Calreticulin upregulation in renal fibrosis

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EuroKUP meeting, Madrid, Spain, 17-19 June 2011
Fibrosis

Fibrosis is defined as the accumulation of extracellular matrix leading to structural and functional alterations of several organs such as lung, heart, kidney, pancreas, liver.

**Kidney Fibrosis**

- is a common feature of Chronic Kidney Diseases [CKD] (which affect 10-12% of the population) and is characterized by progressive loss of kidney function and relentless accumulation and deposition of ECM
- is considered to be an irreversible process that leads to end-stage renal failure and requires expensive and life-long treatments with dialysis or transplantation
- is increasing at a rate of approximately 7% per year among the world population

**Causes of kidney fibrosis**

diabetes, hypertension, infection, inflammation of renal blood vessels and glomeruli, kidney stones, cysts, genetic mutations.
Why do organs in the body develop fibrosis?

A wound-healing response that has gone out of control

45% of all the deaths in the developed world can be attributed to some form of fibroproliferative disease
The Unilateral Ureteral Obstruction (UOO) model of kidney fibrosis

• in vivo model
• encompasses many aspects of other models of kidney fibrosis
• there are features that occur within 1 week
• mimics in a short time a situation that can take years in humans
• leaves one kidney intact
• there is evidence that animal models with UOO are reflective of the molecular changes in human situations
Proteomic analysis

Kypreou K. et al., *Proteomics*, 2008
**Proteomic Approach**

### Classical 2DE

1\textsuperscript{st} dimension: IEF (pI 3-10)

2\textsuperscript{nd} dimension: Gel electrophoresis

PD Quest Image processing Software

Proteins altered in all repeats of the experiment (3 different animals from each group) were selected for further evaluation

Spot excision and tryptic digestion

MALDI-TOF/TOF-MS

MASCOT Software for protein identification
Venn diagram of identified proteins
CALRETICULIN

Sham operated 2 days

Ligated 2 days

Sham operated 8 days

Ligated 8 days
Calreticulin is a multifunctional protein

- First isolated in 1974 as a high-affinity Ca\textsuperscript{2+}-binding protein of the ER

There is no correlation with fibrotic processes yet!
Confirmation of Calreticulin upregulation in fibrotic samples

Quantification of calreticulin protein expression

Quantification of calreticulin mRNA expression

Kypreou K. et al., Proteomics, 2008
Why is Calreticulin upregulated in kidney fibrosis?

- Which is the mechanism of Calreticulin upregulation?
- What is the role of Calreticulin in fibrosis?
1. WHICH IS THE MECHANISM OF CALRETICULIN UPREGULATION?

Transforming Growth Factor-β (TGF-β) induces fibroblasts to synthesize ECM and has long been considered as a central mediator of the fibrotic response.
Transforming Growth Factor-β (TGF-β) induces HK-2 cells to produce Calreticulin

Sham operated  Ligated 2 days  Ligated 8 days

Kypreou K. et al., Proteomics, 2008
TGF-β-mediated Calreticulin induction is accompanied by Epithelial to Mesenchymal Transition in HK-2 cells
Epithelial to Mesenchymal Transition

- **I. Loss of epithelial adhesion**: TGF-β1 induces the loss of epithelial adhesion.
- **II. De novo αSMA expression and actin reorganization**: E-cadherin expression decreases, leading to actin reorganization.
- **III. Enhanced cell migration and invasion**: TBM disruption facilitates cell migration and invasion.
- **IV. TBM disruption by MMP-2**: MMP-2 activity disrupts the tissue basement membrane.
TGF-β induces Calreticulin mRNA expression in HK-2 cells

Quantification of Calreticulin mRNA expression by Real-time RT-PCR

What is upstream of this induction?
TGF-β Signaling Pathway

TGF-β Ligands: TGF-βs, Activins, Nodals

Type II Receptor -> Type I Receptor

RhoA, mDia, ROCK, PAK, Par6, PKC -> Smad2/3

SARA -> Smad2/3

Ras -> Smad1/5/8

Smad7 -> Erk

Smad6, Smad4, Smad2/3, Smad5/8 -> BMP Ligands: BMP-2, -4, -7, MIS

MLC, LIMK, Cofilin -> Actin Polymerization, Stress Fibers

Cell Adhesion

Transcription Factors: AP-1, bZIP, RUNX, Fox, nuclear receptors, IRF-7, TGF, SIPI, Tob (BMP only)

Corepressors: c-SkI/SnoN, c-Myc, E2F1, TGF, SIPI

Coactivators: CBP/p300, SMIF, MSG1, ARCl05
Bioinformatics analysis on Calreticulin promoter

- [www.genomatix.de](http://www.genomatix.de): Gene2Promoter

- **4 organisms:**

- **Promoter sequence:** 2000bp upstream and 1000bp downstream the TSS
Calreticulin promoter sequence contains TF binding sites that act downstream of the TGF-β signaling.
Cloning of the human Calreticulin promoter sequence

-1800 -1500 -1000 -500 +86

Smad -1744
Myc -1080
C/EBP -926
E2F4

TATA -27
YY1 -175
E2F -318
Sp1 -305
Sp1 -251
Sp1 -71

Myc -282
Myc -223

-1500
Smad -1744
Myc -1080
C/EBP -926
E2F4

-1000
Smad -897
Smad -706
Smad -691

-500
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-1000
Smad -897
Smad -706
Smad -691

-500
Smad -291

-500
Smad -291

+1

-1500
-1000
-500
+86

CALR exon 1

pGL3-Basic

KpnI SacI MluI Nhel SmaI XhoI BglII

MCS

Luc
TGF-β induces Calreticulin promoter activity in HK-2 cells

Calreticulin promoter activity upon TGF-β stimulation in HK-2 cells

Normalized luciferase activity (RLU/β-gal)

pGL3-CALR/-TGF-β

pGL3-CALR/+TGF-β

TGF-β regulates Calreticulin at the level of transcriptional activation
Calreticulin promoter is regulated by transcription factors E2F4 and p107

- E2F4 and p107 act downstream the TGF-β signaling pathway
- E2F4 has potential binding sites on Calreticulin promoter
- E2F4 and its cytoplasmic binding partner p107 are cell cycle regulators
Calreticulin promoter includes activator and repressor elements

- The region from -1215 to -1800 contains transcriptional activator element(s)
- The region from -226 to -522 contains transcriptional repressor element(s)
- The fragment -226/+86 harbors the basal/core promoter sequence whose activity is altered by upstream activator and repressor elements
Calreticulin promoter includes activator and repressor elements

Deletion constructs activity in HK2 cells

<table>
<thead>
<tr>
<th>Deletion Constructs</th>
<th>Normalized Luciferase Activity (RLU/β-gal)</th>
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</thead>
<tbody>
<tr>
<td>pGL3-CALR</td>
<td>14000000</td>
</tr>
<tr>
<td>pGL3-(-1215/+86)</td>
<td>12000000</td>
</tr>
<tr>
<td>pGL3-(-522/+86)</td>
<td>10000000</td>
</tr>
<tr>
<td>pGL3-(-226/+86)</td>
<td>8000000</td>
</tr>
<tr>
<td>pGL3-(-165/+86)</td>
<td>6000000</td>
</tr>
<tr>
<td>pGL3-(-66/+86)</td>
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</tr>
</tbody>
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Calreticulin transcription

Activator: -1800 to -1215
Repressor: -1215 to -522
Basal promoter: -522 to -226
TATA: -226 to +1

(+): Activation
(-): Repression

transcription
Calreticulin gene regulation is even more complicated...

...representing the multiplicity of Calreticulin functions
Which is the mechanism of Calreticulin upregulation in fibrosis?

- TGF-β is likely to play a role.

- Other factors should also play a role, taking into consideration the complex nature of both fibrosis and Calreticulin regulation.

- More signaling pathways and transcription factors that upregulate Calreticulin gene expression need to be studied, and corresponded to specific regulatory elements on Calreticulin promoter.
Acknowledgements

**Principal investigators**
- Dr. Aris Charonis
- Dr. Panos Politis

**Lab members**
- Dr. Katerina Kypreou
- Valeria Kaltezioti
- Fani Karagianni
- Panos Kavvadas
- Thanassis Stergiopoulos
- Zozefina Foskolou
- Lila Kaltsa
- Ismini Rozani
- Elena Frangou

**Center for Experimental Surgery**
- Michalis Katsiboulas

**Histochemistry Core Facility**
- Anna Agapaki

**Collaborators**
- Prof. D. Vlahakos, Attikon General Hospital, University of Athens

**Funding**
- BRFAA
- Bodossakis Foundation
- European Social Fund: NSRF-Heracleitus II