Single-Nucleotide Polymorphisms of the Dopamine D2 Receptor Increase Inflammation and Fibrosis in Human Renal Proximal Tubule Cells

Xiaoliang Jiang, Prasad Konkalmatt, Yu Yang, John Gildea, John E. Jones, Santiago Cuevas, Robin A. Felder, Pedro A. Jose, Ines Armando

Abstract—The dopamine D2 receptor (D2R) negatively regulates inflammation in mouse renal proximal tubule cells (RPTCs), and lack or downregulation of the receptor in mice increases the vulnerability to renal inflammation independent of blood pressure. Some common single-nucleotide polymorphisms (SNPs; rs6276, rs6277, and rs1800497) in the human DRD2 gene are associated with decreased D2R expression and function, as well as high blood pressure. We tested the hypothesis that human RPTCs (hRPTCs) expressing these SNPs have increased expression of inflammatory and injury markers. We studied immortalized hRPTCs carrying D2R SNPs and compared them with cells carrying no D2R SNPs. RPTCs with D2R SNPs had decreased D2R expression and function. The expressions of the proinflammatory tumor necrosis factor-α and the profibrotic transforming growth factor-β1 and its signaling targets Smad3 and Snail1 were increased in hRPTC with D2R SNPs. These cells also showed induction of epithelial mesenchymal transition and production of extracellular matrix proteins, assessed by increased vimentin, fibronectin 1, and collagen I α1. To test the specificity of these D2R SNP effects, hRPTC with D2R SNPs were transfected with a plasmid encoding wild-type DRD2. The expression of D2R was increased and that of transforming growth factor-β1, Smad3, Snail1, vimentin, fibronectin 1, and collagen I α1 was decreased in hRPTC with D2R SNPs transfected with wild-type DRD2 compared with hRPTC-D2R SNP transfected with empty vector. These data support the hypothesis that D2R function has protective effects in hRPTCs and suggest that carriers of these SNPs may be prone to chronic renal disease and high blood pressure. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: chronic kidney disease kidney receptors, dopamine D2

Dopamine synthesized in the kidney is necessary for the maintenance of normal renal function and blood pressure.1 Dopamine and dopaminergic drugs have also been shown to regulate the immune response and the inflammatory reaction.2 Mice with intrarenal dopamine deficiency have increased oxidative stress and infiltration of inflammatory cells,3 and decreased renal dopamine production aggravates angiotensin II–mediated renal injury.4 The anti-inflammatory effects of dopamine are mediated, at least in part, by the dopamine D2 receptor (D2R).5,6

Previous work from our laboratory has indicated that the D2R in the kidney has a direct and significant role in regulating the mechanisms involved in the development of renal inflammation and injury, as well as in blood pressure control.6,7 Mice with lack or reduced level of D2R expression and function in the kidney have increased renal expression of proinflammatory and decreased expression of anti-inflammatory cytokines/chemokines, as well as histological and functional evidence of renal inflammation and injury, suggesting that the D2R has protective effects in the kidney by limiting the inflammatory reaction and that impaired function of the D2R results in renal inflammation and organ damage.4

Deficient renal D2R function may be of clinical relevance. Polymorphisms of the DRD2 gene are commonly observed in humans, and some of them have been associated with elevated blood pressure and even hypertension.8,9 Some common single-nucleotide polymorphisms (SNPs; rs6276, rs6277, and rs1800497) in the noncoding region of the human DRD2 gene are associated with decreased D2R expression and function.11,12 We hypothesized that the presence of the DRD2 variants in human renal proximal tubule cells (RPTCs) is associated with decreased D2R protective effects and increased expression of inflammatory and injury markers. To test this hypothesis, we studied RPTCs from

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subjects with and without D₂R SNPs rs6276, rs6277, and rs1800497.

Material and Methods

Cell Culture
RPTCs were isolated from human kidney specimens from patients who had unilateral nephrectomy because of renal carcinoma or trauma. Only the visually and histologically normal pole, distal from the affected part of the kidney, was used to isolate RPTCs. Cells were immortalized, as previously described. A University of Virginia Institutional Review Board–approved protocol was used according to the Declaration of Helsinki.

The phenotypic characteristics of the subjects from whom the cell lines were derived are shown in Table S1 in the online-only Data Supplement. The RPTCs were genotyped for the presence of the rs6276, rs6277, and rs1800497 variants, which are in the noncoding region. Ten cell lines were studied, 5 of them from subjects not bearing SNPs, 3 from subjects heterozygous for both rs6276 and rs6277, and 2 from subjects heterozygous for both rs6276 and rs1800497. The cells were cultured in nonpolarizing conditions as described in Methods in the online-only Data Supplement.

Immunoblotting
RPTC lysates were subjected to immunoblotting as described previously. The primary antibodies used were rabbit polyclonal D₂R (Millipore, Billerica, MA), rabbit polyclonal tumor necrosis factor α (TNFα) (Abcam, Cambridge, MA), mouse monoclonal fibronectin 1 (FN-1; R&D, Minneapolis, MN), mouse anti-human transforming growth factor β (TGF-β) (R&D), and polyclonal anti-GAPDH (Sigma). The densitometry values were corrected for the expression of GAPDH and are shown as percentage of the mean density of the D₂R without SNPs group.

cAMP Accumulation Assay
RPTCs were grown to confluence in 6-well plates and pretreated with 1 μmol/L phorbol 12-myristate 13-acetate (PMA) for 5 minutes in the presence of the phosphodiesterase inhibitor IBMX (1 μmol/L) before treatment with the D₂R agonist quinpirole (1 μmol/L–1 μmol/L/20 min; Sigma). Cell lysates were prepared to determine the protein concentration using the BCA protein assay kit (Thermo Scientific, Rockford, IL) and cAMP concentration using the Cyclic AMP Chemiluminescent Immunoassay Kit (Arbor Assays, Ann Arbor, MI), following the manufacturer’s procedures.

RNA Extraction and cDNA Preparation
Total RNA was purified using the RNeasy RNA Extraction Mini kit (Qiagen, Valencia, CA). RNA samples were converted into first strand cDNA using an RT2 First Strand kit, following the manufacturer’s protocol (SABiosciences-Qiagen).

Quantitative Real-Time Polymerase Chain Reaction
Quantitative gene expression analysis was performed by real-time polymerase chain reaction (ABI Prism 7900 HT, Applied Biosystems, Foster City, CA). The assay used gene-specific primers (SABiosciences-Qiagen) and SYBR Green real-time polymerase chain reaction detection method and was performed as described in the manufacturer’s manual. The primers used are described in Table S2. Data were analyzed using the ∆∆Ct method. 16

Immunofluorescence and Confocal Microscopy
RPTCs, grown on poly-D-lysine–coated coverslips to 50% confluence, were immunostained using a mouse anti–TGF-β antibody (R&D), followed by Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Grand Island, NY). FN-1 was visualized using a mouse monoclonal antibody (Millipore) followed by Alexa Fluor 557 goat anti-mouse IgG antibody (Molecular Probes). Snail1 was visualized with an anti-human Snail1 antibody conjugated with an NL5S7 fluorochrome (R&D Systems). Vimentin was visualized with an anti-human vimentin antibody conjugated with an NL493 fluorochrome (R&D Systems). DAPI was used to visualize the nuclei.

Transient Transfection With a DRD2 Plasmid
RPTCs carrying SNPs were transfected with an empty vector or plasmid harboring wild-type human DRD2 under the control of cytomegalovirus promoter (RC202476, Origene Technologies, Inc.), as described in Methods in the online-only Data Supplement. Three days after transfection, the RPTCs were harvested for protein extraction and immunoblotting, total RNA preparation, and quantitative real-time polymerase chain reaction or processed for immunofluorescence staining.

Treatment With Transforming Growth Factor-β
RPTCs were grown on poly-D-lysine–coated coverslips to 50% confluence. After TGF-β (10 ng/mL; R&D) treatment for 30 hours, cells were immunostained for Snail1, FN-1, and vimentin as described above and subjected to confocal microscopy.

Statistical Analysis
Data are expressed as means±SEM. Comparisons between 2 groups used the Student’s t test. One-way ANOVA followed by Newman–Keuls test was used to assess significant differences in >2 groups. P<0.05 was considered significant.

Results
RPTCs From Subjects Bearing D₂R SNPs Have Decreased Receptor Expression and Function
RPTCs from subjects heterozygous for either rs6276 and rs6277 or rs6276 and rs1800497 SNPs were studied; there were no RPTCs that were homozygous for any of these D₂R SNPs. The D₂R mRNA expression in RPTCs from subjects heterozygous for either the rs6276 and rs6277 or the rs6276 and rs1800497 SNPs was ~50% lower than that in RPTCs from subjects not carrying D₂R SNPs (Figure 1A). Similarly, D₂R protein expression was also ~50% lower in RPTCs from subjects with D₂R SNPs than those without D₂R SNPs (Figure 1B). Because the degree of reduction in D₂R expression was similar in RPTCs with both SNPs genotypes, samples were pooled for further analysis. To determine the functional significance of the decrease in D₂R expression, we evaluated the effect of D₂R stimulation in potentiating the cAMP response to 1 μmol/L PMA. 15 Although D₂R is normally associated with inhibition of cAMP production, it can stimulate adenyl cyclase type 2 activity via protein kinase C in the presence of PMA. 17 The effect of the D₂R agonist quinpirole on the accumulation of cAMP in response to PMA was significantly lower in RPTCs carrying D₂R SNPs than in those not carrying D₂R SNPs (Figure 1C), indicating that the decrease in D₂R expression results in decreased receptor function. These results also indicate that the overall reduction in the expression of D₂R reduces the number of membrane-associated D₂R.

Increased Expression of Proinflammatory Factors in RPTCs With D₂R SNPs
In mouse RPTCs, D₂R downregulation increases the expression of TNF-α and other cytokines and chemokines. 8 The mRNA and protein expressions of TNF-α were also increased (2.5-fold and 1.4-fold, respectively) in RPTCs with D₂R SNPs in comparison with RPTCs with wild-type D₂R.
The expressions of C-reactive protein (1.5-fold), the chemokines monocyte-specific chemokine 3 (1.6-fold) and macrophage inflammatory protein-1β (1.4-fold), nuclear factor kappa B1A (NFkB1A) (1.7-fold), and the cytokine interleukin-10 (1.6-fold) were also increased in RPTCs with D2R SNPs (Figure 2A). The mRNA expressions of the chemokines CCl24 and CXCL14 and the cytokines interleukin-1F7 and interleukin-22 were also found increased in cells with D2R SNPs (Figure S1). These results suggest that in RPTCs the presence of the D2R SNPs imparts an inflammatory phenotype.

TGF-β Expression and Function Are Increased in RPTCs Carrying D2R SNPs

TNF-α induces inflammatory responses leading to renal injury and fibrosis mainly by stimulating the production of TGF-β, a key mediator of fibrosis in the kidney. The mRNA and protein expression of TGF-β was (1.7-fold and 2-fold, respectively) greater in RPTCs with D2R SNPs than RPTCs not carrying D2R SNPs (Figure 3A and 3B). TGF-β immunofluorescence staining was also more intense in RPTCs with D2R SNPs compared with cells without D2R SNPs, but there were no apparent differences in subcellular localization (Figure 3C).

TGF-β signaling is central to renal fibrosis associated with epithelial to mesenchymal transition (EMT) of the renal tubules and synthesis of extracellular matrix. The TGF-β signal is transduced by its transmembrane type I and type II; binding of TGF-β to a heteromeric complex of its type I and type II serine/threonine kinase activates its downstream signaling mediators, Smad2 and Smad3. Phosphorylated Smad2/3 then binds to the common partner Smad4 and is subsequently translocated into the nucleus, where it controls the transcription of TGF-β-responsive genes. Several transcription factors have been identified to mediate TGF-β function, including Snail1, which is upregulated through the...
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TGF-β–Smad3–response gene to complement 32 pathway and has been shown to be important in renal EMT and fibrosis. In agreement with the increased expression of TGF-β, RPTCs carrying D2R SNPs also had increased mRNA expression of Smad3 (1.7-fold) and Snail1 (2.5-fold), in comparison with RPTCs not carrying D2R SNPs (Figure 4A). Although TGF-β signaling is mediated by both Smad2 and Smad3, we have reported that response gene to complement 32, which we have shown to be important in EMT of human renal tubular cells, interacts only with Smad3 in inducing EMT of these cells.

Snail1 immunofluorescence staining was more intense not only in the cytoplasm, but also in the nucleus of RPTCs with D2R SNPs (Figure 4B). Snail1 activates the transcription of the extracellular matrix proteins collagen I α1 (Col 1α) and FN-1, and the expression of the mesenchymal marker vimentin. The mRNA (2.9-fold) and protein expressions (1.8-fold) of FN-1 were increased in RPTCs carrying D2R SNPs (Figure 5A and 5B); FN-1 immunofluorescence was also increased in these cells (Figure 5C), relative to RPTCs not carrying D2R SNPs.

Similarly, mRNA expressions of Col 1α (1.6-fold) and vimentin (1.3-fold) were also increased in cells with D2R SNPs (Figure 5A) as was the immunofluorescence staining for vimentin (Figure 5D). These results indicate that RPTCs with D2R SNPs produce increased levels of extracellular matrix components and express mesenchymal markers.

To confirm the association of the increased TGF-β in the increased profibrotic markers in D2R SNPs, we tested the effects of TGF-β on RPTCs not carrying SNPs. Treatment

Figure 4. SMAD3 and Snail1 expression in renal proximal tubular cells (RPTCs) carrying dopamine D2 receptor (D2R) wild type (WT) or D2R single-nucleotide polymorphisms (SNPs). A, Expressions of SMAD3 and SNAIL1 mRNA in RPTCs from subjects carrying D2R WT or D2R SNPs were quantified by quantitative real-time polymerase chain reaction; GAPDH mRNA was used for normalization of the data, n=5 per group. *P<0.05. B, Laser-scanning confocal images of immunofluorescence staining of Snail1 in RPTCs carrying D2R WT or D2R SNPs. DAPI was used to immunostain the nuclei. Immunostaining was performed in all 10 cell lines (5 WTs and 5 bearing the SNPs). Thirty to fifty cells were observed in each sample.

Figure 5. Fibronectin 1 (FN-1), collagen I α1 (Col 1α), and vimentin expression in renal proximal tubular cells (RPTCs) carrying dopamine D2 receptor (D2R) wild type (WT) or D2R single-nucleotide polymorphisms (SNPs). A, Expressions of FN-1, Col 1α, and vimentin mRNA in RPTC from subjects D2R WT or D2R SNPs were quantified by quantitative real-time polymerase chain reaction; GAPDH mRNA was used for normalization of the data, n=5 per group. *P<0.05. B, RPTC homogenates were immunoblotted using FN-1–specific antibody; GAPDH protein was used for normalization of the data, n=5 per group. *P<0.05, t test. C and D, Laser-scanning confocal images of immunofluorescence staining of FN-1 and vimentin in RPTCs carrying D2R SNPs or not carrying SNPs. DAPI was used to immunostain the nuclei. Immunostaining was performed in all 10 cell lines (5 WTs and 5 bearing the SNPs). Thirty to fifty cells were observed in each sample.

Transient Expression of Wild-Type Human DRD2 Reverses the Fibrotic Phenotype in RPTCs With D2R SPNs

To determine whether the decreased D2R expression was the primary defect in RPTCs carrying D2R SNPs, these cells were transfected with a plasmid harboring cDNA for wild-type human DRD2 under the control of cytomegalovirus promoter or an empty vector. The mRNA (2.8-fold) and protein (2-fold) expressions of D2R were increased in the cells transfected with the wild-type DRD2 plasmid compared with those transfected

Figure 5A B C D

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with the empty vector (Figure 6). Although a direct comparison was not performed, D₂R expression in RPTCs with D₂R SNPs transfected with wild-type DRD2 plasmid seemed to be similar to that in RPTCs carrying no D₂R SNPs. D₂R levels in RPTCs carrying no SNPs were twice as high as those in the RPTCs with D₂R SNPs (Figure 1), whereas transient transfection of the wild-type DRD2 plasmid increased D₂R expression by 2-fold compared with the same cells transfected with an empty vector. This also suggests that there is no overt overexpression of the wild-type DRD2 gene.

Transient expression of wild-type DRD2 in RPTCs with D₂R SNPs decreased both mRNA and protein expression of TGF-β by ≈30% to 40% compared with the same cells transfected with empty vector (Figure 7A and 7B). TGF-β immunostaining was markedly reduced in RPTCs with D₂R SNPs transiently transfected with wild-type DRD2 (Figure 7C). Similarly, mRNA expression of Snail was also decreased (≈75%), and the intensity of Snail immunostaining was reduced in RPTCs with D₂R SNPs transfected with wild-type DRD2 plasmid, compared with the same cells transfected with an empty vector (Figure 7A and 7D). Transient expression of wild-type DRD2 also decreased the mRNA levels of FN-1 (50%), Col 1a (40%), and vimentin (80%) and protein level of FN-1 (60%; Figure 8A and 8B) and decreased the intensity of FN-1 and vimentin immunostaining (Figure 8B and 8C). Taken together, these results show that upregulation of D₂R expression in RPTCs with D₂R SNPs reverses, at least partially, the increase in TGF-β expression and function and blunts the ability of these cells to produce extracellular matrix components and mesenchymal markers.

**Discussion**

Our results show that RPTCs from human subjects carrying D₂R SNPs that result in decreased D₂R mRNA and protein express a proinflammatory and profibrotic phenotype and markers of EMT. This phenotype is partially reversed by increasing D₂R expression by the transient transfection of the wild-type DRD2, indicating that the D₂R has protective effects in these cells. Because both RPTCs and microdissected proximal tubules, in culture, produce significant amounts of dopamine from L-DOPA present in the serum added to the medium and the endogenous D₂R is constitutively activated, these effects are observed in the absence of ligand added to the medium.

Reduced density of D₂Rs attributable to decreased D₂R mRNA stability and synthesis of the receptor has been associated with presence of the rs1800497 (TaqIA A1) allele of the ANKK1 gene (previously reported as located in the DRD2 gene), the rs6276 A/A genotype in exon 8, and the rs6277 (957C>T) in exon 7, in both in vitro and in vivo studies. These polymorphisms are commonly observed with allele frequencies of 0.401 for rs1800497, 0.422 for rs6276, and 0.229 for rs6277 across several populations. These 3
SNPs occur within and adjacent to the DRD2 gene. Because of their close proximity, the SNPs are likely in linkage disequilibrium. This, coupled with the small number of human immortalized RPTCs available, resulted in our being unable to identify cell lines that were homozygous for only 1 DRD2 variant, limitations that have to be acknowledged. Other limitation of this study is the use of immortalized cell lines rather than primary cultures; however, this limitation is minimized by the fact that transformed RPTCs retain many of the functional and morphological characteristics of RPTCs in primary culture.

Perspectives

The prevalence of chronic kidney disease is rising sharply worldwide and affects 13.1% of the population in the United States. Patients with chronic kidney disease represent a population not only at risk of progression to end-organ failure but are also at higher risk for cardiovascular disease.

Hypertension is one of the major causes of renal injury and together with diabetes mellitus accounts for up to 75% of the end-stage renal disease in the United States. Inflammation and oxidative stress are major mediators in the development and progression of renal disease. Low-grade inflammation is associated with cardiovascular disease. Infiltration of inflammatory cells and increased expression of proinflammatory factors are crucial in the development of renal injury, as well as in the induction and maintenance of hypertension. There is increasing evidence that genetic factors contribute to the susceptibility to renal disease associated with essential hypertension, and it has been suggested that hypertension may cause progressive kidney disease only in genetically susceptible individuals. The genetic predisposition to chronic kidney disease is polygenic, but to date only a few genes have been shown to be contributory. Renal susceptibility genes may determine the occurrence and severity of hypertension-induced progressive renal damage. Much of the research in this field has been directed to determining the detrimental factors involved in disease progression. However, the role of factors that prevent inflammation and slow the progression of renal disease has received much less attention. Our results show that individuals carrying D,R polymorphisms that result in decreased D,R expression and function could be more vulnerable to renal injury when challenged with an insult such as elevated blood pressure. Genetic testing could be used to identify the individuals at risk and pharmacological treatment tailored to ameliorate the insult which should result in decreased prevalence of renal injury and chronic kidney disease.

Sources of Funding

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Disclosures

None.

References


What Is New?

- We show that renal proximal tubule cells from subjects carrying common polymorphisms in the DRD2 gene that result in decreased expression of the dopamine D2 receptor have increased levels of proinflammatory factors and extracellular matrix components and express mesenchymal markers.

What Is Relevant?

- Individuals carrying DRD2 polymorphisms may be more vulnerable to renal injury when challenged with an insult such as elevated blood pressure. Genetic testing can be used to identify the individuals at risk and pharmacological treatment tailored to ameliorate the insult, which should result in decreased prevalence of renal injury and chronic kidney disease.

Summary

These results show that renal proximal tubule cells from human subjects carrying dopamine D2 receptor polymorphisms that result in decreased dopamine D2 receptor mRNA and protein express a pro-inflammatory and profibrotic phenotype as well as markers of epithelial mesenchymal transition. Individuals carrying these dopamine D2 receptor polymorphisms could be more vulnerable to renal injury.
Single-Nucleotide Polymorphisms of the Dopamine D2 Receptor Increase Inflammation and Fibrosis in Human Renal Proximal Tubule Cells
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SINGLE NUCLEOTIDE POLYMORPHISMS OF THE DOPAMINE D2 RECEPTOR INCREASE INFLAMMATION AND FIBROSIS IN HUMAN RENAL PROXIMAL TUBULE CELLS.

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Supplemental Methods

Cell culture
Immortalized RPTCs were kept at 37 °C in an atmosphere containing 5% CO2 and cultured to 90–95% confluence in non-polarizing conditions in DMEM-F12 containing (final concentrations): plasmocin (2.5 µg/ml, Fisher Scientific); EGF (10 ng/ml, Sigma), dexamethasone (36 ng/ml, Sigma), triiodothyronine (2 ng.ml; Sigma), insulin-transferrin-selenium (ITS, 1X, Invitrogen); penicillin/streptomycin (1X, Invitrogen); fetal bovine serum (2%, Invitrogen); and G418 (0.4%, EMD Chemicals)\textsuperscript{15}

Transfection
RPTCs carrying SNPs were transfected with an empty vector or plasmid harboring wild-type human \textit{DRD2} under the control of CMV promoter (RC202476, Origene Technologies, Inc.), using Fugene HD transfection reagent (E2311; Promega Corporation) as per the manufacturer’s guidelines. Briefly, 4 µg of plasmid DNA were diluted to 400 µl with serum-free medium and mixed with 16 µl of Fugene HD transfection. The transfection complex was incubated for 15 minutes at room temperature and added drop wise onto the cells in 10 cm tissue culture plates. Three days following transfection, the RPTCs were harvested for protein extraction and immunoblotting, total RNA preparation and RT-qPCR, or processed for immunofluorescence staining. Transfection efficiency was 40-45%.
Table S1. Characteristics of the subjects from whom the cell lines were obtained.

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Table S2: Gene name and catalog number and manufacturer of the primers used for qRT-PCR

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Supplemental Figure S1

Expression of pro-inflammatory factors in RPTCs from subjects carrying D₂R wild-type (WT) or D₂R SNPs.

The mRNA expressions of the chemokines CCL24 and CXCL24, and the cytokines IL1F7 and IL22 in RPTCs from subjects carrying D₂R WT or D₂R SNPs were quantified by qRT-PCR; results and were corrected for expression of GAPDH mRNA, n=5/group. *=P<0.05, t-test.
Supplemental Figure S2

**Snail-1**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**DAPI**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**Merge**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**FN 1**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**DAPI**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**Merge**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**Vimentin**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**DAPI**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**

**Merge**

- **D_{2}R WT**
- **D_{2}R WT +TGFβ**
Supplemental Figure S2

Treatment of RPTCs bearing no D2R SNPs (D2R WT) with TGFβ increased the immunostaining of Snail-1, FN-1, and Vimentin.

RPTCs were grown on poly-D-lysine-coated coverslips to 50% confluence. After TGFβ (10ng/ml; R&D Systems) treatment for 30 hours, the cells were immunostained using a mouse anti-FN-1 antibody (Millipore), followed by Alexa Fluor 557 goat anti-mouse IgG antibody (Molecular Probes, Grand Island, NY). Snail-1 was visualized with an anti-human Snail antibody conjugated with a NL557 fluorochrome (R&D Systems). Vimentin was visualized with an antihuman Vimentin antibody conjugated with a NL493 fluorochrome (R&D Systems). DAPI was used to visualize the nuclei. Immunostaining was performed in all five D2R WT cell lines. Thirty to fifty cells were observed in each sample. Representative images are shown.