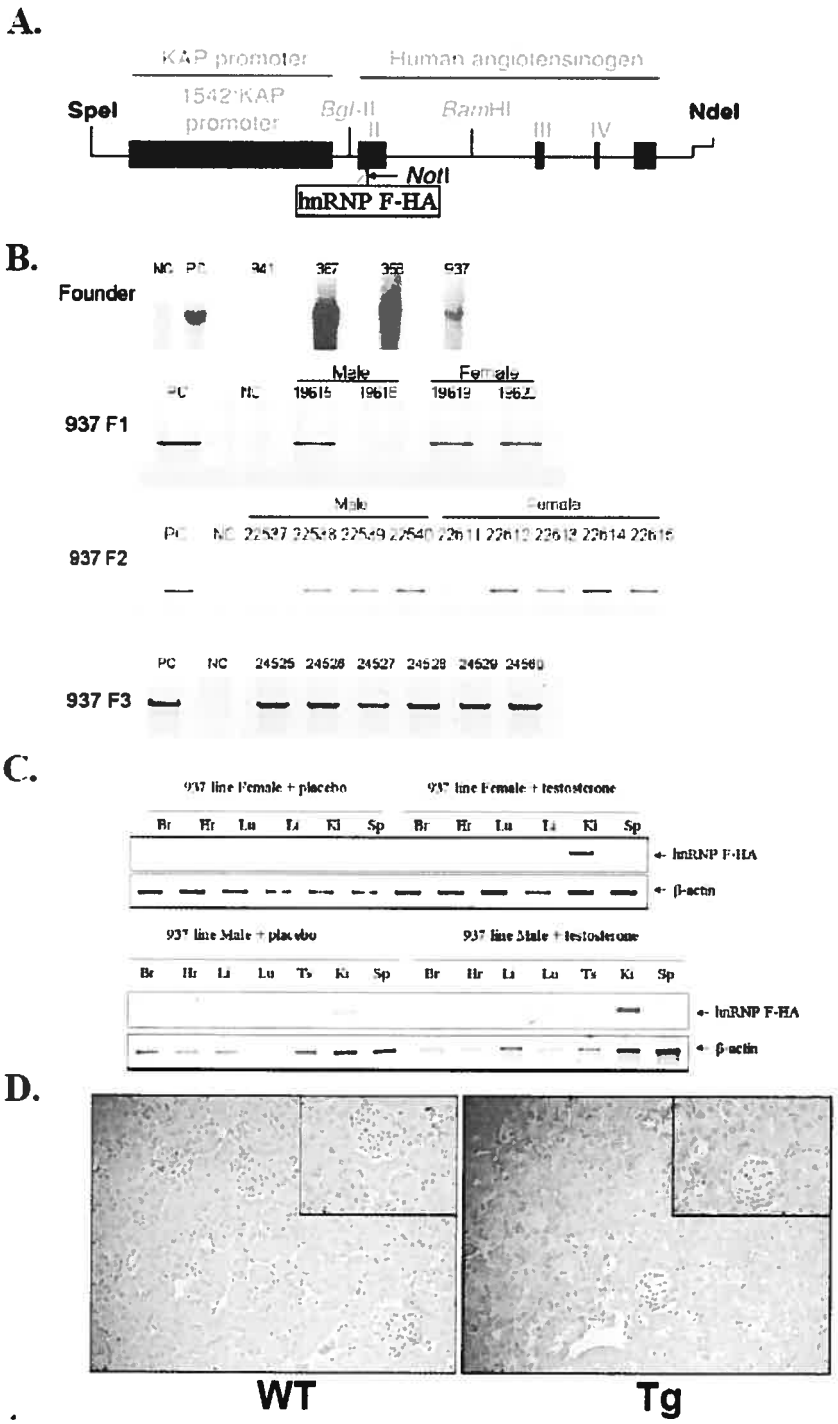

A.
KAP promoter
Human angiotensinogen
SpeI
1542 KAP promoter
Bgl-II
BamHI
NdeI
II
III
IV
NotI
hnRNP F-HA
B.
Founder
NC
PC
941
367
368
937
Male
Female
937 F1
PC
NC
19615
19618
19619
19621
937 F2
Male
Female
PC
NC
22537
22538
22539
22540
22611
22612
22613
22614
22615
937 F3
PC
NC
24525
24526
24527
24528
24529
24530
C.
937 line Female + placebo
937 line Female + testosterone
Br
Hr
Lu
Li
Ki
Sp
← hnRNP F-HA
← β-actin
937 line Male + placebo
937 line Male + testosterone
Br
Hr
Li
Lu
Ts
Ki
Sp
← hnRNP F-HA
← β-actin
D.
WT
Tg

Figure 4

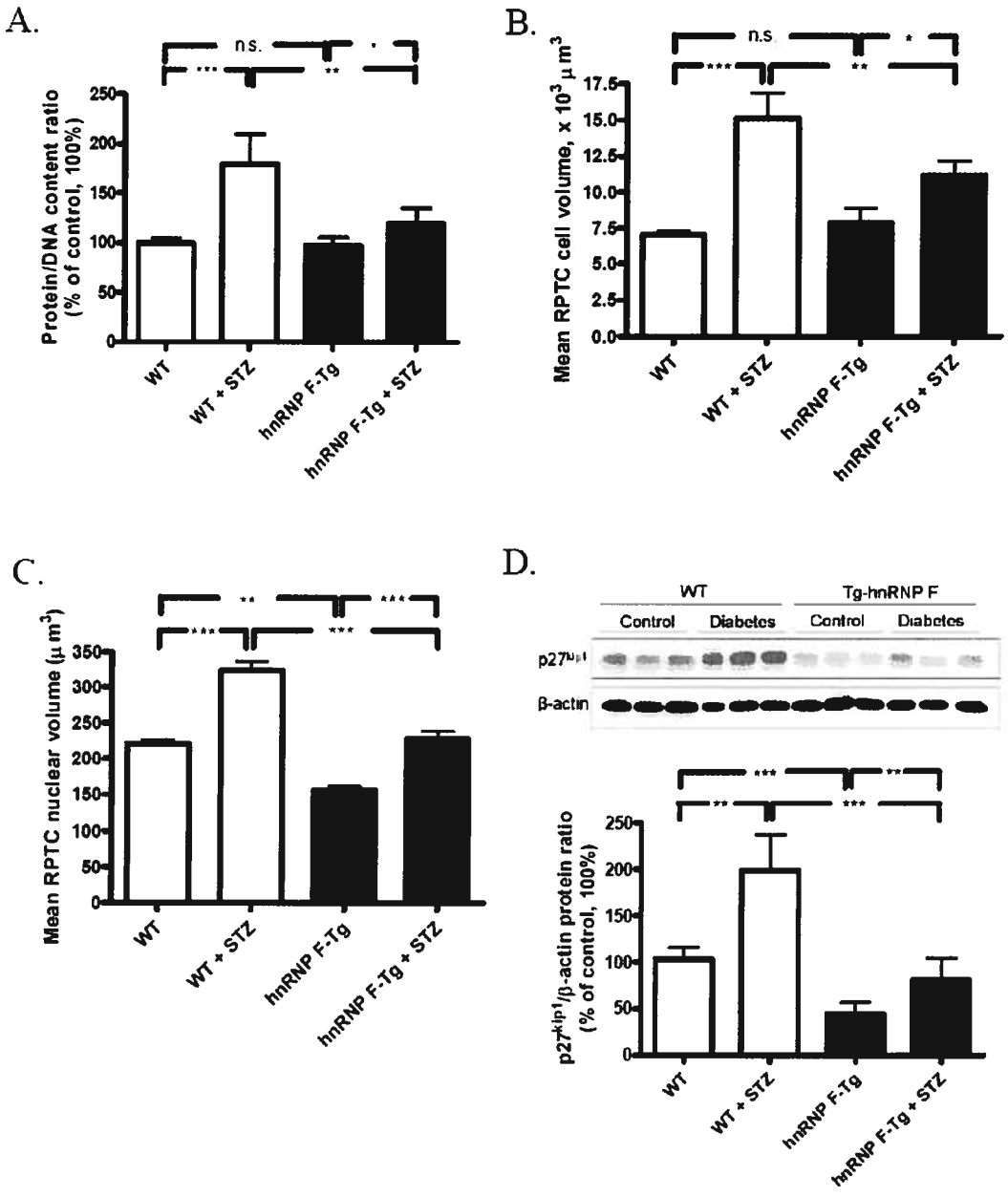
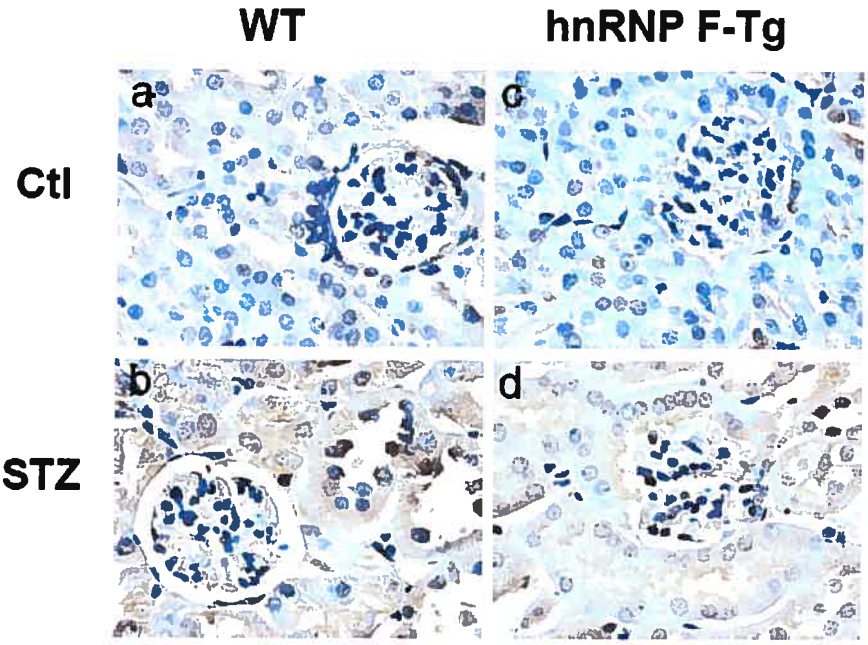

A.
n.s.
***
**
*
Protein/DNA content ratio (% of control, 100%)
250
200
150
100
50
0
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ
B.
n.s.
***
**
*
Mean RPTC cell volume, x 10³ µm³
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ
C.
**
***
***
Mean RPTC nuclear volume (µm³)
350
300
250
200
150
100
50
0
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ
D.
WT
Tg-hnRNP F
Control
Diabetes
Control
Diabetes
p27kip1
β-actin
***
**
**
***
p27kip1/β-actin protein ratio (% of control, 100%)
250
200
150
100
50
0
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ

Figure 5

A.


WT
hnRNP F-Tg
Ctl
STZ
a
b
c
d

B.

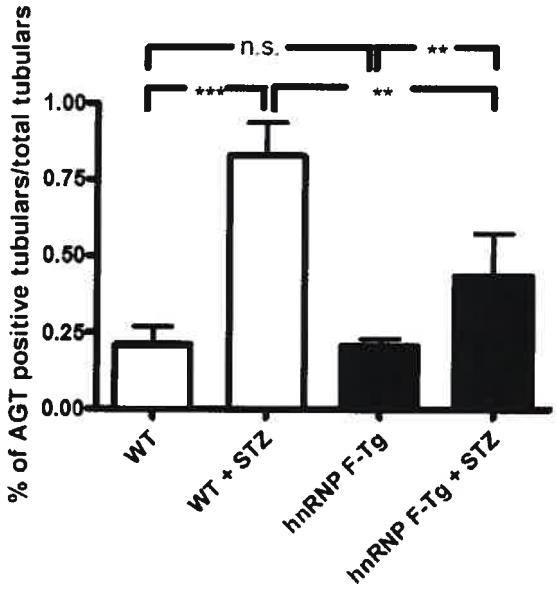
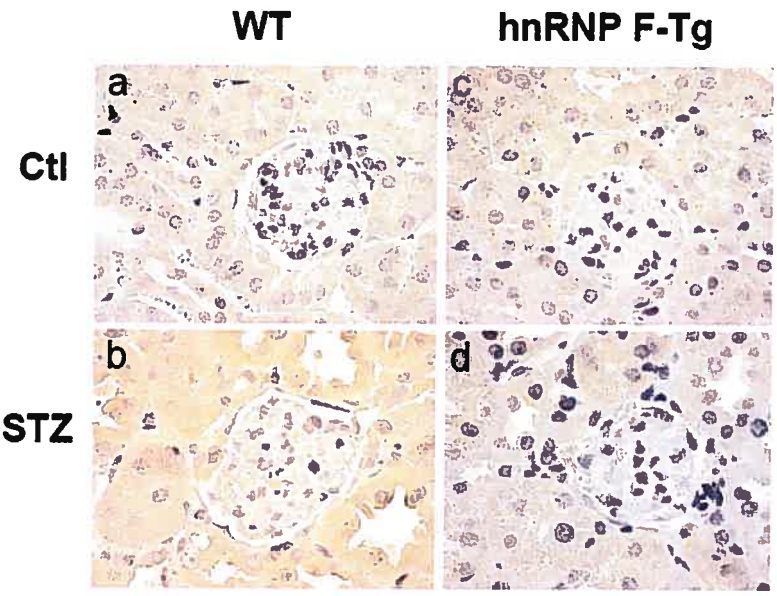

% of AGT positive tubulars/total tubulars
1.00
0.75
0.50
0.25
0.00
n.s.
***
**
**
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ

Figure 6

A.


WT
hnRNP F-Tg
Ctl
STZ
a
b
c
d

B.

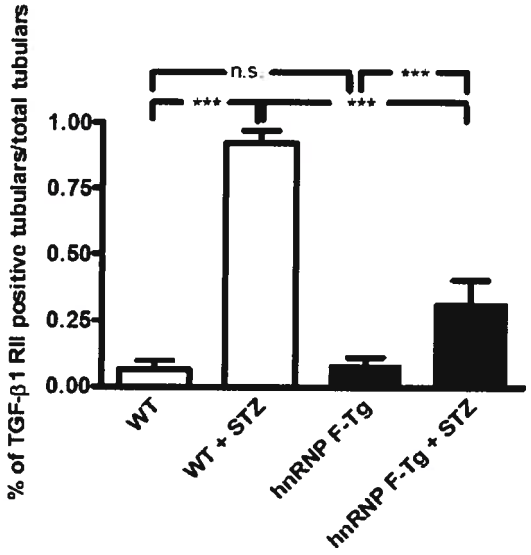

% of TGF-β1 RII positive tubulars/total tubulars
1.00
0.75
0.50
0.25
0.00
n.s.
***
***
***
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ

Figure 7

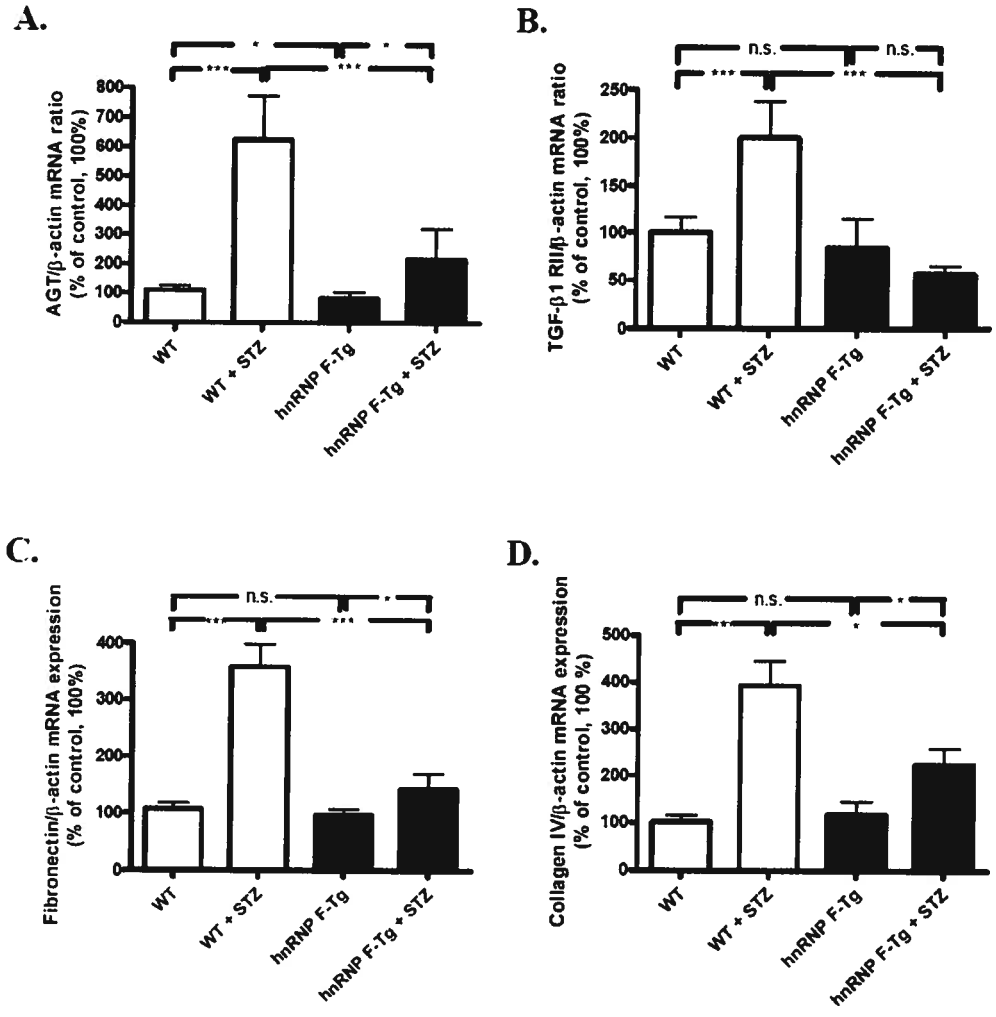

A.
AGT/β-actin mRNA ratio (% of control, 100%)
800
700
600
500
400
300
200
100
0
WT
WT + STZ
hnRNP F-Tg
hnRNP F-Tg + STZ
B.
TGF-β1 RII/β-actin mRNA ratio (% of control, 100%)
250
200
150
100
50
0
n.s.
n.s.
C.
Fibronectin/β-actin mRNA expression (% of control, 100%)
400
300
200
100
0
n.s.
D.
Collagen IV/β-actin mRNA expression (% of control, 100 %)
500
400
300
200
100
0
n.s.

Figure 8

Chapter 5: Discussion

DN is the leading cause of ESRD. RAS activation and hyperglycemia are 2 major risk factors for the development of DN. Previous studies in Dr. Chan's lab have demonstrated that the intrarenal RAS might play an important role in DN development. Since AGT is the sole precursor of the RAS and its regulation is not well-defined, elucidation of the regulation of AGT expression in the kidneys is a major research topic in our lab. We have published several studies on the effects of hyperglycemia and insulin on rAGT gene regulation (35; 131; 132; 165). Our group has identified a novel IRE in the promoter region of the rAGT gene, and showed that 2 nuclear proteins could bind to the IRE. These nuclear proteins could be regulated by glucose and insulin (207). However, their identification has not been completed. Therefore, my Ph.D. studies concentrated on the 2 nuclear proteins, to elaborate their functions in rAGT gene expression and their influence on RPTC hypertrophy in high glucose *in vitro* and *in vivo*.

5.1 Protein-DNA interactions

Proteins often interact with each other, forming transient or stable complexes to exert their biological activities. Some proteins interact specifically or non-specifically with non-protein molecules, such as DNA, RNA, or metabolites. These interactions are critical for cellular functions, especially in gene regulation. Thus, defining the composition of protein complexes and understanding how they are assembled and regulated may yield invaluable insights into protein function.

Proteins that bind DNA have been found to have common folding patterns known as DNA-binding motifs. Each DNA-binding motif is

composed of recognition and stabilization regions (275). A significant number of DNA-binding protein structures have been postulated, along with several structures of their binding site complexes. The α-helix may be best suited to bind major grooves for specific recognition (276). The major classes of binding motifs that have been described are helix-turn-helix, leucine zippers, zinc fingers (ZF), and β-structures (277). Some proteins even indirectly bind to DNA by binding to other proteins before interacting with DNA (278; 279).

5.1.1 General Prospecting Technologies to Elucidate Protein-DNA Interactions

Several methods are commonly used to analyze protein-DNA interactions, including DNase I footprinting, gel mobility shift assay (GMSA), chromatin immunoprecipitation (ChIP), and Southwestern blotting (280; 281). Each of these methods has specific capabilities and limitations.

DNase I footprinting identifies the DNA sequence to which a protein binds. First, a target DNA fragment of about 100-300 bp in length is either PCR-generated or cut from a vector, and then labeled with an isotope before incubation with nuclear proteins. This is followed by controlled digestion with DNase I, which randomly cuts the probe once. The digested DNA is recovered from the reaction and resolved on polyacrylamide gel with a sequencing reaction that employs the same probe as template. The regions bound by proteins are protected from digestion and show up as a blank area on the gel track. The exact protein-bound sequence can be read by comparing the location of the blanks with the sequencing reaction (282;

283). By varying the concentration of DNA-binding protein, binding affinity can be estimated according to the minimum concentration of protein at which a footprint is observed. The disadvantages of this technique are that it is tedious and technically demanding, and DNase I can only be deployed under specific conditions (i.e., in the presence of Mg^{++} and Ca^{++} ions) (281).

GMSA is based on the observation that protein-DNA complexes migrate through non-denaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides (280; 281; 284). GMSA is performed by incubating a purified protein or a complex mixture of proteins (i.e., a nuclear extract) with a labeled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed by electrophoresis on non-denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established in competition experiments with DNA fragments or oligonucleotides containing a binding site either for the protein of interest or for other unrelated DNA sequences. Supershift-GMSA is undertaken by incubating proteins with a labeled DNA fragment containing the putative protein-binding site and an antibody to the putative protein (280; 281). A "super-complex" of DNA-protein antibody has significantly reduced mobility compared to a DNA-protein complex when it is subjected to electrophoresis in non-denaturing gel. This method provides information on the mass of the bound protein, and the results are easier to see than those of footprint assay. Although useful for basic research, gel-shift and supershift assays have low sensitivity, so that a large number of controls are required. Furthermore, gel-shift and supershift assays are not

quantitative. They can detect the presence or absence of a particular DNA-binding protein, but they cannot localize the binding site or predict the molecular size of the binding proteins.

ChIP is typically performed to test whether a protein binds to a candidate promoter in living cells. The principle behind this assay is that DNA-bound proteins in living cells can be cross-linked to the chromatin with which they interact. This is usually accomplished by gentle formaldehyde fixation. After fixation, the cells are lysed, and DNA is broken by sonication into pieces 0.2-1 kb in length (280; 281). Once the proteins are immobilized on the chromatin and the chromatin is fragmented, whole protein-DNA complexes can be immunoprecipitated with an antibody specific for the protein in question. DNA from the isolated protein/DNA fraction can then be purified. The identity of the DNA fragments isolated in a complex with the protein of interest can then be determined by PCR, using primers specific for the DNA regions hypothetically bound by the protein.

ChIP has the advantage of measuring protein-DNA interactions in their natural genomic state. Moreover, ChIP can provide direct information on the histone acetylase status of specific chromatin regions. It also minimizes the chances of chromatin rearrangements during preparation and precipitation. Its disadvantages are that a large number of cells are usually required, and the data may represent an average of multiple functional states in the cell population. In addition, specific antibodies are needed for each protein, and negative results do not necessarily mean that a given factor is not associated with the site (285).

Southwestern blotting was first described by Bowen and colleagues as a method of identifying and characterizing DNA-binding proteins by their ability to bind to a specific oligonucleotide probe (286). Nuclear protein extracts are typically separated electrophoretically on sodium dodecyl sulphate (SDS)-polyacrylamide gel and then transferred to nitrocellulose for screening with a probe. The disadvantages of this technique are that it requires relatively large amounts of nuclear protein, there are problems with protein degradation during isolation, and it is difficult to achieve efficient electrophoretic separation and transfer of a wide range of proteins of different molecular sizes (287).

During the first year of my Ph.D. studies, I tried to clone nuclear proteins that bind to the IRE of rAGT from a cDNA library constructed from IRPTCs. I constructed several cDNA libraries ranging from 300 to 1.5 kbp. Unfortunately, I did not obtain any candidate clones in the second screening (unpublished results). We changed our strategy by first separating nuclear proteins by 2D gel electrophoresis, and then identifying candidate proteins by Southwestern blotting employing radioactive rAGT-IRE as a probe. After we identified positive spots, we sequenced them by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). This powerful proteomics technology has provided us with very good data, and we have been able to identify the 2 nuclear proteins as hnRNP F and hnRNP K (Chapters 2 and 3).

5.1.2 Advantages and Disadvantages of 2D Gel Electrophoresis Combined with MS

2D gel electrophoresis is a technique by which proteins are separated according to their isoelectric point (pI) and mass. It is used as a component of proteomics and is the initial step in the isolation of proteins for further characterization by MS (288). The technique separates proteins in 2 steps: 1) isoelectric focusing (IEF), which separates proteins according to their pI, and 2) SDS-polyacrylamide gel electrophoresis (PAGE), which separates proteins according to their MW. In this way, complex mixtures consisting of thousands of proteins can be resolved, and the relative amount of each protein determined.

The procedure involves placing the sample in a gel with a pH gradient and applying a potential difference across it. In the electrical field, the protein migrates along the pH gradient until it carries no overall charge. The location of the protein in the gel indicates its pI.

There are 2 methods for creating the pH gradient: carrier ampholites and immobilized pH gradient (IPG) gels. In our lab, IEF is performed with commercial IPGs for highly-reproducible results. IEF is the most critical step in the 2D electrophoresis process. The proteins must be solubilized in a buffer without charged detergents, usually a highly-concentrated urea solution that splits the proteins into individual components, but also denatures them, which ensures reliable running in IEF. To obtain high quality data, it is essential to achieve low ionic strength conditions before IEF. Since different types of samples vary in their ion content, it is necessary to adjust the IEF buffer and the electrical profile for each type of sample.

Voltage volume is another problem in running IEF. It appears that higher voltage yields better results. The correct voltage for running strips depends on the length of the strip; Bio-Rad recommends 600 V/cm. This means that for running an 18-cm strip, a maximum of around 10,000 V is required. However, tests of several conditions are recommended to find the best resolution. In this study, we had to run 2 gels under the same condition at the same time to get an identical protein profile, which facilitated comparison with Southwestern blotting.

In recent years, MS has emerged as a powerful tool to quickly and efficiently identify proteins in biological samples (289; 290). However, the application of MS to large biomolecules and synthetic polymers has been limited because of the high volatility and thermal instability of the materials (291). These problems have been overcome to a great extent through the development of soft ionization techniques, such as MALDI-MS (292-294). Coupled with an isolation technique (i.e., 2D electrophoresis) to purify a specific protein of interest, MS can rapidly and reliably identify the structure of a protein.

MALDI is based on an ultraviolet-absorbing matrix pioneered by Hillenkamp et al. (293). The matrix and polymer are mixed at a molecular level in an appropriate solvent with ~104 molar excess of the matrix. The solvent prevents aggregation of the polymer. The matrix mixture is placed on a sample probe tip. The solvent is removed in vacuum, leaving co-crystallized polymer molecules homogeneously dispersed with matrix molecules. When the pulsed laser beam is tuned to the appropriate frequency, energy is transferred to the matrix, which is partially vaporized,

carrying intact polymers into the vapor phase and charging the polymer chains (294). Multiple laser shots improve the signal-to-noise ratio and peak shapes, increasing the accuracy of molar mass determination. In a linear time-of-flying (TOF) analyzer, the distributions of molecules emanating from a sample are imparted to identical translational kinetic energies after being subjected to the same electrical potential energy difference. The digitized data generated from successive laser shots are summed, yielding a TOF mass spectrum, which is a recording of the detector signal as a function of time. The time of flight for a molecule of mass (m) and charge (z) to travel is proportional to (m/z)1/2. This relationship can serve to calculate ion mass, through which conversion of the TOF mass spectrum to a conventional mass spectrum of mass-to-charge axis can be achieved (295).

The protein to be analyzed must be purified by an appropriate approach, which often includes an affinity chromatography step. In this study, I isolated the nuclear proteins from high glucose-stimulated IRPTCs, as our previous results showed that high glucose enhances the binding profile of IREBPs to the rAGT IRE. Then, I separated the proteins by 2D gel electrophoresis.

5.2 Advantages and Disadvantages of IRPTCs

The proximal tubule of the mammalian kidney is composed of polarized epithelial cells endowed with specific ion transport capacities and biochemical functions distinct from those of cells in the more distal segments of the renal tubule (296-298). Primary cultures of proximal cells

retain most of their properties during the first 2 or 3 weeks in culture, but usually lose their differentiated functions when they are sub-cultured (299; 300). Renal proximal tubule (RPT)-like established cell lines, such as LLC-PK1 and OK cells, have been used to analyze glucose and ion transport regulation (301; 302) as well as intrarenal AGT expression (303; 304), and to study processes controlling membrane protein sorting (305). However, the precise origin of these cells remains unclear, since LLC-PK1 cells, for example, have both proximal and distal tubule cell functions (297; 302; 306).

The IRPTCs in this study were established by Ingelfinger's group. The IRPTC 93-p-2-1 cell line was derived from 4- to 6-week-old WKY male rats with an origin-defective SV40 plasmid (307; 308). IRPTC 93-p-2-1 is a rapidly-growing and stable PTC line, which makes it useful in experiments requiring a large amount of material. This IRPTC line polarizes and expresses enzymes and proteins that are normally present in PTCs, thus displaying a highly-differentiated phenotype. Cell line 93-p-2-1 also expresses all components of the RAS: AGT, renin, ACE, and AT1 and AT2 receptors (308).

One of the disadvantages of the IRPTC 93-p-2-1 cell line is that it cannot be 100% identical to PTCs *in vivo.* For example, renal AGT mRNA expression varies in comparison to that of rat RPTCs *in vivo.* There are differences in AGT steady-state mRNA levels and regulation by sodium diet in SHR compared to normotensive WKY rats *in vivo* (307; 309).

5.3 Molecular Mechanisms of hnRNP F and hnRNP K on AGT Gene Expression

Accumulating evidence strongly suggests molecular linkage between transcription by RNA poly II and pre-mRNA processing (310; 311). hnRNPs associated with RNA poly II transcripts in the nucleus have important roles in mRNA biogenesis (312-316). hnRNP F, H/H', and the 2H9 family are a subgroup of hnRNPs (317; 318). This family shares almost 75% sequence homology (319). Rat and human hnRNP F cDNAs encode 415 amino acids and exhibit 99% homology (317). The structure of hnRNP F/H/H' contains 3 RBDs and a GY-rich region between RBD-II and RBD-III. The GY-rich region has been suggested to bind to double-stranded DNA (320). By analyzing its structure in the EMBL-EBI database, we found a ZF in this region (Figure 9). The ZF is a ~26-amino-acid residue peptide forming a structure that binds to specific sequences in DNA (321; 322). The ZF motif consists of an α-helix followed by a turn and 2 short β-strands. The β-strands form a small loop resembling a finger, and the fold sandwiches a zinc ion (323; 324). The α-helix and the first β-strand contain conserved cysteine and histidine residues that coordinate the zinc metal ion, which is important for stabilizing the protein fold (325). Whether this motif is responsible for hnRNP F-binding to the rAGT IRE is still not well-defined. Further experiments should be performed to demonstrate the role of this region in DNA-binding ability (see Chapter 6 on Research Perspectives).

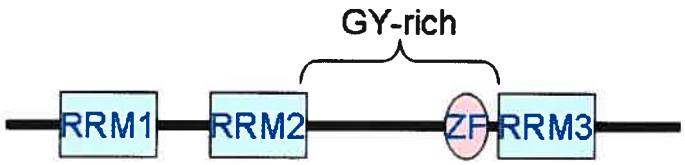


Figure 9. Structure of hnRNP F.

hnRNP F co-immunoprecipitates with TBP and RNA poly II, forming complexes with nuclear cap-binding protein complex (CBC) (320; 326; 327), suggesting that hnRNP F participates in gene regulation. Studies have also shown that hnRNP F/H/H' engages in alternative splicing of c-src (237), β-tropomyosin gene (232), thyroid hormone receptor gene (328), and Bcl-x gene (231) as well as in the 3-end processing of pre-mRNA in B-cell differentiation (329). Thus, evidence indicates that the hnRNP F/H/H' family plays an important role in both gene transcription and splicing.

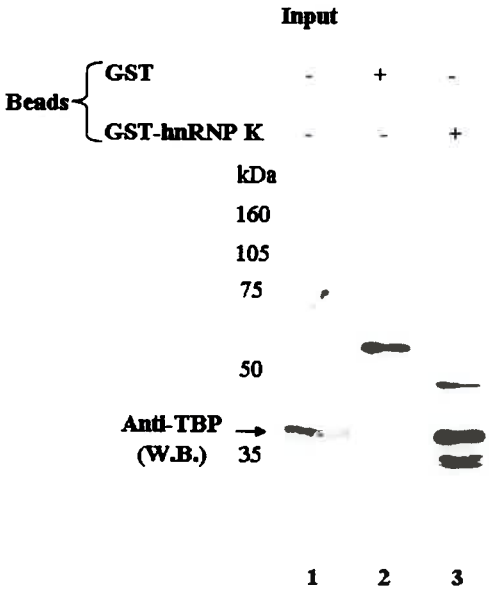


Figure 10. Interaction of hnRNP K with TATA-Box Binding Protein (TBP).

hnRNP K was first discovered as a hnRNP particle (330). It has an apparent MW of 65 kDa in SDS-PAGE (331). hnRNP K, localized in the nucleus, cytoplasm and mitochondria, has been implicated in chromatin-remodeling, transcription, splicing, and translation processes (332). Indeed, it has been suggested that hnRNP K binds to RNA via highly-conserved KH domains (333). K binds both double- and single-stranded DNA motifs (CT element, 5 d-TCCC) within the promoter of c-myc (334), c-src (253), c-fos, and elongation initiation factor 4E gene (335), forming complexes with Sp1 and TBP to enhance gene transcription (217; 246; 251). This evidence suggests that hnRNP K may also be involved in transcription. In fact, hnRNP K expression can both activate and repress RNA poly II promoters (246; 254). hnRNP K has been found to play the role of a transcriptional repressor in suppressing the transcription of thymidine kinase (336), the neuronal nicotinic acetylcholine receptor β4 subunit (247), and osteocalcin (337). We have discovered that hnRNP K suppresses AGT gene transcription and binds with TBP (see Chapter 4 and Figure 10), supporting the notion that it is a suppressor protein. hnRNP K also participates in the alternative splicing of β-tropomyosin (338), stabilizes renin mRNA via binding to the 3-untranslated region of renin mRNA (339), and regulates 15-lipoxygenase gene translation via binding with elongation factor-1α (249; 250). Thus, hnRNP K is a multifunctional protein that interacts with DNA, RNA, and transcriptional and translational molecules to alter the *in vivo* rates of gene transcription and translation (332).

Studies in our lab have demonstrated that high glucose activates PKC and p38 MAPK signaling and phosphorylates CREB and ATF-2 (35; 132). The phosphorylated CREB and ATF-2 form a heterodimer, bind to CRE in the rAGT promoter, recruit p300/CREB-binding protein (p300/CBP), and stimulate rAGT gene expression. To counterbalance the action of CREB/ATF-2, high glucose also stimulates hnRNP F and K gene transcription, subsequently suppressing AGT gene transcription via protein phosphorylation and translocation into the nucleus. Studies have shown that insulin alters hnRNP K-binding to DNA and RNA through phosphorylation (340). ERK phosphorylation also drives the cytoplasmic accumulation of hnRNP K and inhibition of mRNA translation in HeLa cells (341). These results raise several possibilities concerning the mechanism(s) of hnRNP F and hnRNP K action on the inhibition of AGT gene expression. First, phosphorylated hnRNP K may interact with phosphorylated hnRNP F to form a heterodimer, and then bind with single-stranded AGT-IRE. The bound heterodimer competes with CREB/ATF-2 heterodimer for binding with TBP and RNA poly II, which attenuates AGT gene expression. This notion is supported by the data in my second article and unpublished results (as shown in Figure 10) that co-transfection of hnRNP F and hnRNP K into IRPTCs further suppresses AGT mRNA expression. Second, the phosphorylated hnRNP F/K heterodimer may interact directly with p300/CBP, altering the conformation of p300/CBP to release bound CREB/ATF-2, and subsequently suppress AGT gene transcription. Finally, the bound hnRNP K/F heterodimer could exhaust the availability of other unidentified nuclear transcriptional activating factors via protein-protein

interactions, thus attenuating AGT gene transcription. Clearly, more studies are needed along these lines.

Taken together, our data demonstrate that hnRNP F and hnRNP K could both bind to rAGT-IRE and TBP as well as suppress rAGT transcription. The inhibitory effect of hnRNP F and hnRNP K may be mediated directly or via protein-protein interactions with transcriptional factors.

5.4 Renopathological Significance of Suppressing AGT Gene Expression via hnRNP F or hnRNP K in Hyperglycemia

Renal hypertrophy and matrix protein synthesis are associated with the upregulation of TGF-β1 gene expression in diabetic rats and mice (342-344). There is compelling evidence that Ang II-induced RPTC hypertrophy and the stimulated synthesis of collagen type IV are mediated by increased TGF-β1 transcription and production (345-351). This was demonstrated by the elegant studies of Wolf et al. (350) and Sharma et al. (344), who reported that neutralizing anti-TGF-β1 antibodies significantly reduced Ang II-stimulated collagen production and hypertrophy in murine PTCs *in vitro* and *in vivo,* respectively.

Previous investigations in our lab have shown that both basal TGF-β and collagen α1 (type IV) mRNA levels in IRPTCs stably transfected with pRSV/rAGT antisense are lower than in non-transfected IRPTCs and IRPTCs stably transfected with pRC/RSV or pRSV/rAGT sense (352). These data suggest that TGF-β1 and collagen α1 (type IV) mRNA

expression in IRPTCs depends, at least in part, on the presence of endogenous Ang II. Thus, renal Ang II acts in an autocrine manner to stimulate TGF-β1 expression and, subsequently, TGF-β1 enhances cellular hypertrophy and collagen α1 (type IV) expression in RPTCs. Therefore, we hypothesized that either hnRNP F or hnRNP K regulates AGT expression, and subsequently attenuates RAS activation and the progression of renopathology in hyperglycemia, that is, RPTC hypertrophy.

Evidence suggests that the intrarenal RAS plays a key role in hyperglycemia-induced cell hypertrophy via the activation of TGF-β1 signaling (350; 353). Our data have revealed that stable transfectants overexpressing hnRNP F or hnRNP K prevent the high-glucose stimulation of AGT, TGF-β1 and TGF-β1 RII mRNA expression, and circumvent high glucose-induced cellular hypertrophy, as determined by total protein content, [^{3}H]-leucine incorporation, and $p27^{kip1}$ protein expression in RPTCs (Chapter 4 and unpublished data in Figure 11). These findings are consistent with our previous observations that hnRNP F and hnRNP K suppress AGT gene transcription in RPTCs (354; 355) and that decreased AGT gene expression prevents cellular hypertrophy in high glucose (352).

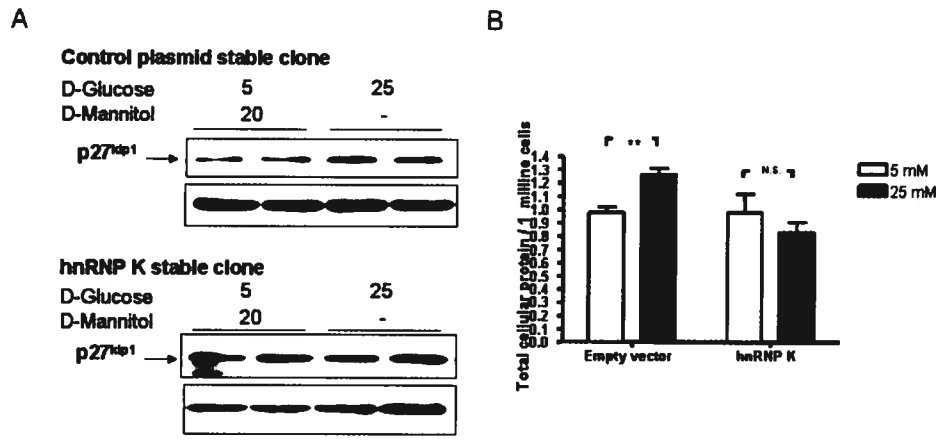


Figure 11. hnRNP K overexpression prevents cellular hypertrophy in high glucose.

Since I did not prove *per se* that the effects on TGF-β and TGF-β RII expression, protein/DNA ratios, cellular volume and $p27^{kip1}$ protein expression are mediated uniquely by the intrarenal RAS, further experiments in kidney-specific AGT knockout mice are required. To obtain tissue-specific gene knockout, the Cre recombinase/loxP (Cre/loxP) system is most widely utilized, for which 2 different mouse lines have to be produced separately. In 1 of these 2 lines, exon 2 of mAGT is flanked by 2 loxP sites by homologous recombination techniques in embryonic stem cells. The loxP sites are short-sequence elements (34 bp) containing 2 13-bp inverted repeats flanking an 8-bp asymmetric spacer. In the second mouse line, generated by standard oocyte injection, Cre recombinase expression is driven by the core promoter of a kidney-specific gene, choosing KAP2 or Sglt2 (sodium-glucose transporter 2) promoter. Mating of these 2 different mouse lines will yield offspring where Cre recombinase is solely expressed in the targeted tissue or cell type in which it will excise the floxed AGT fragment. Analyzing the TGF-β signal pathway and PTC

hypertrophy after diabetes induction, we speculate that the TGF-β signal pathway and PTC hypertrophy will be attenuated or completely blocked in diabetic AGT knockout compared to diabetic wild type mice. Thus, we can validate that the intrarenal RAS is partly or uniquely mediating the TGF-β signal pathway and PTC hypertrophy.

In hnRNP F-Tg mice, we showed that hnRNP F fails to prevent the increased blood glucose and kidney/body weight ratio occurring in diabetic Tg mice. (N.B.: The kidney/body weight ratio in hnRNP F-Tg mice tends to be lower than in non-Tg controls, but the difference is not statistically significant.) However, hnRNP F overexpression clearly prevents attenuation of the protein/DNA ratio, cellular and nuclear volumes, and $p27^{kip1}$ protein expression in RPTCs of diabetic hnRNP F-Tg mice compared to diabetic non-Tg controls. In addition, although DN has traditionally been considered to be primarily a glomerular disease, it is now widely accepted that the diabetic kidney fails not only as a result of glomerular lesions but also because of tubulointerstitial changes, whereas diabetic glomerulosclerosis alone does not lead to chronic renal failure (356; 357). These observations suggest that although glomerular injury plays a primary role in patients with proteinuria, the long-term outcome is determined by events in the renal interstitium. In this study, we also demonstrated that AGT, TGF-β1 RII, fibronectin, and collagen α1 mRNAs are significantly elevated *in vivo* and *in vitro*. However, the heightened expression of these mRNAs is significantly attenuated when hnRNP F is overexpressed (see results in Chapter 4).

Taken together, the present experiments make novel observations that AGT suppression in the RAS through hnRNP F or K can prevent high glucose-induced RPTC hypertrophy both *in vitro* and *in vivo*.

5.5 Physiological Significance of Suppressing Local RAS Gene Expression via hnRNP F and hnRNP K in Diabetes

Our lab has reported previously that AGT mRNA expression is increased at least 3-fold in RPTs of STZ-induced diabetic mice. To avoid the 'nephrotoxicity' induced by STZ (358) and to demonstrate the physiological roles of hnRNP F and hnRNP K *in vivo*, we used spontaneously diabetic Akita mice, an optimal model for type 1 diabetes, and db/db mice, an optimal model for type 2 diabetes. The Akita mouse model has a mutated insulin gene2, which is inherited by an autosomal dominant trait and confers a decrease in the number of β-cells of the pancreatic islets (271).

Our preliminary data disclosed that Akita mice (10 weeks old) develop hyperglycemia and an increase (>3-fold, $P < 0.01$) of AGT mRNA expression in RPTCs compared to wild-type controls (see unpublished data in Figure 12A). In contrast, hnRNP F and K mRNA expression is significantly decreased (>20% lower, $P < 0.05$) in RPTs of diabetic Akita mice compared to wild-type controls. Furthermore, we found that AGT mRNA is increased at least 5-fold ($P < 0.005$) in RPTs of db/db diabetic mice, whereas hnRNP F and hnRNP K mRNA levels are reduced at least 25% in comparison to heterozygous db/m$^+$ ($P < 0.05$) (see unpublished data in Figure 12B).

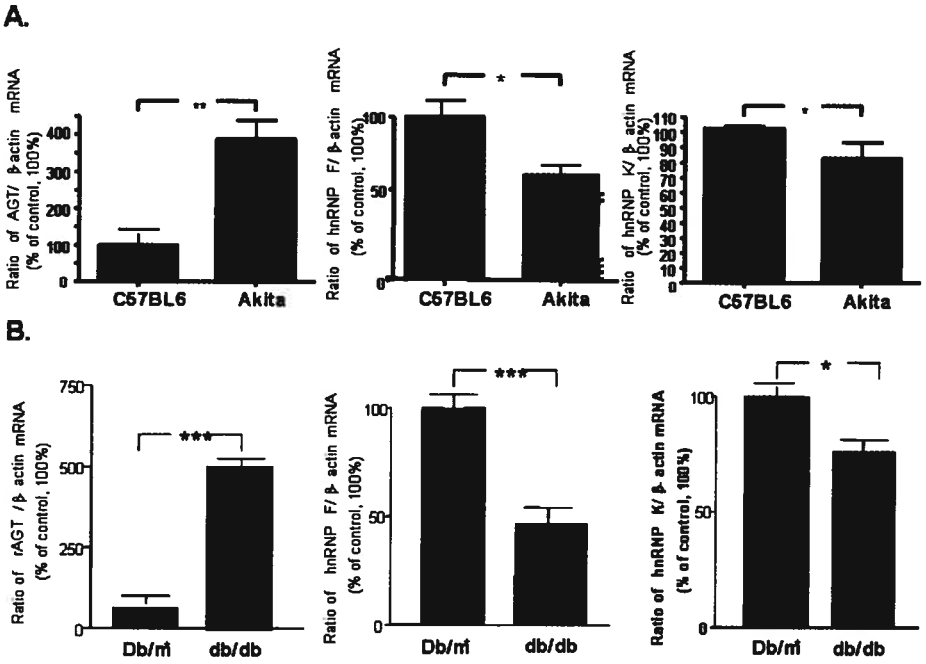


Figure 12. Endogenous hnRNP F and K mRNA expression in diabetic animal models.

High glucose stimulated and insulin suppressed hnRNP F and K expression in PTCs. Interestingly, hnRNP F and K overexpression prevented the high-glucose effect on AGT mRNA expression in IRPTCs (354; 355). In contrast, hnRNP F and K downexpression by siRNA enhanced the high-glucose stimulation of AGT mRNA expression in IRPTCs (355). This, combined with preliminary results, showed that hnRNP F and K expression is significantly lower in RPTs of Akita and db/db mice (Figures 12A and 12B). These data demonstrated that hnRNP F and K proteins may be pivotal in counterbalancing the high-glucose stimulation of AGT and TGF-β1 gene expression in diabetic kidneys. Dysregulation of hnRNP F and/or K expression could enhance high-glucose stimulation of intrarenal AGT gene expression and subsequent kidney injury in diabetes. Thus, it is conceivable that hnRNP F and K proteins may be regulated differently in humans predisposed to DN than in those who are less

susceptible to nephropathy. Clearly, more research is needed to define the physiological role(s) of hnRPN F and hnRNP K proteins on ANG gene expression in the diabetic kidney.

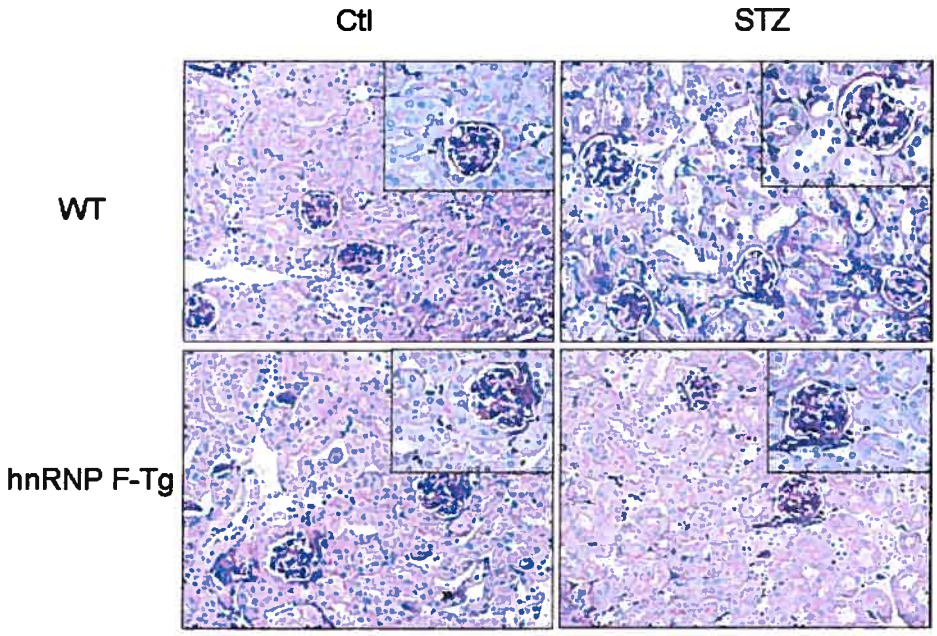


Figure 13. PAS staining of kidneys.

To elucidate the potential implications of hnRNP F and K on renal function and development of histological changes of diabetic nephropathy we have performed PAS staining to analyze the changes in glomerular morphology (Figure 13). We also measured serum creatinine concentration as a marker of renal function (Table II in Chapter 4). There was no significant change in glomerular morphology and serum creatinine in the 4 groups of mice. Since the animals were studied under 4 weeks of diabetes, it is conceivable that we may not see latent effects on the progression of nephropathy, i.e. decreased renal function, glomerular changes, and glomerular homeostasis. To address this question, longterm experiments

are needed, i.e. at least 12 weeks of diabetes. Given that hnRNP F overexpression suppresses AGT gene expression and tubular hypertrophy in diabetes, we anticipate that tubular damage might be attenuated and subsequently ameliorate glomerular damage in diabetic hnRNP F-Tg mice. Nevertheless, more studies are needed to confirm this possibility. Tubular hypertrophy is a good predictor of DN. Awazu et al. (359) and Wolf et al. (360) have reported that albuminuria, glomerular sclerosis and mesangial, endothelial, podocyte and PTC hypertrophy are attenuated by $p27^{kip1}$ knockout in STZ-diabetic mice. These studies provide an *in vivo* link between renal hypertrophy and the progression of DN.

Our findings support the notion that the suppressive action of hnRNP F and hnRNP K on AGT expression in RPTs may play an important role in DN in animal models. They also raise the possibility that hnRNP F or hnRNP K dysregulation in the RPTs of Akita or db/db mice could be a major risk factor in kidney injury in diabetes.

5.6 Conclusions

The following conclusions can be drawn from our studies:

- Two nuclear proteins that bind to rAGT-IRE have been identified as hnRNP F and hnRNP K by a combination of Southwestern blotting and proteomics. Both proteins negatively modulate AGT gene expression in IRPTCs. High glucose upregulates and insulin downregulates hnRNP F and hnRNP K expression in IRPTCs. hnRNP F and hnRNP K are detected in various rat tissues that express AGT mRNA.

- hnRNP K interacts with hnRNP F and TBP. Overexpression of both hnRNP F and hnRNP K in IRPTCs suppresses AGT expression.
- hnRNP F overexpression attenuates high glucose-induced RPTC hypertrophy via AGT inhibition in RPTCs both *in vitro* and *in vivo*.
- The expression of endogenous hnRNP F and hnRNP K in RPTs of diabetic animals is lower than in nondiabetic animals, suggesting that hnRNP F and hnRNP K dysregulation may constitute risk factors in the progression of nephropathy in diabetic animals.

Chapter 6: Perspectives of Research

To clearly understand the mechanism of AGT gene regulation under glucose and insulin stimulation via hnRNP F and hnRNP K, several additional experiments should be performed.

6.1 Correlation between hnRNP F/hnRNP K and RAS Gene Expression in Diabetic Animals *in vivo*

The spontaneously diabetic Akita mouse model has an insulin gene2 mutation that is inherited by an autosomal dominant trait with a decrease in the number of β-cells of the pancreatic islets (361). Our preliminary data reveal increased AGT mRNA expression in RPTCs of diabetic Akita mice, db/db mice and BB rats compared to wild-type controls. In contrast, hnRNP F and K mRNA expression is significantly lower in RPTs of diabetic animals. We postulate that AGT mRNA overexpression is due to decreased hnRNP F or K expression in diabetic Akita mice. To test the hypothesis that re-introduction of hnRNP F in RPTs could rescue or prevent renal injury, male heterozygous Akita mice will be crossbred with homozygous female hnRNP F-Tg mice to produce hybrid Tg mice. The hybrids will be identified by Southern blotting of genomic DNA for KAP-hnRNP F transgene expression and by PCR of mutated insulin gene2.

Briefly, non-Tg Akita littermates and Tg Akita (KAP-hnRNP F) mice will be divided into 4 subgroups: 1. non-treated diabetic group; 2. insulin-treated diabetic group; 3. perindopril/losartan-treated diabetic group; 4. perindopril/losartan/insulin-treated diabetic group. All females but not males will also receive a testosterone pellet. At 2 and 6 months after treatment, the animals will be sacrificed after measurement of BP, blood glucose, GFR, and

the urinary albumin/creatinine ratio. Body and kidney weights will be recorded. Trunk blood will be collected for plasma AGT and Ang II analysis. The left kidney of each mouse will be removed and processed immediately by histology and immunohistochemistry of hnRNP F, hnRNP K, AGT, TGF-β1, collagen 1α (type IV) protein and cellular hypertrophy, as described in Chapter 4. The right kidney will be harvested for RPT isolation, total RNA extraction, and quantitative RT-PCR for AGT, TGF-β1, and collagen 1α (type IV) mRNA expression (see Chapter 4 for details).

The functional role of hnRNP F and K will also be tested by conditional knockout or siRNA technologies. These experiments will demonstrate whether hnRNP F is important in regulating ANG gene expression, RAS activation and subsequent renal injury in diabetes *in vivo*.

6.2 Identification of Transcriptional Factors Interacting or Associating with hnRNP F and hnRNP K *in vitro*

Experiments have shown that hnRNP F and K bind to TBP, associate with RNA poly II, and interact directly with nuclear CBC (320; 326; 327), suggesting that hnRNP F and K could modulate gene transcription via interaction with basal transcription factors. In Chapter 5, our preliminary data from pull-down assay have revealed that hnRNP K interacts with TBP. To confirm whether hnRNP F and K interact directly with TBP, RNA poly II and CBC as well as with other unidentified transcription factors in IRPTCs, we will incubate cellular nuclear proteins from hnRNP F and K stable clones with an equal amount of proteins of GST-hnRNP F or GST-hnRNP K or a combination of both in the absence or presence of ribonucleases in pull-down

assays. The immunoprecipitates will be subjected to Western blotting with antibodies against TBP, RNA poly II, and p20/80 CBC.

As some other factors could interact with hnRNP F or K, we will perform MALDI-MS. We will sequence the peptide in immunoprecipitates from GST-hnRNP F and GST-hnRNP K or from ChIP. Then, we will clearly know which other proteins are involved in transcription in association with hnRNP F and K *in vitro*. These data should discern details of AGT transcription mechanisms.

Meanwhile, we do not know the exact mechanisms whereby high glucose stimulates AGT expression through hnRNP F or K. We hypothesize that high glucose stimulates hnRNP F and K nuclear translocation via phosphorylation in RPTCs (Chapter 5).

6.3 Gene Chip Microarrays to Identify Downstream Genes that are Differentially Regulated by hnRNP F and hnRNP K in RPTCs

Both hnRNP F and hnRNP K could be involved in different cellular roles. It is not known whether other genes regulated by hnRNP F and K also have functions in AGT expression. To establish whether other genes besides AGT could be modulated by hnRNP F or K, we will isolate RNA from stable transfectants of hnRNP F and hnRNP K, or RPTCs from hnRNP F-Tg mice for gene chip microarray assay. Gene chip microarray is widely used to screen gene expression profiles under different conditions. Through microarray technology, we can acquire valuable information about

other genes regulated by hnRNP F and hnRNP K, which should help us know more about hnRNP F and hnRNP K functions.

6.4 Potential Gene Therapy with hnRNP F and hnRNP K

As mentioned above, AGT mRNA overexpression might be due to decreased hnRNP F or K expression in diabetic animals. The introduction of hnRNP F or K could prevent or attenuate hyperglycemic or other effects in diabetes, raising the possibility of developing gene therapy of the disease. Since data from clinical trials have disclosed that ARBs and ACEi are unable to cure diabetes in patients but significantly slow disease progression, combined treatment of RAS blockers and hnRNP F or K introduction may be successful. Obviously, more studies are needed to explore these possibilities.

Data from my studies have opened up a new area of research into renal RAS regulation. 2D gel electrophoresis, Southwestern blotting and MS for the identification of transcription factors represent powerful tools to clone unknown functional DNA-binding proteins. The kidney-specific expression of hnRNP F in Tg mice is an appropriate approach to investigating the functional roles of hnRNP F in the diabetic kidney *in vivo*. Our studies should garner novel information on the regulation of intrarenal AGT gene expression and RAS activation in the pathogenesis of DN.

Chapter 7: References

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1. Identification de l'étudiant et du programme

Chih-Chang Wei,
Ph.D. en Sciences biomédicales

2. Description de l'article

Chih-Chang Wei, Deng-Fu Guo, Shao-Ling Zgang, Julie R. Ingelfinger and John S.D. Chan: Heterogenous nuclear ribonucleoprotein F modulates angiotensinogen gene expression in rat kidney proximal tubular cells. J Am Soc Nephrol 16 :616-628, 2005.

3. Déclaration de tous les coauteurs autres que l'étudiant

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Chih-Chang Wei inclue cet article dans sa thèse de doctorat qui a pour titre Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat.

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1. Identification de la revue / Identification of the journal

Journal of Biological Chemistry
NIDDK, National Institutes of Health
Building 8, Room 223
Bethesda, MD 20892-0830
Tel:301-496-2562
[redacted]

2. Identification de l'éditeu[redacted] des éditeurs / Identification of the editors

Dr. Herbert Tabor

3. Identification de l'article / Identification of article

Chih-Chang Wei, Shao-Ling Zgang, Yun-Wen Chen, Deng-Fu Guo, Julie R. Ingelfinger, Karol Bomsztyk and John S.D. Chan: Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells. J Biol Chem 281 :25344-25355, 2006

L'étudiant Chih-Chang Wei est autorisé à inclure l'article ci-dessus dan sa thèse de doctorat qui a pour titre Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat.
(The student Chih-Chang Wei is authorized to include this article in his Ph.D. thesis which the title is "Cloning and characterisation of insulin responsive element-binding protein (IREBP) of angiotensinogen gene in rat.)

Éditor Signature Date

msn® Hotmail®

[redacted]

Printed: July 19, 2007 1:48:17 PM

From : [redacted]
Sent : July 18, 2007 3:04:18 PM
To : [redacted]
Subject : Re: Fw: REQUEST FOR PERMISSION

Dear Chih-Chang Wei,
As per this email you have permission to reprint:

Chih-Chang Wei, Shao-Ling Zgang, Yun-Wen Chen, Deng-Fu Guo, Julie R. Ingelfinger, Karol Bomsztyk and John S.D. Chan: Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells. J Biol Chem 281 :25344-25355, 2006

For your Ph.D. thesis. There is no fee for this use.

Sincerely,
Nancy

Nancy Rodnan , Director of Publications
American Society for Biochemistry and Molecular Biology
9650 Rockville Pike, Bethesda, MD, 20814
Ph. 301-634-7145
www.asbmb.org

www.jbc.org www.mcponline.org www.jlr.org

----- Forwarded by Mail To ASBMB/ASBMB on 07/18/2007 02:49 PM -----
"chihchang wei" <chihchangwei@hotmail.com>

07/18/2007 01:19 PM

To [redacted]
cc
Subject REQUEST FOR PERMISSION

Dir Sir,

I would like to request permission to reprint the manuscript list below as part of my Chih-Chang Wei) Ph.D. thesis.

Chih-Chang Wei, Shao-Ling Zgang, Yun-Wen Chen, Deng-Fu Guo, Julie R. Ingelfinger, Karol Bomsztyk and John S.D. Chan: Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells. J Biol Chem 281 :25344-25355, 2006

Could you please sign on page 2 and email me back.

Thank you very much for your attention and assistance.

Yours truly,

Chih-Chang Wei

ANNEXE II

ACCORD DES COAUTEURS ET PERMISSION DE L'ÉDITEUR

A) Déclaration des coauteurs d'un article

Lorsqu'un étudiant n'est pas le seul auteur d'un article qu'il veut inclure dans son mémoire ou dans sa thèse, il doit obtenir l'accord de tous les coauteurs à cet effet et joindre la déclaration signée à l'article en question. Une déclaration distincte doit accompagner chacun des articles inclus dans le mémoire ou la thèse.

1. Identification de l'étudiant et du programme

Chih-Chang Wei,
Ph.D. en Sciences biomédicales

2. Description de l'article

Chih-Chang Wei, Fang Liu, Shao-Ling Zgang, Julie R. Ingelfinger and John S.D. Chan: Heterogenous Nuclear Ribonucleoprotein F Overexpression Attenuates Angiotensinogen Expression and Renal Proximal Tubular Cell Hypertrophy in Transgenic Mice. 2007, soumis pour publication.

3. Déclaration de tous les coauteurs autres que l'étudiant

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Chih-Chang Wei inclue cet article dans sa thèse de doctorat qui a pour titre Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat.

[redacted] July 11 2007

July 12, 2007

07/11/07

John S.D. Chan, Signature, Date

ANNEXE II

ACCORD DES COAUTEURS ET PERMISSION DE L'ÉDITEUR

A) Déclaration des coauteurs d'un article

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[redacted] July 11 2007

July 12 2007

9-20-07

07/11/07

DEMANDE D'AUTORISATION DE RÉDIGER PAR ARTICLES

Annexe III

1. Identification de l'étudiant

   Chih-Chang Wei
   [redacted]

2. Nom de l'unité académique

   Faculté de médecine

3. Nom du programme

   Programme Sciences biomédicales

4. Liste des articles proposés

   Chih-Chang Wei, Deng-Fu Guo, Shao-Ling Zgang, Julie R. Ingelfinger and John S.D. Chan: Heterogenous nuclear ribonucleoprotein F modulates angiotensinogen gene expression in rat kidney proximal tubular cells. J Am Soc Nephrol 16:616-628, 2005.

   Chih-Chang Wei, Shao-Ling Zgang, Yun-Wen Chen, Deng-Fu Guo, Julie R. Ingelfinger, Karol Bomsztyk and John S.D. Chan: Heterogeneous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells. J Biol Chem 281:25344-25355, 2006

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5. Signature et déclaration de l'étudiant concernant les articles

   [redacted]

6. Avis du directeur de recherche

   [redacted]

7. Décision ou recommandation et signature du directeur du programme

   [redacted]

   Da[redacted] 23 juillet 2007

To whom it may concern

We, the undersigned, agree and consent that Mr. Chih-Chang Wei can use the following articles in his Ph.D thesis entitled"Clonage et caractérisation des protéines liant l'élément de réponse à l'insuline (IREBP) du gène de l'angiotensinogène chez le rat "to be submitted to the Faculité des études supérieures, Université de Montréal.

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Name	Signature	Date
John S.D. Chan..	[redacted]	le 31 mai 2007
Deng-Fu Guo....		05-06-2007
Julie R. Ingelfinge		
Karol Bomsztyk...		
Shao-Ling Zhang		le 31 mai 2007
Yun-Wen Chen...		01-06-2007
Fang Liu.....		31/May/2007

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John S.D. Chan	[redacted]	le 31 mai 2007
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Julie R. Ingelfing		June 6, 2007
Karol Bomsztyk		
Shao-Ling Zhang		le 31 mai 2007
Yun-Wen Chen		31-05-2007
Fang Liu		31/May/2007

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Fang Liu.....		31/May/2007