

Chronic Exposure to Nanoparticulate TiO_2 Causes Renal Fibrosis Involving Activation of the Wnt Pathway in Mouse Kidney

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ABSTRACT: Chronic exposure to nano- TiO_2 may induce renal fibrosis, and the mechanism of this process is not well understood. Therefore, in this study, mice were administered nano- TiO_2 by intragastric feeding for 9 months, and the urinary levels of nephrotoxicity biomarkers, activation of the Wnt pathway, and markers of the epithelial-to-mesenchymal transition (EMT) in the kidneys were investigated. The findings suggested that exposure to nano- TiO_2 increased the level of renal titanium accumulation, urinary levels of kidney injury molecule-1 (1.18 ± 0.13 - to 3.60 ± 0.41 -fold), clusterin (1.40 ± 0.16 - to 5.14 ± 0.58 -fold), and osteopontin (0.71 ± 0.08 - to 2.41 ± 0.29 -fold), and increased levels of renal inflammation and fibrosis. Furthermore, nano- TiO_2 increased the level of expression of Wnt ligands (Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt9a, Wnt10a, and Wnt11, 0.09 ± 0.02 - to 4.84 ± 0.52 -fold), Wnt receptors Frizzled (Fz1, Fz5, and Fz7, 0.37 ± 0.04 - to 8.57 ± 0.91 -fold), and coreceptors low-density lipoprotein receptor-related proteins 5 and 6 (0.73 ± 0.09 - to 5.27 ± 0.56 -fold) in the kidney. Wnt signaling components induced by nano- TiO_2 were corroborated by decreased levels of expression of Wnt antagonist-related markers (Dkk1, Dkk2, Dkk3, Dkk4, and sFRP/FrzB, -0.06 ± 0.01 - to -0.87 ± 0.09 -fold) and increased levels of expression of Wnt target genes (Abcb1b, cyclin D1, and Myc, 0.03 ± 0.01 - to 2.73 ± 0.28 -fold) and EMT markers Colla1, Fn, Twist, and α -SMA (0.06 ± 0.02 - to 5.80 ± 0.61 -fold). These findings indicate that nano- TiO_2 induced renal fibrosis that may be mediated via Wnt signaling.

KEYWORDS: nanoparticulate titanium dioxide, nephrotoxicity biomarkers, chronic inflammation, renal fibrosis, Wnt pathway

INTRODUCTION

Nano- TiO_2 is used in the food and agricultural industries to improve the taste, color, and texture of foods and to improve food packaging materials.^{1,2} TiO_2 and several of its composites are used in food products as additives and in several health care products.³ In particular, nano- TiO_2 (E171) is normally used as a coloring agent in confectionery products, baked foods, beverages, dairy products, and several processed foods.³ However, Food and Drug Administration (FDA) regulations do not address the particle size of additives in various foodstuffs, although a growing body of evidence has shown that there are potential hazards to human health resulting from food additives.⁴ Because of the unique physical and chemical properties of nano- TiO_2 , the International Agency for Research on Cancer (IARC) classified pigment-grade TiO_2 as a group 2B carcinogen ("possibly carcinogenic to humans") in 2006, and as it may pose a significant health risk, it has received an increased level of attention.^{5,6} As the frequency of use of this material has increased recently, it is necessary to determine the potential biological effects of nano- TiO_2 .

Recently, several studies have reported the deleterious effects of nano- TiO_2 in animal kidneys; for instance, exposure to nano- TiO_2 increased blood urea nitrogen (BUN) and creatinine (Cr) levels and caused swelling in the renal glomerulus and dilatation and proteinic liquids in the renal tubules of mice.^{7,8} Our previous studies also suggested that exposure to nano- TiO_2 led to renal injuries, including infiltration of inflammatory cells, a

reduction in the number of renal glomeruli, necrosis of renal tubular epithelial cells, or disorganization of the renal tubules and dysfunction.^{9–12} The studies mentioned above suggested that renal proximal tubules are sensitive to nanotoxic insults. Importantly, it has been suggested that chronic renal inflammation leads to renal fibrosis.¹³ Numerous studies have also demonstrated that the Wnt signaling pathway plays a key role in the development of organ fibrosis.¹⁴ Nevertheless, the question of whether chronic renal inflammation caused by chronic exposure to nano- TiO_2 leading to nano- TiO_2 -induced fibrosis involves the Wnt pathway remains unanswered.

The canonical Wnt pathway plays critical roles in human organogenesis and tumorigenesis and is involved in renal development and the initiation of several renal diseases.¹⁵ The Wnt pathway consists of the Wnt ligands (Wnts), Wnt receptors, and Wnt antagonists.¹⁶ Wnt ligands function with transmembrane Frizzled (Fz or Fzd) receptors and their coreceptors, and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and -6, respectively). Wnt genes have been demonstrated to be modulated by the Wnt pathway that is closely associated with cell proliferation, survival, and migratory factors Abcb1b, Myc, and cyclin D1.¹⁷ The Wnt

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pathway is strictly regulated by Wnt antagonists, including Wnt inhibitory protein 1 (WIF1), Frizzled related protein (sFRP), and Dickkopf (Dkk). Binding of WIF to Wnts and binding of sFRP to both Wnts and Fzs cause negative modulation in both the canonical and noncanonical Wnt pathways. Binding of Dkks to the Fz–LRP complex impairs its function, acting as specific inhibitors of the canonical Wnt pathway.¹⁸ Abnormal Wnt gene expression is associated with the epithelial-to-mesenchymal transition (EMT). Importantly, many oncogenic pathways may activate EMT, thus causing EMT metastatic potential in epithelial cancer cells,¹⁹ which is related to the upregulation of mesenchymal markers, including Twist and fibronectin, in the majority of premetastatic tumors.²⁰ However, tissue fibrosis is tight EMT activation, which may also be initiated by tissue damage and inflammatory processes.²¹ Furthermore, EMT has been suggested to result in the increases in the levels of fibroblasts, collagen, and other matrix components at chronic inflammatory sites, involving progressive organ fibrosis.²² Furthermore, changes in the expression of Wnts, their receptors, and antagonists were demonstrated to be associated with renal interstitial fibrosis induced by unilateral ureteral obstruction.¹⁴ Therefore, we hypothesized that nano-TiO₂-induced chronic renal inflammation may subsequently lead to renal fibrosis and is associated with the Wnt pathway in mouse kidney.

Nano-TiO₂ is a common additive in food and personal care products, and their presence in daily life is increasing. Dietary exposure to TiO₂ in Western populations is 1–3 mg of TiO₂ [kg of body weight (bw)]⁻¹ day⁻¹ on average for children under the age of 10 years and approximately 0.2–1 mg of TiO₂ (kg of bw)⁻¹ day⁻¹ for other age groups. Several reports (e.g., NIOSH) recommend exposure limits of 2–3 mg/m³ for fine TiO₂ and 0.3–1 mg/m³ for ultrafine (including nanoscale engineered) TiO₂.²² Assuming that 36% of food-grade TiO₂ is smaller than 100 nm in at least one dimension, this exposure limit decreases to approximately 0.1 mg of nanoscale TiO₂ person⁻¹ day⁻¹.²³ Therefore, the long-term oral ingestion of the smaller dose of TiO₂ may be associated with potential kidney toxicity. In this study, female mice were continuously exposed to 1.25, 2.5, or 5 mg of nano-TiO₂/kg administered by gavage according to FDA guidelines for 9 consecutive months. The expression levels of Wnt pathway-related markers and EMT markers in mouse kidney were investigated to determine the mechanism of renal fibrosis caused by chronic exposure to nano-TiO₂.

MATERIALS AND METHODS

Chemicals. The preparation of nano-anatase TiO₂ was described by Yang.²⁴ It was fully characterized as previously described by Yang et al.²⁴ and in our previous studies²⁵ and is listed in Table 1.

Table 1. Characteristics of Nano-TiO₂^{24,25}

phase	particle size (nm)	surface area (m ² /g)	Mean hydrodynamic diameter (nm)	ζ potential (mV)
anatase	5–6	174.8	208–330 (mainly 294)	9.28

Animals and Treatment. CD-1 (ICR) female mice (4 weeks old, 20 ± 2 g), obtained from the Animal Center of Soochow University (China), were used in this study and were fed with sterilized food and distilled water *ad libitum* and housed in stainless steel cages (five mice per cage). All mice were kept at standardized laboratory conditions (24 ± 2 °C, 60 ± 10% humidity, and a 12 h light/dark cycle). After being acclimated to the laboratory conditions for one week, the mice were

fasted for 3 h prior to nano-TiO₂ administration. All experiments were conducted using the recommendations for the ethical conditions approved by the Care and Use of Animals Committee of Soochow University, which are consistent with international ethics for handling experimental animals.

One hundred mice were divided into four groups (*n* = 25 in each group): a control group treated with 0.5% (w/v) hydroxypropylmethylcellulose (HPMC) K4M (Sigma-Aldrich, St. Louis, MO) solvent and three experimental groups treated with 1.25, 2.5, or 5 mg of nano-TiO₂/kg of bw suspended in HPMC solvent. All mice were weighed, and the nano-TiO₂ suspensions of different doses were administered to mice by intragastric feeding every day for 9 months. Food and water intake was carefully recorded during the last week of the 9th months. Food and water intake was calculated according to the Drozdz method.²⁶

Kidney Indices. After the 9 month study period, all mice were weighed and urine was collected in 50 mL conical tubes over 24 h. Urine volumes were measured, and the samples were stored at –80 °C. All mice were lightly anesthetized with ether; blood samples were collected from the heart, and the kidneys were quickly removed and placed on ice. After the kidneys had been weighed, the kidney indices were calculated as the ratio of kidney (wet weight, milligrams) to body weight (grams). Fresh kidneys from five mice in each group were quickly soaked in a 10% formaldehyde solution for histopathological examination. The remaining fresh kidneys were quickly stored in liquid nitrogen and subsequently assayed for titanium content and cytokine expression. Serum was collected by centrifuging the blood samples at 12000*g* for 10 min.

Analysis of Titanium Content. The frozen kidney tissues (*n* = 5 from each group) were thawed; approximately 0.3 g samples were digested, and titanium, sodium, magnesium, potassium, calcium, zinc, and iron concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Elemental X7, Thermo Electron Co., Waltham, MA).^{11,12}

Analysis of Kidney Toxicity Biomarkers. Urinary levels of kidney injury molecule-1 (KIM-1), clusterin (CLU), osteopontin (OPN), β 2-microglobulin, and cystatin C were performed by enzyme-linked immunosorbent assays (ELISAs) using commercial kits (R&D Systems, Minneapolis, MN), following the manufacturer's instructions.

Analysis of Serum Biochemical Parameters. Uric acid (UA), blood urea nitrogen (BUN), creatinine (CR), and urinary protein excretion in serum (*n* = 10 in each group) were assessed using a clinical automatic chemistry analyzer (type 7170A, Hitachi, Tokyo, Japan).

Histopathological Observation of Kidneys. After being fixed in 10% neutral formalin and embedded in paraffin, kidney specimens from five mice in each group were sliced at a thickness of 5 μ m, and the slices were placed on separate glass slides. After being stained with hematoxylin and eosin, the renal samples were routinely evaluated by a histopathologist unaware of the treatments for light microscopy (U-III Multipoint Sensor System, Nikon, Tokyo, Japan).

Assay of Cytokine Expression. Total RNA was extracted from individual kidneys (*n* = 5 in each group) with Tripure Isolation Reagent (Roche) according to the manufacturer's protocol. According to MIQE guidelines, probes and cycling conditions were optimized for polymerase chain reaction (PCR).²⁷ cDNA was reverse transcribed (RT) from total RNA samples using the TaqMan RT reagents (Applied Biosystems) and used for real-time PCR by employing primers designed using Primer Express Software according to its guidelines. Primer sets were used for real-time PCR assays and are listed in Table 2. The probes for *Wnt1*, *Wnt2*, *Wnt3*, *Wnt4*, *Wnt5a*, *Wnt6*, *Wnt7a*, *Wnt9a*, *Wnt10a*, *Wnt11*, *Gsk3 β* , *Fz1*, *Fz5*, *Fz7*, *LRPS*, *LRP6*, *Dkk1*, *Dkk2*, *Dkk3*, *Dkk4*, *SFRP/FrzB*, *Abcb1b*, *cyclin D1*, *Myc*, *Col1a1*, *Fn* (fibronectin), *Twist*, and α -SMA in the kidneys (*n* = 5 in each group) were designed by the manufacturer and purchased from Shingene Co. (Shanghai, China). As an endogenous reference for these PCR quantification studies, GAPDH gene expression was measured. According to the method of Scheife et al.,²⁸ the RT-qPCR data were processed with sequence detection software version 1.3.1.

Table 2. Real-Time PCR Primer Pairs Used in Gene Expression Analysis

gene name	description	primer sequence	primer size (bp)
Refer-GAPDH	mGAPDH-F	5'-TGTGTCGTCGTGGATCTGA-3'	
<i>Wnt1</i>	mGAPDH-R	5'-TTGCTGTTGAAGTCGCAGGAG-3'	150
	mWnt1-F	5'-CCGAGAAACAGCGTTCATCT-3'	
<i>Wnt2</i>	mWnt1-R	5'-GCCTCGTTGTTGAAGGTT-3'	252
	mWnt2-F	5'-ATCTCTTCAGCTGGCGTTGT-3'	
<i>Wnt3</i>	mWnt2-R	5'-AGCCAGCATGTCCTCAGAGT-3'	326
	mWnt3-F	5'-CGCTCAGCTATGAACAAGCA-3'	
<i>Wnt4</i>	mWnt3-R	5'-AAAGTTGGGGAGTTCTCGT-3'	303
	mWnt4-F	5'-AACGGAACCTTGAGGTGATG-3'	
<i>Wnt5a</i>	mWnt4-R	5'-GGACGTCACAAAGGACTGT-3'	345
	mWnt5a-F	5'-CTGGCAGGACTTTCTCAAGG-3'	
<i>Wnt6</i>	mWnt5a-R	5'-CTCTAGCGTCCACGAACCTCC-3'	388
	mWnt6-F	5'-GCAGCAGGACATCCGAGAG-3'	
<i>Wnt7a</i>	mWnt6-R	5'-TCCAGGAGTGCCAGAAGG-3'	166
	mWnt7a-F	5'-ACGCCATCATCGTCATAGGA-3'	
<i>Wnt9a</i>	mWnt7a-R	5'-CACAGTCGCTCAGGTTGCC-3'	225
	mWnt9a-F	5'-GGGTGTGAAGGTGATAAAGGC-3'	
<i>Wnt10a</i>	mWnt9a-R	5'-CAGTGGCTTCATTGGTAGTGC-3'	179
	mWnt10a-F	5'-AGCCTGGAGACTCGGAACAA-3'	
<i>Wnt11</i>	mWnt10a-R	5'-CGCAAGCCTTCAGTTTACCC-3'	148
	mWnt11-F	5'-AAGTTTCCGATGCTCCTATGA-3'	
<i>Gsk3β</i>	mWnt11-R	5'-ATGGCATTACACTTCGTTTCC-3'	129
	m Gsk3β-F	5'-GCCACCATCCTTATCCCTCC-3'	
<i>Fz1</i>	m Gsk3β2-R	5'-GTTATTGGTCTGTCACGGTCT-3'	111
	mFz1-F	5'-TGTGTTGTGGGGCTCAACAAC-3'	
<i>Fz5</i>	mFz1-R	5'-CTTCTCTGCTTGGTGCCTC-3'	159
	mFz5-F	5'-GGCAATGAAGCCATCGCA-3'	
<i>Fz7</i>	mFz5-R	5'-GCCAAGACAAAGCCTCGTA-3'	174
	mFz7-F	5'-AGAGGAGAGACGGTCGCC-3'	
<i>LRP5</i>	mFz7-R	5'-CAGGAAGATGATGGGTCGC-3'	136
	mLRP5-F	5'-AAGACCTGCTTGAGGACAA-3'	
<i>LRP6</i>	mLRP5-R	5'-GAGTGGATAGCCACATCGT-3'	402
	mLRP6-F	5'-GAGCTCATCGGTGACATGAA-3'	
<i>Dkk1</i>	mLRP6-R	5'-GCTCGAGGACTGTCAGGTC-3'	400
	mDkk1-F	5'-GAGGGAAATTGAGGAAAGC-3'	
<i>Dkk2</i>	mDkk1-R	5'-GGTGCACACCTGACCTTCTT-3'	230
	mDkk2-F	5'-CATCCTCACCCACATATCC-3'	
<i>Dkk3</i>	mDkk2-R	5'-GTAGGCATGGTCTCCTTCA-3'	155
	mDkk3-F	5'-TGTGAAGGGAGAGGATGG-3'	
<i>Dkk4</i>	mDkk3-R	5'-TTGTGTAGCCACTGCCTCAG-3'	158
	mDkk4-F	5'-TAGAGTTCGCAGGAGGTGTCA-3'	
<i>FrzB</i>	mDkk4-R	5'-TCCCTGAGGTCTGTTTCC-3'	182
	mFrzB-F	5'-GCTGCCTCTGTCCTCCACTTA-3'	
<i>Abcb1b</i>	mFrzB-R	5'-TCCAAGGTGTCGGAGTTCA-3'	167
	mAbcb1b-F	5'-CCAGGCTGCCAGTGATG-3'	
<i>cyclin D1</i>	mAbcb1b-R	5'-GCCAATACAATGAGCGGTA-3'	167
	mcyclin D1-F	5'-TGAGGAGCAGAAGTGCAGA-3'	
<i>Myc</i>	mcyclin D1-R	5'-CGGCAGTCAGGAAATGGT-3'	161
	mc-Myc-F	5'-CACAACTACGCCGCACCC-3'	
<i>Colla1</i>	mc-Myc-R	5'-GCTTCAGCTCGTCCCTCCTCT-3'	196
	mColla1-F	5'-GAGGGCGAGTGCTGTGCT-3'	
<i>Fn</i>	mColla1-R	5'-GTCCAGGGATGCCATCTCG-3'	145
	mFn-F	5'-GCAATCCTCTGACGGCACA-3'	
<i>α-SMA</i>	mFn-R	5'-CAGTCGGTAGCCTGCTATACG-3'	131
	mα-SM-F	5'-CCCTGAAGAGCATCCGACA-3'	
<i>Twist</i>	mα-SM-R	5'-CTCCAGAGTCCAGCACAATACC-3'	1791
	mTwist-F	5'-AGCGGGTCACTGGCTAACG-3'	
<i>Twist</i>	mTwist-R	5'-GCCGCCAGTTGAGGGTC-3'	133

The levels of Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt9a, Wnt10a, Wnt11, Gsk3 β , Fz1, Fz5, Fz7, LRP5, LRP6, Dkk1, Dkk2, Dkk3, Dkk4, SFRP/FrzB, Abcb1b, cyclin D1, Myc, Colla1, Fn, Twist, and α -SMA in mouse kidney tissues were measured by an ELISA using commercial kits that were selective for each respective protein (R&D Systems), following the manufacturer's protocols.

Statistical Analysis. Values are presented as means \pm the standard deviation (SD). One-way analysis of variance (ANOVA) was performed to compare the differences in means from the multigroup data using SPSS 19 software (SPSS, Inc., Chicago, IL). Dunnett's test was performed when each data set was compared with the solvent control data. The statistical significance for all tests was judged at the $P < 0.05$ probability level.

RESULTS

Food and Water Intake. The quantitative data regarding food and water intake of female mice after intragastric feeding with nano-TiO₂ during the last week of the 9 months are presented in Figure 1. It can be observed that the values of daily food and water intake from nano-TiO₂-exposed groups were decreased compared with those of the control, suggesting

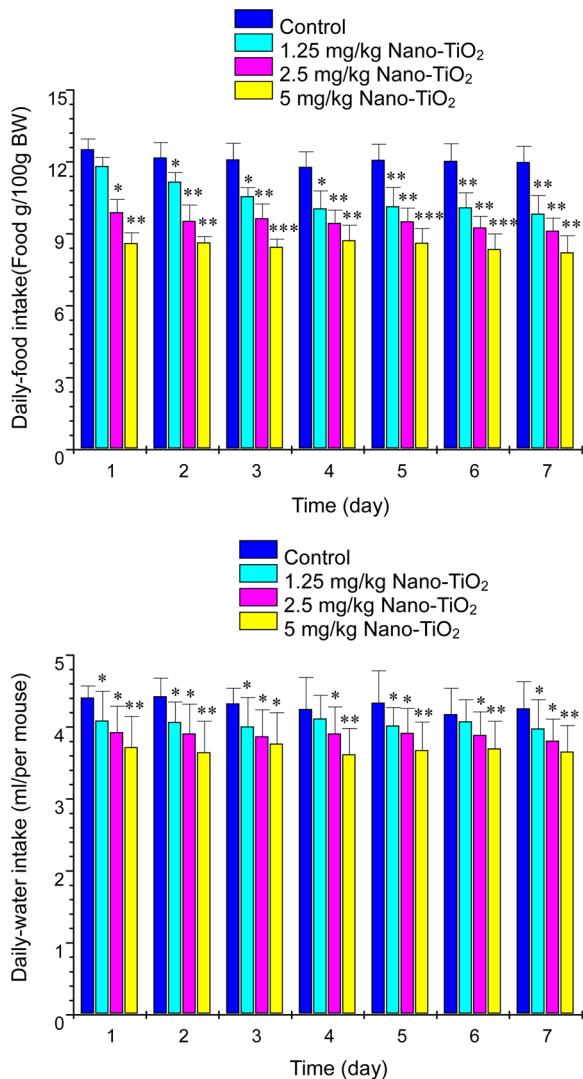


Figure 1. Daily food intake and water intake of female mice after intragastric feeding with nano-TiO₂ during the last week of the 9 months. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. Values represent means \pm SD ($n = 10$).

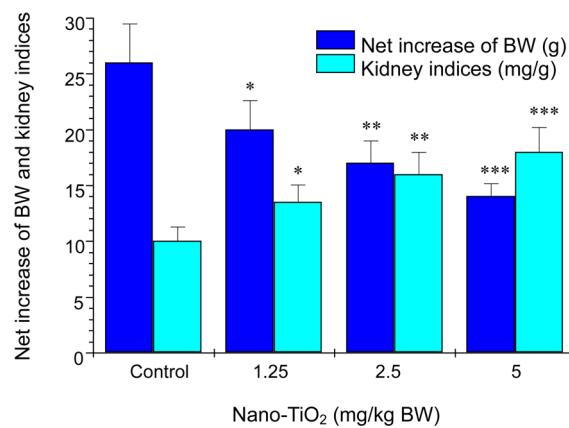


Figure 2. Body weight and kidney indices of female mice after intragastric feeding with nano-TiO₂ for 9 consecutive months. * $P < 0.05$. Values represent means \pm SD ($n = 25$).

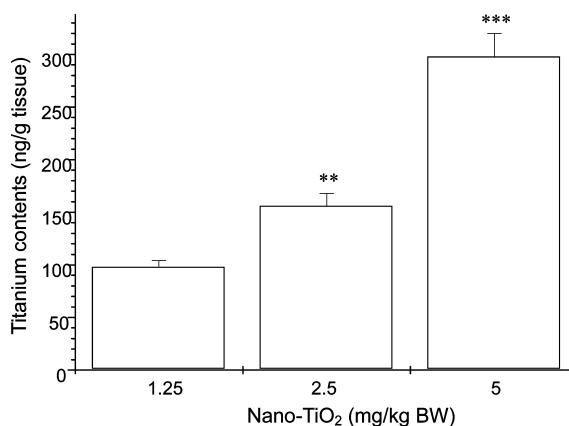


Figure 3. Titanium content of mouse kidneys after intragastric feeding with nano-TiO₂ for 9 consecutive months. ** $P < 0.01$. *** $P < 0.001$. Values represent means \pm SD ($n = 5$).

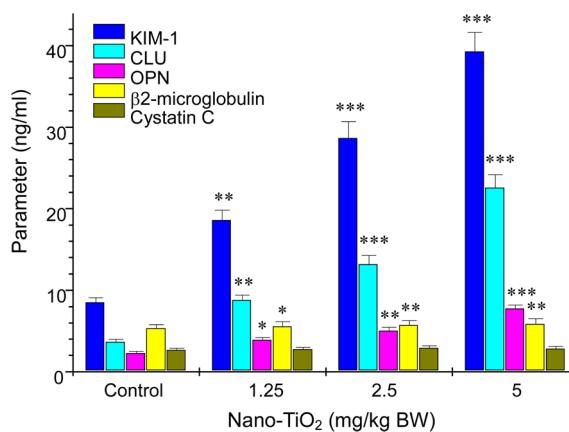


Figure 4. Alterations in nephrotoxicity biomarkers in mouse urine after intragastric feeding with nano-TiO₂ for 9 consecutive months. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. Values represent means \pm SD ($n = 5$).

reductions of 5.6–31.42% for daily food intake and 2.39–17.61% for daily water intake.

Body Weight, Renal Indices, and Titanium Content of Mouse Kidneys. Figure 2 shows the net increase in body weight and renal indices in female mice caused by exposure to nano-TiO₂. Nano-TiO₂ exposure led to significant decreases in

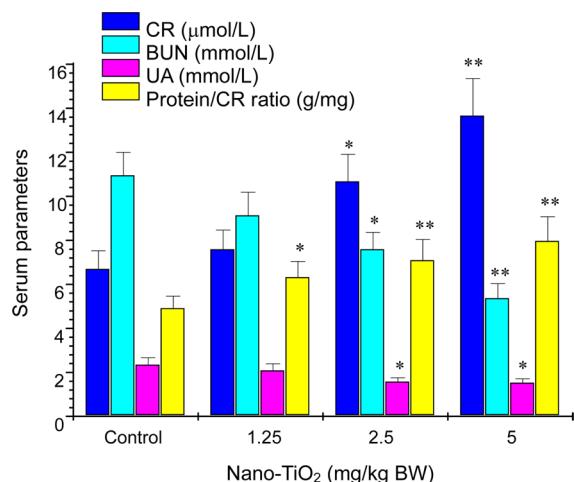


Figure 5. Changes in serum parameters in mice after administration of TiO₂ NPs by intragastric feeding for 9 consecutive months. **P* < 0.05. ***P* < 0.01. Values represent means ± SD (*n* = 10).

body weight and increases in renal indices compared with those of the control group (*P* < 0.05). Furthermore, significant titanium accumulation occurred with an increased nano-TiO₂ dose (Figure 3; *P* < 0.05). The increased renal indices following exposure to nano-TiO₂ may be related to nephrotoxicity and renal injury.

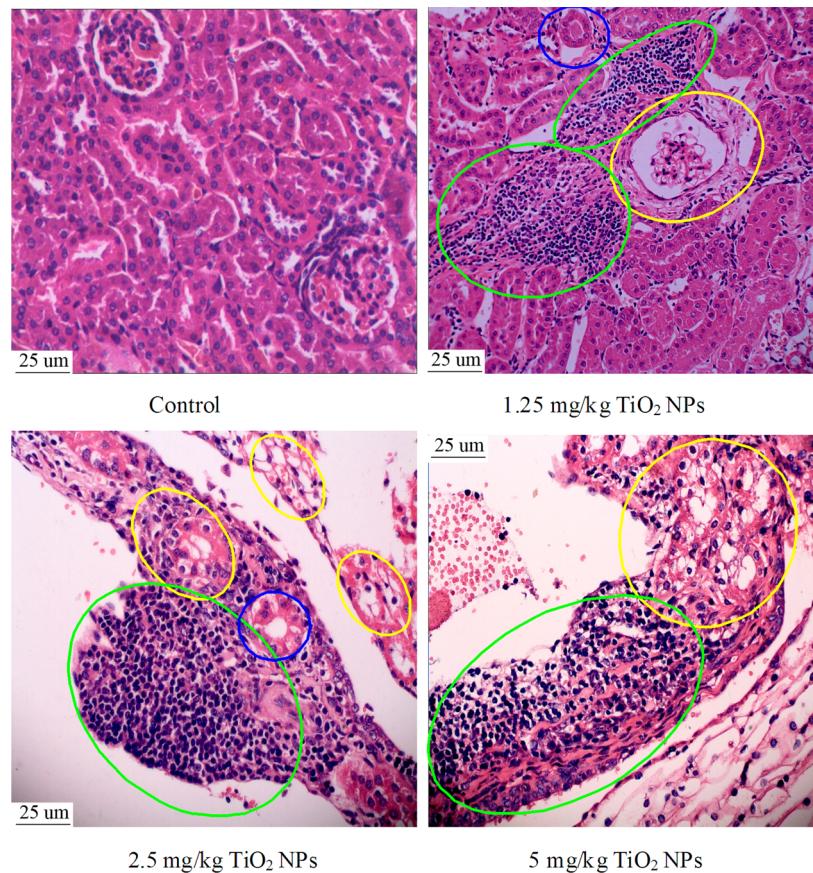


Figure 6. Histopathological observation of kidneys of male mice after intragastric feeding with nano-TiO₂ for 9 consecutive months (*n* = 5). The green ovals denote infiltration of inflammatory cells; the yellow ovals suggest tissue fibrosis or mesh in renal glomerulus, and blue ovals denote the proteinic liquid in the renal tubule.

Table 3. Effect of Nano-TiO₂ on the Level of mRNA Expression of Wnt Ligand Genes in Mouse Kidney after Intragastric Feeding with Nano-TiO₂ for 9 Consecutive Months

	x-fold change in the level of mRNA expression (treatment vs control)		
	1.25 mg of nano-TiO ₂ /kg of bw	2.5 mg of nano-TiO ₂ /kg of bw	5 mg of nano-TiO ₂ /kg of bw
<i>Wnt1</i>	2.08 ± 0.21 ^b	2.34 ± 0.27 ^b	5.02 ± 0.51 ^c
<i>Wnt2</i>	1.88 ± 0.19 ^a	2.61 ± 0.25 ^b	4.51 ± 0.47 ^c
<i>Wnt3</i>	1.12 ± 0.15	1.66 ± 0.18 ^b	2.40 ± 0.22 ^c
<i>Wnt4</i>	1.15 ± 0.16	1.83 ± 0.20 ^a	2.15 ± 0.22 ^b
<i>Wnt5a</i>	1.09 ± 0.14	1.63 ± 0.18 ^a	1.77 ± 0.19 ^a
<i>Wnt6</i>	1.13 ± 0.11	1.85 ± 0.21 ^a	2.24 ± 0.23 ^b
<i>Wnt7a</i>	1.58 ± 0.16 ^a	1.74 ± 0.18 ^a	1.90 ± 0.20 ^a
<i>Wnt9a</i>	1.76 ± 0.18	2.94 ± 0.31	3.96 ± 0.41
<i>Wnt10a</i>	1.98 ± 0.22 ^b	2.38 ± 0.25 ^b	4.13 ± 0.41 ^c
<i>Wnt11</i>	1.10 ± 0.11	1.78 ± 0.19 ^a	3.29 ± 0.34 ^b
<i>Gsk3β</i>	0.86 ± 0.10	0.53 ± 0.07 ^a	0.36 ± 0.05 ^b

^a*P* < 0.05. Values represent means ± SD (*n* = 5). ^b*P* < 0.01. Values represent means ± SD (*n* = 5). ^c*P* < 0.001. Values represent means ± SD (*n* = 5).

Nephrotoxicity Biomarkers. Changes in nephrotoxicity biomarkers in mouse urine induced by exposure to nano-TiO₂ are presented in Figure 4. With an increasing nano-TiO₂ dose, levels of KIM-1, CLU, and OPN were significantly increased by 1.18–3.60-, 1.40–5.14-, and 0.71–2.41-fold (*P* < 0.05),

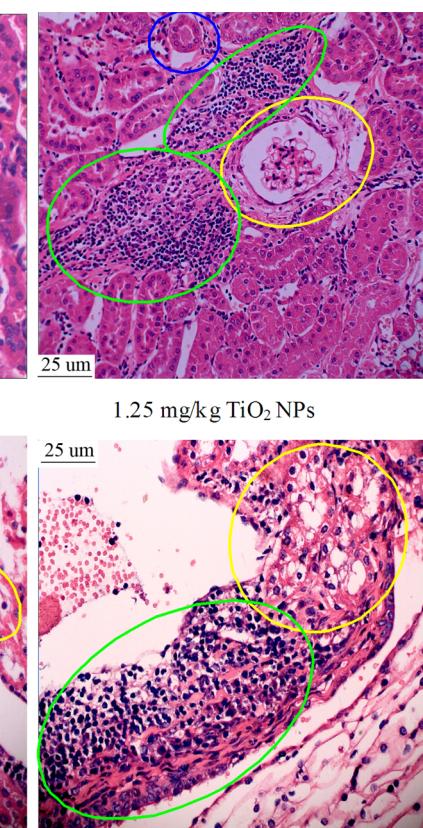


Table 4. Effect of Nano-TiO₂ on the Level of Protein Expression of Wnt Ligand Genes in Mouse Kidney after Intragastric Feeding with Nano-TiO₂ for 9 Consecutive Months

		level of protein expression (ng/g of tissue)			
	control	1.25 mg of nano-TiO ₂ /kg of bw	2.5 mg of nano-TiO ₂ /kg of bw	5 mg of nano-TiO ₂ /kg of bw	
Wnt1	42.3 ± 3.12	88.7 ± 6.44 ^b	136 ± 11.55 ^c	220 ± 18.0 ^c	
Wnt2	54.1 ± 5.71	102 ± 10.2 ^b	170 ± 16.3 ^c	316 ± 25.9 ^c	
Wnt3	140 ± 12.35	207 ± 21.4 ^a	304 ± 29.7 ^b	408 ± 38.2 ^c	
Wnt4	169 ± 15.8	268 ± 25.8 ^b	454 ± 41.3 ^c	653 ± 52.5 ^c	
Wnt5a	206 ± 18.4	326 ± 29.9 ^a	553 ± 49.3 ^b	799 ± 70.2 ^c	
Wnt6	29.6 ± 2.51	53.9 ± 4.31 ^a	99.7 ± 10.1 ^c	141 ± 12.6 ^c	
Wnt7a	63.5 ± 6.27	100 ± 8.53 ^a	132 ± 14.1 ^b	193 ± 20.2 ^c	
Wnt9a	145 ± 15.7	257 ± 26.1 ^a	426 ± 39.8 ^b	574 ± 52.6 ^c	
Wnt10a	38.2 ± 4.08	68.5 ± 7.37 ^a	113 ± 10.8 ^b	158 ± 16.2 ^c	
Wnt11	83.0 ± 7.71	164 ± 15.1 ^b	249 ± 21.3 ^c	418 ± 40.7 ^c	
Gsk3β	262 ± 24.5	211 ± 20.8 ^a	180 ± 15.3 ^b	131 ± 12.2 ^c	

^aP < 0.05. Values represent means ± SD (n = 5). ^bP < 0.01. Values represent means ± SD (n = 5). ^cP < 0.001. Values represent means ± SD (n = 5).

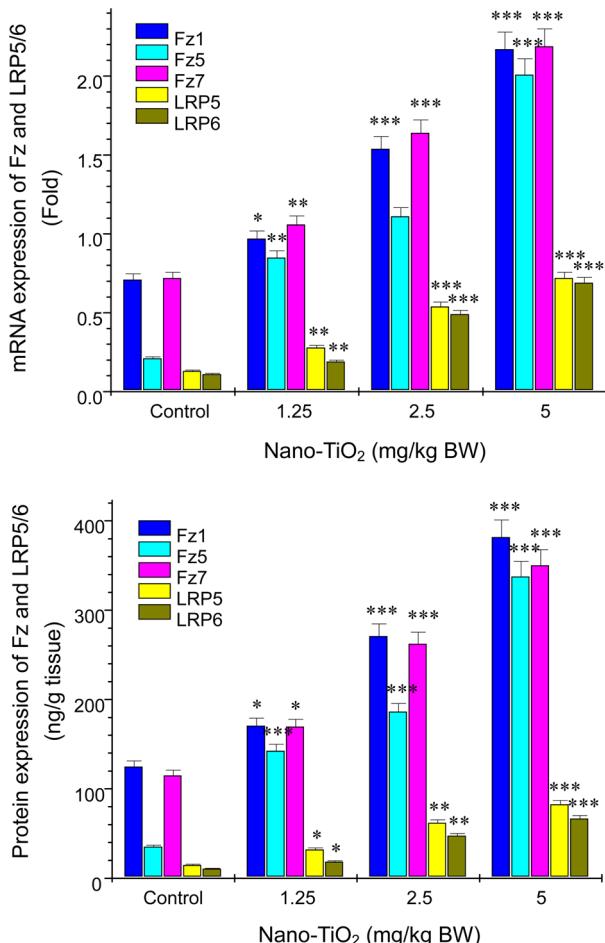


Figure 7. Effect of exposure to nano-TiO₂ on Fz and LRP5 and -6 expression in mouse kidney after intragastric feeding with nano-TiO₂ for 9 consecutive months. ^aP < 0.05. ^bP < 0.01. ^cP < 0.001. Values represent means ± SD (n = 5).

respectively, whereas levels of β 2-microglobulin and cystatin C showed no statistically significant change (P > 0.05).

Serum Biochemical Parameters. To confirm the effects of nano-TiO₂ on renal function, serum levels of CR, UA, and BUN were examined and are shown in Figure 5. It can be seen that the level of CR and the protein/CR ratio increased by 11.32–20.40 and 2.66–62.19%, respectively, and those of UA

Table 5. Effects of Nano-TiO₂ on the Level of mRNA Expression of the Wnt Pathway- and EMT-Related Genes in Mouse Kidney after Intragastric Feeding with Nano-TiO₂ for 9 Consecutive Months

	x-fold change in the level of mRNA expression (treatment vs control)		
	1.25 mg of nano-TiO ₂ /kg of bw	2.5 mg of nano-TiO ₂ /kg of bw	5 mg of nano-TiO ₂ /kg of bw
Dkk1	0.93 ± 0.11	0.55 ± 0.06 ^a	0.38 ± 0.04 ^b
Dkk2	0.94 ± 0.13	0.57 ± 0.07 ^a	0.34 ± 0.04 ^b
Dkk3	0.56 ± 0.06 ^a	0.71 ± 0.07 ^a	0.59 ± 0.06 ^a
Dkk4	0.65 ± 0.07 ^a	0.61 ± 0.05 ^a	0.36 ± 0.03 ^b
sFRP/FrzB	0.51 ± 0.05 ^b	0.40 ± 0.04 ^b	0.29 ± 0.02 ^c
Abcb1b	1.52 ± 0.15 ^a	1.78 ± 0.19 ^a	2.78 ± 0.29 ^b
cyclin D1	1.63 ± 0.16 ^a	1.80 ± 0.19 ^a	1.94 ± 0.20 ^a
Myc	1.03 ± 0.13	1.44 ± 0.16 ^a	2.08 ± 0.23 ^c
Col1a1	2.52 ± 0.28 ^b	2.67 ± 0.29 ^b	4.64 ± 0.45 ^c
Fn	1.28 ± 0.14	1.78 ± 0.18 ^a	2.08 ± 0.23 ^b
Twist	2.29 ± 0.22 ^b	2.71 ± 0.26 ^b	2.93 ± 0.31 ^b
α -SMA	1.25 ± 0.12	1.88 ± 0.20 ^a	2.62 ± 0.28 ^b

^aP < 0.05. Values represent means ± SD (n = 5). ^bP < 0.01. Values represent means ± SD (n = 5). ^cP < 0.001. Values represent means ± SD (n = 5).

and BUN declined by 16.62–50.96 and 11.06–34.94% due to nano-TiO₂ (P < 0.05 or 0.01), respectively, compared with the control.

Histopathological Evaluation. The histological changes in the kidney specimens are shown in Figure 6. Unexposed kidney samples presented intact architecture and regularly arranged renal cells, while those from the mice exposed to increasing concentrations of nano-TiO₂ exhibited severe pathological changes, including infiltration of inflammatory cells, dilatation and filling proteinic liquids in the renal tubule, degeneration of tubular cells, tubular dilatation, fibrosis, cell abscission, and tissue necrosis. The results suggested that chronic exposure to TiO₂ NPs resulted in significant pathological changes in the kidneys, which may be related to expression of renal fibration-related markers.

Expression of Wnt Ligands. The effects of nano-TiO₂ on the expression of Wnt ligands are listed in Tables 3 and 4. The results suggested that the level of expression of all Wnt ligands in the nano-TiO₂-exposed kidneys, including Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt9a, Wnt10a, and

Table 6. Effects of Nano-TiO₂ on the Level of Protein Expression of the Wnt Pathway- and EMT-Related Genes after Intragastric Feeding with Nano-TiO₂ for 9 Consecutive Months

	level of protein expression (ng/g of tissue)			
	control	1.25 mg of nano-TiO ₂ /kg of bw	2.5 mg of nano-TiO ₂ /kg of bw	5 mg of nano-TiO ₂ /kg of bw
Dkk1	84.6 ± 7.05	63.2 ± 5.35 ^a	59.6 ± 4.76 ^a	43.4 ± 3.49 ^b
Dkk2	77.9 ± 8.01	67.9 ± 5.76	52.4 ± 4.03 ^a	35.7 ± 3.29 ^b
Dkk3	63.9 ± 6.57	44.2 ± 4.69 ^a	32.7 ± 3.21 ^b	28.9 ± 2.65 ^b
Dkk4	115 ± 8.08	101 ± 7.67	79.1 ± 5.51 ^a	51.8 ± 4.22 ^b
sFRP/FrzB	79.5 ± 6.73	32.4 ± 3.49 ^a	28.9 ± 2.82 ^a	10.1 ± 1.45 ^c
Abcb1b	15.1 ± 1.27	16.9 ± 1.38	27.4 ± 2.55 ^b	29.7 ± 3.77 ^b
cyclin D1	19.6 ± 1.61	29.1 ± 2.72 ^a	45.6 ± 4.27 ^b	73.2 ± 6.73 ^c
Myc	88.5 ± 7.59	98.8 ± 10.3	120 ± 11.5 ^a	252 ± 23.9 ^c
Colla1	35.8 ± 2.91	51.5 ± 4.76 ^a	94.0 ± 8.59 ^b	243 ± 23.1 ^c
Fn	165 ± 13.78	192 ± 18.1 ^a	289 ± 26.7 ^b	318 ± 30.9 ^b
Twist	7.84 ± 0.59	16.3 ± 1.79 ^a	19.7 ± 2.05 ^a	35.8 ± 5.75 ^c
α-SMA	77.3 ± 6.56	81.7 ± 8.42	142 ± 13.05 ^b	216 ± 19.4 ^c

^aP < 0.05. Values represent means ± SD (n = 5). ^bP < 0.01. Values represent means ± SD (n = 5). ^cP < 0.001. Values represent means ± SD (n = 5).

Wnt11, was significantly elevated by 0.09–4.84-fold, whereas the level of Gsk3β expression was reduced by 14–64% with an increased nano-TiO₂ dose (P < 0.05).

Expression of Fz and Coreceptors LRP5 and -6. Figure 7 shows that exposure to nano-TiO₂ significantly induced the expression of all Wnt receptors Fz- and coreceptors LRP5 and -6-related markers in the kidneys, including Fz1, Fz5, Fz7, and LRP5 (0.37–8.57-fold) and LRP6 (0.73–5.27-fold).

Expression of Wnt and EMT Target Genes. Tables 5 and 6 show that with an increased nano-TiO₂ dose, the expression of all Wnt antagonist-related markers, such as Dkk1, Dkk2, Dkk3, Dkk4, and sFRP/FrzB, was notably inhibited by –0.06- to –0.87-fold. In addition, the levels of expression of Wnt and EMT target genes, such as Abcb1b, cyclin D1, Myc, Colla1, Fn, Twist, and α-SMA, were markedly elevated by 0.03–2.73- and 0.06–5.80-fold in the kidneys with an increased nano-TiO₂ dose, respectively.

DISCUSSION

The FDA's Critical Path Initiative sought to identify more sensitive and predictive biomarkers of nephrotoxicity that can specifically detect adverse changes in renal integrity before kidney function is compromised. ELISA measurements can identify numerous urinary protein biomarkers (such as KIM-1, CLU, OPN, cystatin C, and β2-microglobulin) and serum parameters, including CR, UA, and BUN, which were qualified by the FDA and European Medicines Agency (EMA) as prognostic prepathological indicators of nephrotoxicity in preclinical rat studies.^{29,30} The study presented here suggests that administration of 1.25, 2.5, and 5 mg of nano-TiO₂/kg of bw by intragastric feeding for 9 consecutive months increased the level of nano-TiO₂ accumulation in mouse kidney. This led to severe nephrotoxicity, shown by significant elevations in levels of urinary KIM-1, CLU, and OPN, serum CR and proteinuria, and reductions in serum UA and BUN, together with decreased body weight, inflammatory responses, fibrosis, and tissue necrosis. Reductions in levels of eating, drinking, and activity in mice caused by nano-TiO₂ were also observed; therefore, loss of body weight may be associated with loss of appetite. A decreased level of food intake is due to excessive ROS,³⁰ and exposure to nano-TiO₂ promoted the over-production of ROS in mouse kidney.^{10–13} Our previous studies indicated that exposure to nano-TiO₂ for 3 or 6 consecutive months resulted in renal inflammation in mice; however, renal

fibrosis was not observed.^{9–13} Renal inflammation and fibrosis in mice following exposure to nano-TiO₂ for 9 consecutive months may be due to modulation of the Wnt pathway.¹⁴

The Wnt pathway has been shown to play an important role in kidney development; in adult mouse kidney, however, Wnts and their receptors (Fzs) are also expressed.¹⁴ Therefore, we examined the alterations in Wnt ligands and Wnt receptors (Fzs) in mouse kidney following exposure to nano-TiO₂. Levels of all Wnt ligands, including Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt9a, Wnt10a, and Wnt11, were elevated following exposure to nano-TiO₂. As suggested, Wnt3a may be involved in various cancers³¹ and is associated with β-catenin activation, thus driving transcription from the LEF-1 promoter.³² As secretory proteins, Wnts proteins can bind to their respective receptors and execute their function in a paracrine or autocrine manner. He et al. observed that Fz receptors are expressed in tubular epithelial cells in vivo and guessed that these receptors may be the main targets of Wnt signaling in renal fibrosis.¹⁴ Our findings suggest that exposure to nano-TiO₂ markedly upregulated the expression of Fz1, Fz5, and Fz7 in the kidneys. These results support the hypothesis that nano-TiO₂ promotes renal inflammation and fibrosis by triggering the Wnt pathway as chronic exposure to nano-TiO₂ caused significant alterations in these Wnts and Fzs.

In this study, our data suggest that chronic exposure to nano-TiO₂ significantly decreased the level of expression of Dkk1, Dkk2, Dkk3, Dkk4, and sFRP/FrzB as antagonists of the Wnt pathway in the kidneys. As suggested, epigenetic silencing of Wnt antagonistic genes such as sFRP/FrzB,³³ Dkks,³⁴ and WIF1 is closely associated with poor prognosis in renal cell carcinoma patients.³⁵ The reduction in the level of antagonist expression in the kidneys caused by exposure to nano-TiO₂ may promote renal inflammation and fibrosis in mice.

As the expression of all Wnts and Fzs was greatly induced in the kidneys by nano-TiO₂, the question of whether β-catenin modulated transcription and the canonical Wnt pathway activated by nano-TiO₂ was also examined. Our findings indicate that nano-TiO₂ significantly elevated the level of expression of the Wnt genes c-Myc, cyclin D1, and Abcb1b in mouse kidney. It was previously demonstrated that both cyclin D1 and Abcb1b expression was involved in renal cancer and the aberrant Wnt/β-catenin pathway.³⁶ As EMT markers, fibronectin, collagen I, and α-SMA are closely related to tissue regeneration or fibrosis; in particular, these EMT markers play

pivotal roles in the development of renal fibrosis under various stresses and chronic inflammation, while Twist is associated with cancer generation and cancer cell metastasis.^{37–39} Expression of Twist and fibronectin was demonstrated to be modulated by the β -catenin pathway.^{40,41} Because of the activation of the Wnt/ β -catenin pathway caused by exposure to nano-TiO₂, we observed significantly increased levels of expression of fibronectin, α -SMA, and Twist in the kidneys. Importantly, collagen I has been shown to be very abundant in renal fibrosis¹⁴ and to be a critical modulator of kidney fibrosis.⁴¹ Our data also suggest that nano-TiO₂ significantly elevated the level of collagen I expression in the kidney.

In conclusion, this study shows that administration of nano-TiO₂ resulted in significant increases in urinary KIM-1, CLU, and OPN levels in mice. Furthermore, exposure to nano-TiO₂ activated the Wnt pathway through directly increasing the levels of expression of Wnts, Fz receptors, and EMT markers and decreasing the levels of expression of Wnt antagonists, thus resulting in renal inflammation and fibrosis.

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Notes

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REFERENCES

- Hendren, C. O.; Mesnard, X.; Droege, J.; Wiesner, M. R. Estimating production data for five engineered nanomaterials as a basis for exposure assessment. *Environ. Sci. Technol.* **2011**, *45*, 2562–2569.
- Skocaj, M.; Filipic, M.; Petkovic, J.; Novak, S. Titanium dioxide in our everyday life; is it safe? *Radiol. Oncol.* **2011**, *45*, 227–247.
- Weir, A.; Westerhoff, P.; Fabricius, L.; Hristovski, K.; von Goetz, N. Titanium dioxide nanoparticles in food and personal care products. *Environ. Sci. Technol.* **2012**, *46*, 2242–2250.
- Elsaesser, A.; Howard, C. V. Toxicology of nanoparticles. *Adv. Drug Delivery Rev.* **2012**, *64*, 129–137.
- Sang, X. Z.; Li, B.; Ze, Y. G.; Hong, J.; Ze, X.; Gui, S. X.; Sun, Q. Q.; Liu, H. T.; Zhao, X. Y.; Sheng, L.; Liu, D.; Yu, X. H.; Hong, F. S. Toxicological effects of nanosized titanium dioxide-induced spleen injury in mice. *J. Agric. Food Chem.* **2013**, *61*, 5590–5599.
- Hong, J.; Wang, L.; Zhao, X. Y.; Yu, X. H.; Sheng, L.; Xu, B. Q.; Liu, D.; Zhu, Y. T.; Long, Y.; Hong, F. S. Th2 factors may be involved in the TiO₂ NP-induced hepatic inflammation. *J. Agric. Food Chem.* **2014**, *62*, 6871–6878.
- Wang, J. X.; Zhou, G. Q.; Chen, C. Y.; Yu, H. W.; Wang, T. C.; Ma, Y. M.; Jia, G.; Gao, Y. X.; Li, B.; Sun, J.; Li, Y. F.; Jia, F.; Zhao, Y. L.; Chai, Z. F. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicol. Lett.* **2007**, *168*, 176–185.
- Chen, J. Y.; Dong, X.; Zhao, J.; Tang, G. P. In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitoneal injection. *J. Appl. Toxicol.* **2009**, *29*, 330–337.
- Liu, H. T.; Ma, L. L.; Zhao, J. F.; Liu, J.; Yan, J. Y.; Ruan, J.; Hong, F. S. Biochemical toxicity of mice caused by nano-anatase TiO₂ particles. *Biol. Trace Elem. Res.* **2009**, *129*, 170–180.
- Zhao, J. F.; Wang, J.; Wang, S. S.; Zhao, X. Y.; Yan, J. Y.; Ruan, J.; Li, N.; Duan, Y. M.; Wang, H.; Hong, F. S. The mechanism of oxidative damage in nephrotoxicity of mice caused by nano-anatase TiO₂. *J. Exp. Nanosci.* **2010**, *5* (5), 447–462.
- Gui, S. X.; Zhang, Z. L.; Zheng, L.; Sun, Q. Q.; Sang, X. Z.; Liu, X. R.; Gao, G. D.; Cui, Y. L.; Cheng, Z.; Cheng, J.; Tang, M.; Hong, F. S. The molecular mechanism of kidney injury of mice caused by exposure to titanium dioxide nanoparticles. *J. Hazard. Mater.* **2011**, *195*, 365–370.
- Gui, S. X.; Li, B. Y.; Zhao, X. Y.; Sheng, L.; Hong, J.; Yu, X. H.; Sang, X. Z.; Sun, Q. Q.; Ze, Y. G.; Wang, L.; Hong, F. S. Renal injury and Nrf2 modulation in mouse kidney following exposure to titanium dioxide nanoparticles. *J. Agric. Food Chem.* **2013**, *61*, 8959–8968.
- Lee, S. B.; Kalluri, R. Mechanistic connection between inflammation and fibrosis. *Kidney Int.* **2010**, *78* (Suppl.119), S22–S26.
- He, W.; Dai, C.; Li, Y.; Zeng, G.; Monga, S. P.; Liu, Y. Wnt/ β -catenin signaling promotes renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **2009**, *20*, 765–776.
- Banumathy, G.; Cairns, P. Signaling pathways in renal cell carcinoma. *Cancer Biol. Ther.* **2010**, *10*, 658–664.
- McCoy, K. E.; Zhou, X.; Vize, P. D. Non-canonical wnt signals antagonize and canonical wnt signals promote cell proliferation in early kidney development. *Dev. Dyn.* **2011**, *240*, 1558–1566.
- Vlad, A.; Rohrs, S.; Klein-Hitpass, L.; Muller, O. The first five years of the Wnt targetome. *Cell. Signalling* **2008**, *20*, 795–802.
- Niehrs, C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* **2006**, *25*, 7469–7481.
- Kalluri, R. EMT: When epithelial cells decide to become mesenchymal-like cells. *J. Clin. Invest.* **2009**, *119*, 1417–1419.
- Yang, J.; Mani, S. A.; Donaher, J. L.; Ramaswamy, S.; Itzykson, R. A.; Come, C.; Savagner, P.; Gitelman, I.; Richardson, A.; Weinberg, R. A. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **2004**, *117*, 927–939.
- Lee, J. M.; Dedhar, S.; Kalluri, R.; Thompson, E. W. The epithelial–mesenchymal transition: New insights in signaling, development, and disease. *J. Cell Biol.* **2006**, *172*, 973–981.
- Outlines Guidance on Handling Titanium Dioxide (TiO₂); National Institute for Occupational Safety and Health: Atlanta, 2011.
- Baan, R.; Straif, K.; Grosse, Y.; Secretan, B.; El Ghissassi, F.; Cogliano, V. Carcinogenicity of carbon black, titanium dioxide, and talc. *Lancet Oncol.* **2006**, *7*, 295–296.
- Yang, P.; Lu, C.; Hua, N.; Du, Y. Titanium dioxide nanoparticles co-doped with Fe³⁺ and Eu³⁺ ions for photocatalysis. *Mater. Lett.* **2002**, *57*, 794–801.
- Hu, R. P.; Zheng, L.; Zhang, T.; Cui, Y. L.; Gao, G. D.; Cheng, Z.; Chen, J.; Tang, M.; Hong, F. S. Molecular mechanism of hippocampal apoptosis of mice following exposure to titanium dioxide nanoparticles. *J. Hazard. Mater.* **2011**, *191*, 32–40.
- Drozdz, A. Food habits and food assimilation in mammals. In *Methods for ecological bioenergetics*; Grodzinski, W., Klekowski, R. Z., Duncan, A., Eds.; Blackwell: Oxford, U.K., 1975; pp 333–337.
- Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L.; Vandesompele, J.; Wittwer, C. T. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55* (4), 611–622.
- Schefe, J. H.; Lehmann, K. E.; Buschmann, I. R.; Unger, T.; Funke-Kaiser, H. Quantitative real-time RT-PCR data analysis: Current concepts and the novel “gene expression’s CT difference” formula. *J. Mol. Med.* **2006**, *84*, 901–910.
- FDA, European Medicines Agency to Consider Additional Test Results When Assessing New Drug Safety Collaborative Effort by FDA and EMEA Expected to Yield Additional Safety Data. U.S. Food and Drug Administration: Silver Spring, MD, 2008.
- Benani, A.; Troy, S.; Carmona, M. C.; Fioramonti, X.; Lorsignol, A.; Leloup, C.; Casteilla, L.; Pénicaud, L. Role for mitochondrial reactive oxygen species in brain lipid sensing: Redox regulation of food intake. *Diabetes* **2007**, *56*, 152–160.
- Katoh, M. WNT3-WNT14B and WNT3A-WNT14 gene clusters (review). *Int. J. Mol. Med.* **2002**, *9*, 579–584.
- Filali, M.; Cheng, N.; Abbott, D.; Leontiev, V.; Engelhardt, J. F. Wnt-3A/ β -catenin signaling induces transcription from the LEF-1 promoter. *J. Biol. Chem.* **2002**, *277*, 33398–33410.

- (33) Kawakami, K.; Yamamura, S.; Hirata, H.; Ueno, K.; Saini, S.; Majid, S.; Tanaka, Y.; Kawamoto, K.; Enokida, H.; Nakagawa, M.; Dahiya, R. Secreted frizzled-related protein-5 is epigenetically downregulated and functions as a tumor suppressor in kidney cancer. *Int. J. Cancer* **2011**, *128*, 541–550.
- (34) Hirata, H.; Hinoda, Y.; Nakajima, K.; Kawamoto, K.; Kikuno, N.; Ueno, K.; Yamamura, S.; Zaman, M. S.; Khatri, G.; Chen, Y.; Saini, S.; Majid, S.; Deng, G.; Ishii, N.; Dahiya, R. Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. *Int. J. Cancer* **2011**, *128*, 1793–1803.
- (35) Kawakami, K.; Hirata, H.; Yamamura, S.; Kikuno, N.; Saini, S.; Majid, S.; Tanaka, Y.; Kawamoto, K.; Enokida, H.; Nakagawa, M.; Dahiya, R. Functional significance of Wnt inhibitory factor-1 gene in kidney cancer. *Cancer Res.* **2009**, *69*, 8603–8610.
- (36) Alao, J. P. The regulation of cyclinD1 degradation: Roles in cancer development and the potential for therapeutic invention. *Mol. Cancer* **2007**, *6*, 24.
- (37) Kalluri, R.; Neilson, E. G. Epithelial–mesenchymal transition and its implications for fibrosis. *J. Clin. Invest.* **2003**, *112*, 1776–1784.
- (38) Polyak, K.; Weinberg, R. A. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stemcell traits. *Nat. Rev. Cancer* **2009**, *9*, 265–273.
- (39) Zeisberg, M.; Neilson, E. G. Biomarkers for epithelial–mesenchymal transitions. *J. Clin. Invest.* **2009**, *119*, 1429–1437.
- (40) Grasl, D.; Kuhl, M.; Wedlich, D. The Wnt/Wg signal transducer β -catenin controls fibronectin expression. *Mol. Cell. Biol.* **1999**, *19*, 5576–5587.
- (41) Howe, L. R.; Watanabe, O.; Leonard, J.; Brown, A. M. Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. *Cancer Res.* **2003**, *63*, 1906–1913.