ORIGINAL ARTICLE

In vitro versus *in vivo* models of kidney fibrosis: Time-course experimental design is crucial to avoid misinterpretations of gene expression data

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Background: *In vitro* models are common tools in nephrology research. However, their validity has rarely been scrutinized. **Materials and Methods:** Considering the critical role of transforming growth factor (TGF)-β and hypoxia pathways in kidney fibrosis, kidney-derived cells were exposed to TGF-β and/or hypoxic conditions and the expression levels of some genes related to these two signaling pathways were quantified in a time-course manner. Furthermore, a unilateral ureteral obstruction mouse model was generated, and the expressions of the same genes were assessed. **Results:** In all *in vitro* experimental groups, the expression of the genes was noisy with no consistent pattern. However, in the animal model, TGF-β pathway-related genes demonstrated considerable overexpression in the ureteral obstruction group compared with the sham controls. Interestingly, hypoxia pathway genes had prominent fluctuations with very similar patterns in both animal groups, suggesting a periodical pattern not affected by the intervention. **Conclusion:** The findings of this study suggest that *in vitro* findings should be interpreted cautiously and if possible are substituted or supported by animal models that are more consistent and reliable. Furthermore, we underscore the importance of time-course evaluation of both case and control groups in gene expression studies to avoid misconceptions caused by gene expression noise or intrinsic rhythms.

Key words: Chronic kidney failure, hypoxia pathway, rhythmic behavior, transforming growth factor-β pathway, time-course analysis

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INTRODUCTION

The complex and chronic nature of noncommunicable diseases has made them a major health challenge all over the world. Investigations to discover novel therapeutics have been seriously followed and disease models are indispensable tools in preclinical settings. Animal models provide the opportunity to investigate the disease process in the context of a natural environment.[1] However, these studies are challenging to perform not only due to ethical issues but also their difficulty and high cost. On the other hand, *in vitro* studies are easy to manage, cheap, and scalable. Nevertheless, to what extent an *in vitro* model is capable of recapitulating the real process in the human body is a matter of debate. Indeed, microenvironment has a great impact on cellular physiology which is missed in *ex vivo* settings, leading to changes in cell identity.[1] It is demonstrated that upon isolation and primary culture of mesenchymal stem cells from different postnatal organs, they acquire almost identical properties.[2]

Chronic kidney disease (CKD) is a prototype of complex disorders with a prevalence of near 8% in the global population and suboptimal therapies.[3] Renal fibrosis with characteristic histopathologic features, including tubular cell injury, tubulointerstitial fibrosis, and glomerulosclerosis, is the main manifestation of CKD.

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[4] The two essential driving forces of CKD progression are transforming growth factor (TGF)-β and hypoxia pathway which have been extensively investigated to understand the disease mechanisms. In this regard, various disease models have been developed for simulating CKD progression in order to investigate the underlying mechanisms and drug screening. Among various animal models, unilateral ureteral obstruction is the most popular model of renal fibrosis that recapitulates histopathologic features as CKD progression.[4] In parallel, multiple studies have treated kidney-derived cells with TGF-β or hypoxic culture conditions to investigate the molecular basis of the disorder.[5-9] However, the robustness of these simplified *in vitro* models is a matter of debate. To address this issue, we have here developed *in vitro* and *in vivo* models of kidney fibrosis to assess the potential of each strategy in terms of simulating the molecular underlying events. To account for the dynamic nature of the processes, gene expression measurements were performed in a time-course manner.

MATERIALS AND METHODS

Determination of key factors in transforming growth factor-beta and hypoxia pathways

Upon binding of a homodimer of TGF-β ligand to the transmembrane receptor II (TGFBR2), it is activated and forms a heterotetrameric complex with TGF-beta receptor I (TGFBR1). Subsequently, TGFBR1 is phosphorylated by serine/threonine kinase activity of TGFBR2, phosphorylates, and activates regulatory Smads (Complex of SMAD2/3).[10] In the cytoplasm, this complex is connected to Co-Smad (Smad4) and translocated to the nucleus. Finally, this transcription factor complex leads to the expression of extracellular matrix proteins such as COL1A1.[11] The activity of this pathway is controlled by the negative feedback effect of SMAD7, SKI/SNON complex, and SMURF2 proteins.[12]

The main regulatory element of the hypoxia pathway is the hypoxia-inducible factor (HIF) family containing three known members as HIF1, HIF2, and HIF3.[13] In the presence of oxygen, HIF is deactivated by hydroxylation in an asparagine residue by hypoxia-inducible factor 1, alpha subunit inhibitor (HIF1AN), or two proline residues by EGLN2, EGLN3, and EGLN1. The hydroxylation in asparagine halts HIF interaction with CBP and P300, whereas proline hydroxylation leads to HIF interaction with E3 ubiquitin ligase VHL and subsequently degradation. In the hypoxic condition, hydroxylation is inhibited which results in HIF translocation into the nucleus and expression of a large set of genes such as extracellular matrix proteins.[14,15] Similar to TGF-β, the hypoxia pathway has a negative regulatory loop formed by E3 ubiquitin-protein ligase Siah1.

Following a detailed survey on the role of different components in each pathway, we selected *TGF-β1*, *SMAD3*, and *SMAD7* for *TGF-β* signaling and *HIF1a*, *EGLN1*, *EGLN3*, *HIF1AN*, and *SIAH2* for hypoxia signaling as the main regulatory components to be evaluated for gene expression analysis. COL1A1 was also selected as a common gene for both pathways.

Human kidney cell isolation and cell culture

Pieces of about 1 cm^3 were extracted from the kidney cortex of a patient subjected to total nephrectomy. The tissue was minced with a surgical blade and incubated in collagenase I (Sigma, St Louis, USA) for 45 min. The cellular content was harvested by centrifugation in 300 g for 5 min and after washing with phosphate-buffered saline (PBS; GIBCO-BRL, Grand Island, USA) was cultured in Dulbecco's modified eagle medium (Bioidea, Tehran, Iran) containing 10% fetal bovine serum (GIBCO-BRL), as well as penicillin G (100 U/mL; Sigma) and streptomycin (100 mg/mL; Sigma) at 37°C, in 5% CO_2, and 20% O_2. After 24 h, the nonadherent cells were discarded. The hypoxic condition was obtained by culturing the cells in an aerobic incubator (Memmert, Germany) with 1% O_2. TGF-β treatment was applied by recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA) at 5 ng/mL. After expanding cells for about 3 weeks, they were cultured in four different conditions: normoxia, hypoxia, normoxia + TGF-β, and hypoxia + TGF-β. Two flasks were considered for each time point in different conditions and the cells were harvested on days 0, 1, 3, 5, 7, 9, 11, and 13 for gene expression analysis.

Animal model of unilateral ureteral obstruction

Male C57BL/6 mice aged 6–8 weeks were obtained from the Pasteur Institute of Iran (Tehran, Iran). Animal care and experiments were conducted according to the institutional guide for the Care and Use of Laboratory Animals.

Ketamine and xylazine (both from Alfasan, Woerden, Netherland) were injected intraperitoneally for anesthesia induction at the doses of 115 and 11.5 mg/kg, respectively. During anesthesia and operation, the mice were kept on a 37.5°C plate. After a mid-abdominal incision, the left ureter was isolated from the surrounding tissues, double ligated, and the incision was sutured. Sham operation was performed with the same procedure, except for the ligation of the ureter. Mice were harvested 1, 3, 6, 9, 12, 15, 18, and 21 days after surgery. For each time point, two unilateral ureteral obstruction (UUO) and two sham-operated mice were allocated. Furthermore, four untreated mice were used as normal controls. After sacrificing with cervical dislocation, a part of each kidney was kept in 10% formalin for histopathological study and another part was sustained in liquid nitrogen for RNA extraction.

Histopathological analysis

After fixation in 10% formalin, kidney tissues were embedded in paraffin, and then, five-micrometer coronal sections were stained with hematoxylin and eosin (H and E) and Masson's trichrome. Histopathological examination was performed in a blinded manner.

Real-time polymerase chain reaction

Kidney sections preserved in liquid nitrogen were minced, suspended in 500 µL of cold RNX-plus (Sinagen, Tehran, Iran), and lysed by microhomogenizer (Micro Smash MS-100, Japan) for 5 min. Then, 1 mL of excessive RNX-plus was added to the lysed tissues for RNA extraction according to the manufacturer's instructions. This reagent was also used for RNA extraction from *in vitro* expanded cells. RNA purity and concentration were measured by BioSpectrometer® (Eppendorf, Hamburg, Germany). Random hexamer-primed cDNA synthesis was carried out using RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Rockford, USA).

Human and mouse exon junction primers [Table 1] for nine critical genes of both hypoxia and TGF-β pathways were designed by AlleleID version 7.6 (Primer Biosoft, Palo Alto, USA). Primer specificity was assessed by NCBI BLAST, and the expression level was assessed by Maxima™ SYBR Green quantitative polymerase chain reaction (qPCR) Master Mix (Thermo Scientific) and Rotor-Gene 6000 Real-Time PCR Machine (Corbett Life Science, Concorde, Australia). The temperature profile was as follows: an initial step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. For quantitation of real-time PCR results, the Pfaffl method was applied. For the *in vitro* study *ACTB* and *GAPDH* and in the *in vivo* study *Tfrc* and *Hprt* were considered as the internal controls. The data were analyzed using REST 2009 software.

RESULTS

To assess the kinetics of hypoxia and TGF-β pathways, HK cells were cultured in four conditions: normoxia, hypoxia, normoxia + TGF-β stimulation, and hypoxia + TGF-β stimulation. All groups were followed for 13 days and were harvested for RNA extraction and cDNA synthesis every other day. Gene expressions were quantified relative to untreated cells on day 0. According to our previous study,[16] *GAPDH* and *ACTB* were chosen as the most stable housekeeping genes (data not shown).

Table 1: The sequence of primers for human and mouse genes

	Gene Name	Human	Mouse
TGF-β pathway	TGFβ1	F:CTCGCCAGAGTGGTTATCTT	F: ATTCCTGGCGTTACCTTG
		R:GTAGTGAACCCGTTGATGTC	R:GCTGATCCCGTTGATTTC
	SMAD3	F: CGGAGACACATCGGAAGAG	F:GCATGGACGCAGGTTCTC
		R:CGAACTCCTGGTTGTTGAAG	R:GTAGGTGACTGGCTGTAGGT
	SMAD7	F:GGCTTTCAGATTCCCAACTTCT	F:GGCTTTCAGATTCCCAACTT
		R:AGCTGACTCTTGTTGTCCG	R:GTCTTCTCCTCCCAGTATGC
	COL1A1	F: TGGAGCAAGAGGCGAGAG	F:AACAGCGTAGCCTACATGG
		R: CACCAGCATCACCCTTAGC	R:CGGTGTGACTCGTGCAG
Hypoxia pathway	HIF1α	F:AGTTCACCTGAGCCTAATAGTC	F:TTGGCAGCGATGACACA
		R:GTCTAAATCTGTGTCCTGAGTAG	R:CGATGAAGGTAAAGGAGACATT
	EGLN1	F:ATGCTACAAGGTACGCAATAAC	F:GGGACGCCAAGGTAAGTG
		R:TTACCGACCGAATCTGAAGG	R:CTCTCGCTCGCTCATCTG
	EGLN3	F:CCTCTTACGCAACCAGATATG	F:TGCCACCAGGTACGCTAT
		R:GCACGGTCAGTCTTCAGT	R:GCACACCACAGTCAGTCTT
	HIF1AN	F:AGGAAGCACCAGGACATG	F:GGAGAAGAGCGGTTGTATCT
		R:AAGTCCACCTGGCTCTGT	R:TCATCATAGTGAGCAGGTGTC
	SIAH2	F:CCTGTAAGTATGCCACCAC	F:GCCCTAACGCCCAGCATCAG
		R:AGGAAGCACCAGGACATG	R:AACAGCCCGTGGTAGCATACTTAC
House keeping genes	GAPDH	F:GTCCACTGGCGTCTTCAC	_
		R:AGGCATTGCTGATGATCTTGA	_
	ACTB	F:GAAGATCAAGATCATTGCTCCT	_
		R:AAGTCATAGTCCGCCTAGAAG	_
	TFRC	_	F:TGCATTGCGGACTGTAGAG
		_	R:CCCACCAAACAAGTTAGAGAAT
	HPRT	_	F:CGTCGTGATTAGCGATGATG
		_	R:AGTCTTTCAGTCCTGTCCATAA

TGF=Transforming growth factor; SMAD3=Smad family member 3; SMAD7=Smad family member 7; *COL1A1*=Collagen type I α1; HIF=Hypoxia-inducible factor; *Siah2*=Siah E3 ubiquitin protein ligase 2; EGLN: Egl-9 family hypoxia-inducible factor; HIF1AN: Hypoxia-inducible factor 1, alpha subunit inhibitor. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ACTB: Actin beta; TFRC: Transferrin receptor; HPRT: Hypoxanthine phosphoribosyl transferase

Collagen type I α1 (*COL1A1*), as the hallmark of TGF-β stimulation, shows about 10-fold increment in TGF-β-stimulated conditions, which validates our assay [Figure 1]. However, the other genes did not demonstrate any meaningful pattern and their fluctuations were in the order of housekeeping genes. As UUO in rodents, models progressive renal fibrosis, in the next step of this study, we assessed the expression profile of these genes in the mice model of UUO.

Male C57BL/6 mice were subjected to surgery for UUO or sham operation and then sacrificed at distinct time points up to 3 weeks [Figure 2]. Histopathological examination of the kidneys revealed the validity of this model according to parameters, such as glomerular and interstitial injuries. Increased mesangial matrix and diffuse glomerular sclerosis as well as diffuse tubulointerstitial fibrosis and tubular atrophy were evident after 21 days in UUO kidneys compared with the sham and normal groups [Figure 2].

Gene expression analysis demonstrated that TGF-β pathway genes, including *Tgfb1*, Smad family member 3 (*Smad3*), Smad family member 7 (*Smad7*), and *Col1a1*, were significantly overexpressed in UUO mice. Notably, this increasing trend was not homogenous during the course as local declines were observed in some time points. Variations of these genes in the sham group were minimal. Unexpectedly, hypoxia pathway-related genes, hypoxia-inducible factor 1 alpha subunit (*Hif1a*), egl-9 family hypoxia-inducible factor 1 (*Egln1*), egl-9 family hypoxia-inducible factor 3 (*Egln3*), hypoxia-inducible factor 1, alpha subunit inhibitor (*Hif1an*), and Siah E3 ubiquitin protein ligase 2 (*Siah2*) remained unchanged in UUO mice compared with the sham group. Interestingly, these genes had spontaneous variations with similar patterns in both groups, suggesting that these patterns are related to inherent periodic fluctuations rather than random noise in gene expression [Figure 2].

DISCUSSION

Different kinds of *in vitro* and *in vivo* models have been developed to the model acceleration of fibrosis in the kidney. Nevertheless, to the best of our knowledge, no study has mentioned the reproducibility of these two different approaches. Considering the critical role of TGF-β and hypoxia pathways in promoting kidney fibrosis,[17] kidney-derived cells were exposed to TGF-β or hypoxic conditions and the expressions of nine genes related to these pathways were measured in a time-course manner. In a parallel experiment, the same genes were evaluated in a UUO mouse model of kidney fibrosis. The expressions of genes in cell culture were noisy with no prominent pattern. In contrast, the TGF-β pathway genes demonstrated clear upregulation in the kidneys of UUO mice. Our data also provided preliminary evidence for a rhythmic pattern in the expression of hypoxia pathway-related genes.

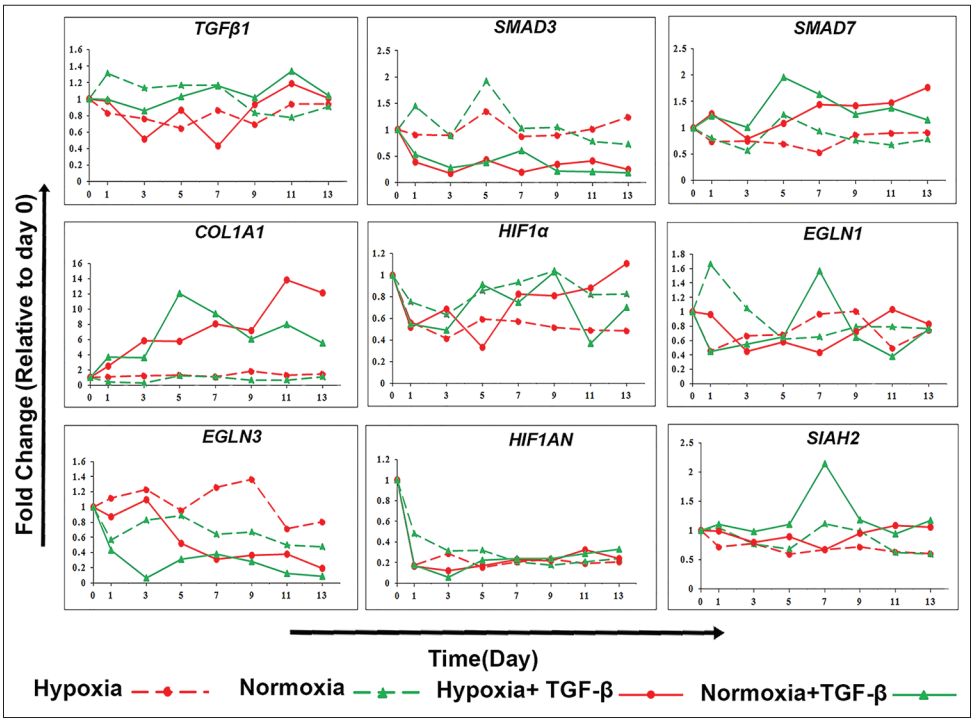

Figure 1: The expression level of genes related to TGF-β and hypoxia pathways were followed in a time-course manner in four culture conditions: Hypoxia, normoxia, hypoxia + TGF-β, and normoxia + TGF-β. TGF-β = Transforming growth factor beta

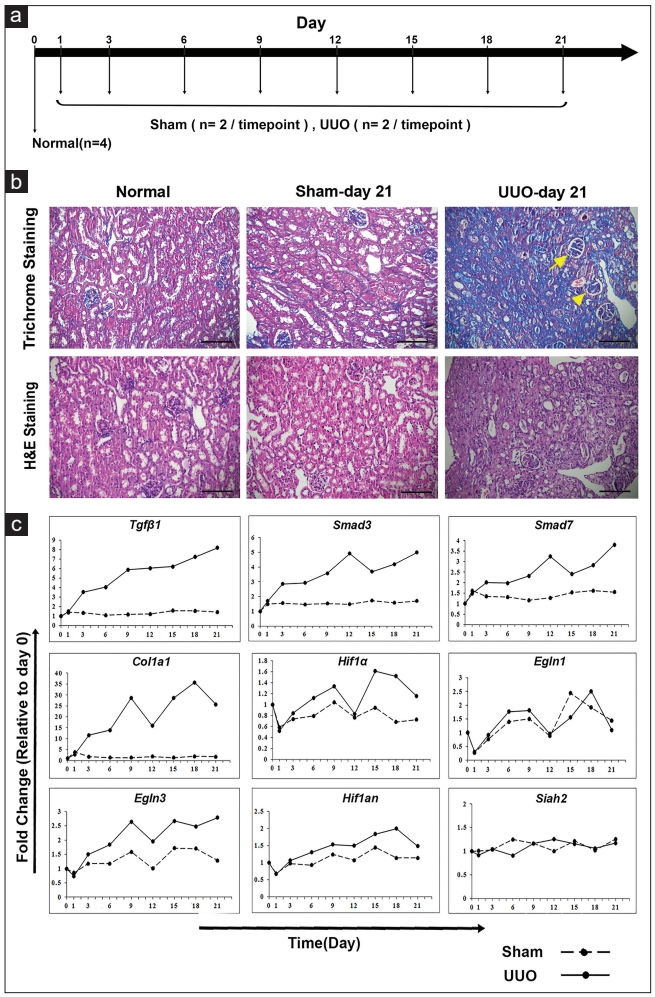

Figure 2: The expression level of TGF-β and hypoxia pathway-related genes in mouse model of UUO. (a) Experimental scheme. (b) Trichrome and H and E-stained kidney sections after 21 days of obstruction. Blue fibers indicate diffuse tubulointerstitial fibrosis in UUO samples compared with the sham and normal groups. Renal tubules are atrophic in UUO on day 21. Diffuse glomerular sclerosis (arrow) and increased glomerular mesangial matrix (the arrow head) are also visible. Scale bars: 100 μm. (c) The expression levels of genes related to TGF-β and hypoxia pathways were followed in a time-course manner over 21 days in UUO and sham-operated mice. TGF-β = Transforming growth factor beta; UUO = Unilateral ureteral obstruction

We did not observe illustrative responses to *in vitro* treatments. Cell physiology is largely regulated by the microenvironment and so *in vitro* expanded cells may respond to stimuli in a totally different manner compared to their *in vivo* counterparts.[1] Silliman and Wang have compared different *in vitro* and *in vivo* models of adaptive immunity and concluded that because of the complexity of this system and the presence of multiple cells and components that interact in specific time points, *in vitro* modeling would be misleading.[18] The limitation of *in vitro* settings for modeling biomedical phenomena is not only related to their inability to consider the effects of the natural microenvironment and the interactions of surrounding cells but also to the genetic aberrations that may occur in cultured cells. Indeed, we and other investigators have shown that chromosomal abnormalities are frequently observed in *ex vivo* expanded cells.[19-22] Similarly, a recent study has reported rapid genetic diversification of several cancer cell lines due to clonal selection imposed by cell culture conditions. This genetic heterogeneity was reflected in gene expression patterns, cell phenotypes, and responsiveness to drugs.[23] Taken together, *in vitro* findings should be cautiously interpreted and extrapolated to clinical settings.

We have here assessed gene expression profiles at different time points. Almost all biological processes are time dependent;[24] therefore, time-course studies are matched better with this dynamism, preventing misconceptions from single time point assessments.[25-27] In such studies, pattern recognition is a critical issue for data analysis and contemplation.[28] As we have discussed in our previous study, in the analysis of time-course gene expression data, some parameters should be considered such as the magnitude of difference between the test and control groups, the proportion of time points that one group has higher expression levels than the other group, and reproducibility of the data to discriminate the noisy fluctuations from biological expression changes.[29]

Another advantage of this study is that not only UUO but also the sham group were followed over time. This design allows the actual responses to treatments to be distinguished from inherent variations. Indeed, hypoxia-related genes in UUO mice were differentially expressed compared with day 0; however, when the trends of UUO and sham were simultaneously inspected, it was found that these alterations are intrinsic rather than a response to the treatment. In line with our observations, it has recently been shown that hypoxia pathway genes are affected by the circadian rhythms.[30-32] Therefore, in agreement with our previous study,[29] the current results underscore the importance of temporal assessment of all experimental groups in gene expression studies.

CONCLUSION

Overall, the results of this study indicate the consistency of gene expression patterns in the animal model of kidney injury compared with the *in vitro* counterparts. We also highlight the significance of time-course experimental design to provide a reliable image of dynamic biological processes.

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Conflicts of interest

There are no conflicts of interest.

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