UNRAVELING CHRONIC KIDNEY DISEASE: EVIDENCE FROM TGF-β-INDUCED IN VITRO AND IN VIVO FIBROSIS MODELS AND META-ANALYSES

PhD thesis

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1. INTRODUCTION

Chronic kidney disease (CKD) is a significant health challenge that affects 13.4 percent of the global adult population. It is a leading cause of mortality, and patients often face irreversible structural and functional losses in their kidneys. However, recent research has shed light on several promising avenues for tackling kidney fibrosis. These include exploring TGF-β-induced renal fibrosis models, which have helped researchers gain a deeper understanding of the molecular basis of this condition. Dysregulation of autophagy and miRNAs also appear to play critical roles in kidney injury besides well-known fibrotic and inflammatory processes, and further study in this area may yield new insights into the development of diagnostic markers and therapeutic agents. In addition to novel therapeutic approaches, repurposing existing medications is a timeless strategy for enhancing health outcomes — for example, the anti-diabetic PPARγ agonist pioglitazone is known to have anti-fibrotic effects.

This thesis will elucidate four projects focused on different aspects of kidney fibrosis: 1) HK-2 cell response to TGF-β1 in six cell culture media, 2) PPARγ agonist, pioglitazone effect on pro- and anti-fibrotic markers, miRNA dysregulation and autophagy function during TGF- β induced kidney fibrosis, 3) two systematic reviews and meta-analyses on miRNA dysregulation during CKD - identification of most dysregulated miRNAs and diagnostic accuracy of miRNAs.

The HK-2 cells are often used to study EMT *in vitro*. However, different studies have used different types of cell culture media, which might cause inconsistent experimental results. To address this issue, we conducted a comparative study investigating how different culture media affect the HK-2 cells when exposed to TGF-β1.

PPARγ plays a crucial role in maintaining normal kidney function and homeostasis. Recently, our research group studied the anti-fibrotic effect of PPARγ agonist in TGF-β transgenic mice by examining pro-fibrotic markers. However, we have yet to address the impact of PPARγ agonists on primary tubular epithelial cells (TECs), renal autophagy, and miRNA dysregulation during fibrosis. By highlighting miRNA dysregulation, we aim to elucidate the effect of PPARγ on pro- and anti-fibrotic markers and autophagy in TGF-β-induced kidney tubular cells and tissue from TGF-β transgenic mice.

MiRNAs have emerged as potential markers and therapeutic agents for kidney diseases. However, with over 2000 miRNAs in humans and mice, it is unclear which ones play a key role in kidney diseases. We aimed to identify the most dysregulated miRNAs in human CKD and murine models of CKD, along with their molecular pathways, and to assess the diagnostic accuracy of miRNAs in human CKD from peer-reviewed articles using two different meta-analysis approaches.

2. OBJECTIVES

We aimed to answer the following questions.

1. How does cell culture medium formulation affect TGF-β1-induced EMT in HK-2 cells?
2. How does pioglitazone treatment impact TGF-induced kidney fibrosis involving inflammation, autophagy, and miRNA dysregulation in vitro and in vivo?
3. What are the most dysregulated miRNAs in human and murine CKD, and what are their enriched molecular pathways?
4. What is the diagnostic accuracy of miRNAs in CKD?

3. METHODS

HK-2 cell culture: HK-2 cells were cultured in six different growth media (Table 1). The cells were then treated with 10 ng/ml of recombinant human TGF-β1 for 24 hours, and microphotographs of treated and untreated cells were taken. Finally, the cells were harvested for total RNA isolation or protein extraction.

Primary TEC Isolation and Cell Culture: A 4-week-old C57B1/6J male mouse was euthanized, and both kidneys were quickly removed after median laparotomy under aseptic conditions. TECs with passage numbers 6 to 8 were used. The day after the cell seeding, the medium was changed to serum-free DMEM/F12 and the cells were subjected to pre-treatment with 5 μM pioglitazone in 0.1% DMSO or DMSO alone (controls). After 24 hours of serum starvation, recombinant human TGF-β1 (10 ng/mL) was added and incubated for 24 hours (n = 3–4/group).

Table 1. Cell culture media and their formulation.

Medium abbreviations	Formulation
DMEM 2%	DMEM with 1000 mg/L glucose, L-glutamine, sodium bicarbonate (Invitrogen, Carlsbad, CA), and 2% fetal bovine serum (Invitrogen), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)
DMEM 5%	DMEM with 1000 mg/L glucose, L-glutamine, sodium bicarbonate (Invitrogen, Carlsbad, CA), and 5% fetal bovine serum (Invitrogen), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)
DMEM 10%	DMEM with 1000 mg/L glucose, L-glutamine, sodium bicarbonate (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Invitrogen), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)
DMEM/F12 10%	DMEM/F12 medium contains a 1:1 mixture of DMEM medium and Ham's F12 medium, With L-glutamine, 15 mM HEPES, sodium bicarbonate (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Invitrogen), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)
PTEC (hormonally defined)	DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (Invitrogen), 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 40 ng/mL hydrocortisone, 5 pg/mL triiodo-1-thyronine, 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)
KSFM	Keratinocyte Serum-Free Growth Medium (Invitrogen, Carlsbad, CA) supplemented with 0.05 mg/ml Bovine Pituitary Extract, 5 ng/ml Human Recombinant epidermal growth factor (Invitrogen, Carlsbad, CA), 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen)

Animal experiments: Ten-week-old male TGF-β1 transgenic mice on C57Bl6/J genetic background (B6-Alb/TGF-β1(Cys^{223,225}Ser) backcrossed in our laboratory and wild-type C57Bl6/J males of the same age (controls, CTL, n = 6) were kept in a standard specific pathogen-free environment at the Semmelweis University NET GMO Animal Facility (Approval, PE/EA/948-4/2018). Transgenic mice were separated into two groups: one received standard rodent chow (TGF, n = 6), and the other received pioglitazone-containing rodent chow (TGF + Pio, n = 6) for chronic treatment (20 mg/kg/day, adjusted to average food consumption). The mice were euthanized with isoflurane after 6 weeks, and the kidneys were collected and frozen in liquid nitrogen or fixed in a 4% buffered formalin solution.

RNA Isolation and Quantitative RT-PCR Analysis: RNA was reverse transcribed using specific kits, and PCR reactions were performed using the SensiFast SYBR Green PCR Master Mix. The target gene expression was normalized to the appropriate housekeeping gene and expressed relative to a control sample.

Immunoblot: Proteins were extracted from cells, loaded on gels, and transferred to membranes. The membranes were incubated with primary and secondary antibodies and visualized using a chemiluminescence detection kit. Data was evaluated using Image Studio Lite 5.2 software.

Immunocytochemistry: After methanol fixation, permeabilization with 0.25% Triton-X, and cells blocked with primary (overnight at 4 °C) and secondary antibodies. Next, coverslips were mounted with Vectashield containing DAPI. The protein expression of EGR1 and TGF-β for HK-2 cells and EGR2, vimentin, and SQSTM for primary TECs were assessed using immunofluorescence. Cells were visualized and photographed under UV light using a Leica DMR-HC microscope at 400 × magnification.

Renal Histology and Immunohistochemistry: Masson's trichrome stain is used for formalin-fixed and paraffin-embedded kidney slices. Glomerulosclerosis and tubulointerstitial damage were measured using a semi-quantitative scale. SQSTM1 immunofluorescent staining was done on paraffin-embedded sections. ImageJ v1.53 software was used to analyze the vimentin and SQSTM1-positive stained area at 400x magnification.

Statistical analysis: Experimental data are presented as mean ± SD. Statistical analysis was performed using IBM SPSS. The Shapiro-Wilk test was performed to analyze the normal distribution of the data. For the HK-2 experiments, pairwise comparisons were conducted using the independent samples Kruskal-Wallis and Mann-Whitney U test and Bonferroni multiple comparison corrections, as indicated. Pearson's correlation was used to test the relationship between continuous and discrete variables. For the primary TECs and TGF-β transgenic mice experiments, oneway ANOVA and Holm–Sidak post hoc tests were used. The significance level was $p < 0.05$ at the 95% confidence level.

Meta-analysis – systematic search: Methods were specified in a protocol registered with the PROSPERO International Prospective Register of Systematic Reviews (CRD42021283763 and CRD42021282785).

Meta-analysis eligibility criteria: The first meta-analysis included patients with CKD and a murine experimental model (kidney fibrosis) with healthy control groups. The s***econd meta-analysis included*** individuals with (case group) and without CKD (healthy and diseased control groups) who had undergone miRNA detection test by qRT-PCR, and diagnostic accuracy (AUC, ROC curve, sensitivity (SEN) and specificity (SPE)) of miRNAs is presented.

Meta-analysis statistical analysis: The first meta-analysis used robust rank aggregation (RRA) and the vote-counting method to identify the most dysregulated miRNAs. Next, gene set enrichment analysis was

conducted utilizing the DIANA-miRPath v4.0 tool. The second meta-analysis involved univariate and multivariate analyses of AUC and pooled SEN and SPE with ROC plot visualization for single and panel miRNAs in different kidney diseases. The statistical analysis was performed using the R software.

4. RESULTS

4.1. Results 1 (EMT in HK-2 cells)

First, we observed the effect of six culture medium formulations on cell morphology. Cells without TGF-β1 showed epithelial morphology and cells treated with TGF-β1 (10 ng/ml) for 24 hours were elongated in all culture media. However, HK-2 cells cultured in DMEM 5% FBS appeared round-shaped and polygonal, but cells in KFSM looked smaller and did not divide as much as cells cultured in FBS-supplemented media.

In TGF-β-induced HK-2 cells, the *TGFB1* gene, protein, and mesenchymal markers were highly expressed (**Fig. 1**). At the mRNA level, the EMT-related changes were consistent across all culture media. However, the vimentin protein expression was elevated in all media except DMEM 5% FBS by immunoblotting. TGF-β1 partially induced the mRNA expression of *FN* in PTEC 2%, DMEM F12 10%, and KFSM) and the protein expression in almost all media except DMEM/F12 10% and KFSM. Still, by gene expression and immunoblot analysis, we observed TGF-β1 protein expression induced by TGF-β1 treatment independent of the culture medium (**Fig. 1**).

CTGF, *COL4A1*, and EGR2 expressions were significantly elevated in the EMT model, whereas *PPARG* decreased in all culture media. Among the inflammatory markers, *IL6* mRNA was not induced in TGF-β1-treated HK-2 cells cultured in PTEC 2% medium in addition to the DMEM 2%. The expression of the complement C3 protein-coding gene, *C3*, decreased in KSFM and increased in DMEM 5% but did not change in any other media upon TGF-β1 treatment.

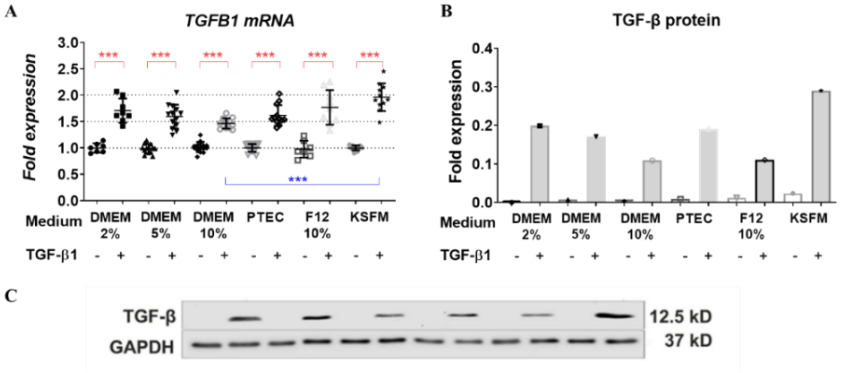

Figure 1. TGFB1 mRNA and protein (*TGF-β*) expression of HK-2 cells in different medium formulations. *A. TGFB1 mRNA, B.* TGF-β1 *protein expression, and C. Immunoblot. Data are shown as mean ± SD; Kruskal-Wallis test, $p < 0.05$*, $p < 0.01$** and $p < 0.001$***, red: control vs. TGFb groups (n = 7–16/group), blue: TGFb vs. TGFb.*

Interestingly, medium formulations had a substantial impact on pro-fibrotic *EGR1* expression. TGF-β1 treatment significantly increased *EGR1* mRNA expression only in DMEM 5% and DMEM/F12 10% media but decreased *EGR1* in PTEC 2% and DMEM 10%. Based on the

differential expression of mRNAs in culture medium formulations, we investigated the expression pattern of EGR1 in TGF-β-induced HK-2 cells by immunofluorescence. TGF-β1 induced EGR1 translocation to the nucleus, indicating transcriptional activation in DMEM F12 10% and KFSM media. Cells grown in other media (DMEM 2%, 5%, 10%, and PTEC 2%) showed only cytoplasmic overexpression of EGR1.

4.2. Results 2 (PPARγ agonist in kidney fibrosis)

Chronic pioglitazone treatment significantly reduced the extent of TGF-β-induced glomerulosclerosis and reversed tubulointerstitial damage induced by TGF-β (**Fig. 2A-C**).

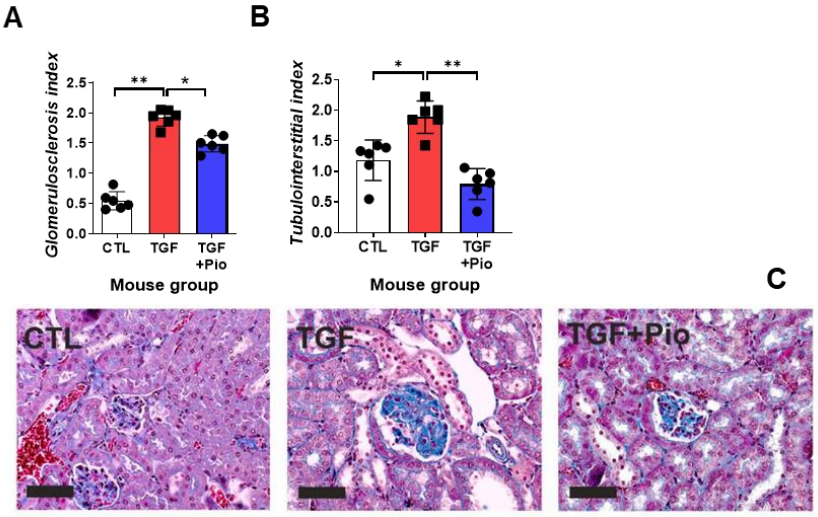

Figure 2. Mouse kidney immunohistochemistry *is shown in (A) glomerulosclerosis index, (B) tubulointerstitial index, (C) dilatation, and epithelial cell desquamation.*

TGF-β1 induction increased the expression of pro-fibrotic mRNAs (*Tgfb1, Ctgf, Col1a1, Runx1, Mmp2, and Acta2*) in primary TECs, which were effectively inhibited by pioglitazone therapy (**Fig.3**).

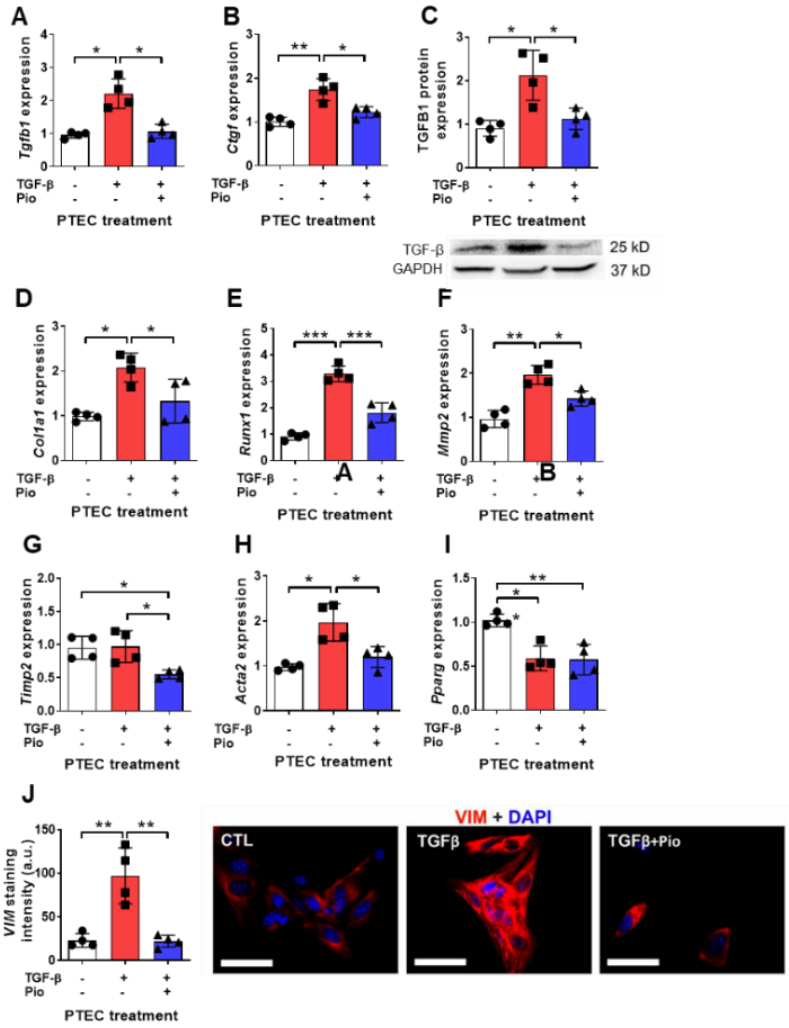

Figure 3. The effect of pioglitazone on the fibrotic response and EMT of primary murine TECs. *Bar charts represent TECs treated with TGF-*

*β1 for 24 h (10 ng/mL) and pioglitazone (5 μM) (n = 4/group): expression of mRNAs. Mean ± SD, One-way ANOVA, and Holm–Sidak post hoc test: *: p < 0.05; **: p < 0.01; ***: p < 0.001.*

While TGF-β1 did not enhance the mRNA expression of the *Mmp2* inhibitor *Timp2*, pioglitazone decreased its expression to lower levels than the control (**Fig.3G**). The protein expression of TGF-β1 and vimentin was also induced in the TGF-β1 group and decreased in pioglitazone treatment in vitro (**Fig.3J**). We observed similar results on *Mmp2* and *Runx1* in the kidney of TGF-β transgenic mice. *Timp2* was induced in the TGF-β transgenic mice and completely abolished by pioglitazone treatment (data is not shown).

Interestingly, the nearly 50% reduction in *Pparg* expression upon TGF-β1 administration was not influenced by pioglitazone treatment *in vitro* (**Fig. 3I**). In contrast, pioglitazone treatment in vivo practically normalized the TGF-β-induced massive reduction of renal *Pparg* expression.

Pioglitazone had beneficial effects on *Egr1* and *Stat3* mRNA expression in primary TECs (data is not shown). Pioglitazone treatment reduced the up-regulation of TGF-β1-induced *Egr2* mRNA and EGR2 protein expression both in vitro and in vivo.

Pioglitazone has effectively reduced miR-199a-3p, miR-199a-5p, and miR-130a in addition to the miR-21-5p (already known pro-fibrotic

miRNA) in the primary TECs and kidneys of TGF-β transgenic mice when TGF-β induced them (**Fig. 4**).

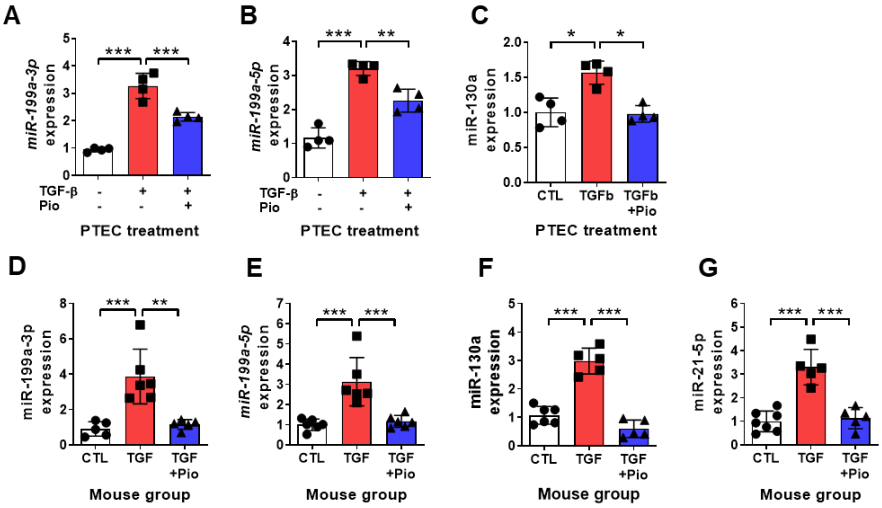

Figure 4. Pioglitazone attenuates the TGF-β induced dysregulation of pro-fibrotic miRNAs in vitro and in vivo. *Charts A-C represent primary TEC treatment groups (n = 4/group; TGFβ: 10 ng/mL; Pio: 5 μM pioglitazone):* ***(A)*** *miR-199a-3p,* ***(B)*** *miR-199a-5p and* ***(C)*** *miR-130a. Charts D-G represent mouse groups (n = 6/group): CTL: wild-type controls, TGF: untreated TGF-β transgenic mice; TGF + Pio: pioglitazone-treated (20 mg/kg/day, p.o.) TGF-β transgenic mice:* ***(D, E)*** *miR-199a,* ***(F)*** *miR-130a, and* ***(G)*** *miR-21-5p. Target miRNA expressions were normalized to U6 snRNA. Data are presented as mean ± SD, One-way ANOVA, and Holm–Sidak post hoc test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.*

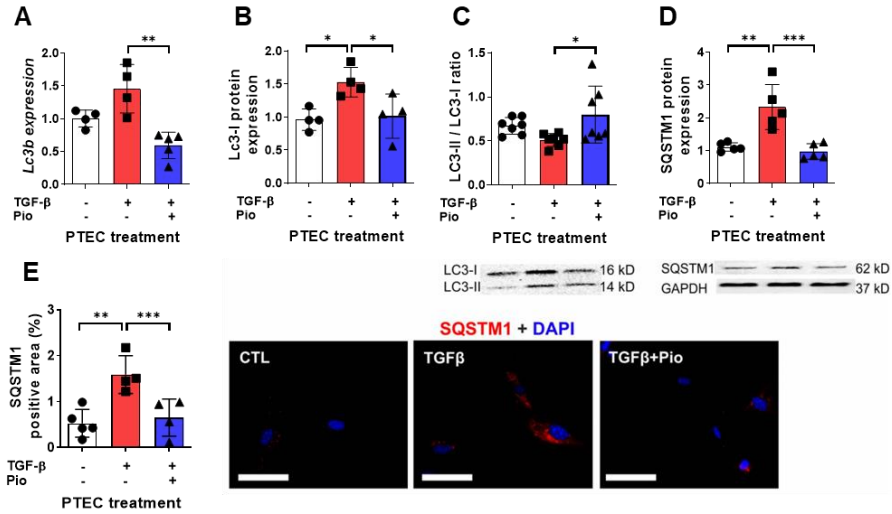

Figure 5. Pioglitazone protects primary TEC from TGF-β-induced autophagy dysfunction in vitro. *Charts represent primary TEC treatment groups. (n = 4/group; TGF-β1: 10 ng/mL; Pio: 5 μM pioglitazone):* ***(A)*** *Lc3b mRNA,* ***(B)*** *LC3-I protein,* ***(C)*** *LC3-II/LC3-I ratio,* ***(D, E)*** *SQSTM1 protein. Target gene expressions were normalized to 18S rRNA. SQSTM1 staining was evaluated as the percentage of positively stained cytoplasmic area using ImageJ v1.53 (SQSTM1: red; nuclear staining (DAPI, blue), 400× magnification, white scale bars represent 20 μm). Data are presented as mean ± SD, One-way ANOVA, and Holm–Sidak post hoc test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.*

We measured the levels of LC3 and SQSTM1 protein to evaluate the impact of pioglitazone on impaired autophagy in renal tubules both in vitro and in vivo. Results showed that autophagy degradation was not

stimulated (decreased LC3-II/I ratio and accumulation of SQSTM1) despite the increased expression of LC3 in primary TECs exposed to TGF-β1 and in the kidneys of TGF-β transgenic mice (**Fig. 5**). However, long-term administration of pioglitazone restored the impaired autophagy induced by TGF-β. In TGF-transgenic mice, kidneys showed increased *LC3b* and *Sqstm1* mRNA expression levels, but this didn't increase the autophagy degradation rate (data is not shown).

Pioglitazone has been found to have an anti-inflammatory effect. In vitro and in vivo, pioglitazone reduced TGF-β1 induced *Ccl2*, *Lgals3*, and pro-inflammatory cytokine *Il6* mRNA overexpression (data is not shown). Renal C3 expression increased in TGF-β transgenic animals, but it is unlikely attributable to modifications in tubular cells (data is not shown).

4.3. Results 3 (miRNA in kidney diseases, meta-analysis)

First systematic review and meta-analysis:

38 human (762 cases, 671 controls) and 12 murine (70 cases, 66 controls) eligible studies were analyzed. Down-regulated miRNAs in human CKD were: urinary miR-181a-5p; blood miR-451a, miR-106b-5p; renal miR-486-3p, miR-486-5p, miR-451a. The most up-regulated miRNAs were urinary miR-27a-3p, circulating miR-1260b, tissue miR-155-5p and miR-21-5p. Mouse kidneys had down-regulated miR-20a-5p, miR-709, miR-429-3p and up-regulated miR-21a-5p, miR-223-3p, miR-342-3p, miR-214-3p, and miR-199a-5p. Additionally, early CKD stages depicted

up-regulated urinary miR-27b-3p and circulating miR-122-5p. Enrichment analysis revealed apoptosis, TNF-α signaling via NF-kB, G2M checkpoint, EMT, TGF-β signaling, hypoxia, and cholesterol homeostasis known to be involved in CKD pathogenesis as the most affected pathways in both species.

4.4. Results 4 (miRNA in kidney diseases, meta-analysis)

Second systematic review and meta-analysis:

Eighty-seven articles (from 2013 to 2022) reported 238 single and 34 panel miRNA results, which were included in the analysis. We analyzed data from 8351 CKD patients, 2989 healthy individuals, and 4331 diseased controls. Among single miRNAs, pooled SEN was 0.82, and SPE was 0.81 for DN vs. diabetes mellitus (DM). SEN and SPE were 0.91 and 0.89 for DN vs. healthy control, respectively. MiR-192 was the most frequently reported in DN, with pAUC of 0.91 and SEN and SPE of 0.89 and 0.89, respectively, compared to healthy controls. Panel miRNAs outperformed single miRNAs (pAUC of 0.86 vs 0.79, $p<0.05$). SEN and SPE of panel miRNAs were 0.89 and 0.73 in DN vs. DM, respectively. In lupus nephritis (LN) vs. systemic lupus erythematosus (SLE), the SEN and SPE of single miRNAs were 0.84 and 0.81, respectively. Urinary miRNAs tended to be more effective than blood miRNAs ($p=0.06$).

5. CONCLUSIONS

1. The behavior of HK-2 cells during TGF-β1-induced in vitro EMT can be affected by the cell culture medium formulations, which should be carefully selected in translational research.

2. Pioglitazone has potential anti-fibrotic effects in TGF-β induced kidney fibrosis and can regulate pro-fibrotic transcription factors, miRNA, inflammation, and macroautophagy function.

3. Dysregulated miRNAs are common during CKD and are mostly enriched in the pathways involved in kidney pathogenesis.

4. Panel miRNAs have better diagnostic accuracy than single miRNAs in CKDs.

The novelty of the thesis:

- Highlighting the variety of cell culture medium-related biases in the TGF-β-induced EMT model *in vitro*.
- miR-199-3p and miR-199-5p are involved in TGF-β-induced kidney fibrosis *in vitro* and *in vivo*. The antifibrotic effect of PPARγ might be related to miR-199 and its target genes.
- Using systematic review and meta-analysis approaches to identify most dysregulated miRNAs from studies focused on hypothesis-free assays and identifying targeted molecular pathways are relatively new methods in translational nephrology.

6. PUBLICATIONS

Publications related to the thesis:

1. **Garmaa G**, Manzéger A, Haghighi S, Kökény G. HK-2 cell response to TGF-β highly depends on cell culture medium formulations. Histochemistry and Cell Biology. 2024;161(1):69-79. Impact factor, journal quartile – 2.3, Q1.
2. Manzéger A, **Garmaa G**, Mózes MM, Hansmann G, Kökény G. Pioglitazone Protects Tubular Epithelial Cells during Kidney Fibrosis by Attenuating miRNA Dysregulation and Autophagy Dysfunction Induced by TGF-β. Int J Mol Sci. 2023;24(21). Impact factor, journal quartile – 5.6, D1.
3. **Garmaa G**, Bunduc S, Kói T,. Hegyi P, Csupor D. Ganbat D,. Dembrovszky F, Meznerics F.A, Nasirzadeh A,. Barbagallo C, Kökény G. A Systematic Review and Meta-Analysis of microRNA Profiling Studies in Chronic Kidney Diseases. Non-Coding RNA 2024; 10 (3)30 - Impact factor, journal quartile – 4.3, Q1.
4. **Garmaa G**, Rita N, Kökény G. Panel miRNAs are potential diagnostic markers for chronic kidney diseases: a systematic review and meta-analysis. (accepted in BMC Nephrology). Impact factor, journal quartile – 2.2, Q2.

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Publications not related to the thesis:

1. A. Tóth-Mészáros, **G. Garmaa**, P. Hegyi, A. Bánvölgyi, B. Fenyves, P. Fehérvári, et al. The effect of adaptogenic plants on stress: A

systematic review and meta-analysis. Journal of Functional Foods 2023 Vol. 108 Pages 105695, DOI: https://doi.org/10.1016/j.jff.2023.105695. Impact factor, journal quartile – 5.6, Q1.

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