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# Gene expression analysis in LLC-PK1 renal tubular cells by atrial natriuretic peptide (ANP): correlation of homologous human genes with renal response

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**Summary**

We used human DNA microarray to explore the differential gene expression profiling of atrial natriuretic peptide (ANP)-stimulated renal tubular epithelial kidney cells (LLC-PK1) in order to understand the biological effect of ANP on renal kidney cell's response. Gene expression profiling revealed 807 differentially expressed genes, consisting of 483 up-regulated and 324 down-regulated genes. The bioinformatics tool was used to gain a better understanding of differentially expressed genes in porcine genome homologous with human genome and to search the gene ontology and category classification, such as cellular component, molecular function and biological process. Four up-regulated genes of ATP1B1, H3F3A, ITGB1 and RHO that were typically validated by real-time quantitative PCR (RT-qPCR) analysis serve important roles in the alleviation of renal hypertrophy as well as other related effects. Therefore, the human array can be used for gene expression analysis in pig kidney cells and we believe that our findings of differentially expressed genes served as genetic markers and biological functions can lead to a better understanding of ANP action on the renal protective system and may be used for further therapeutic application.

## Introduction

Atrial natriuretic peptide (ANP) is a member of the natriuretic peptide family that consists of 28 amino acid residues. It is synthesized, stored, and released by atrial myocytes of the heart in response to atrial distension, as well as to stimulation by angiotensin II, endothelin, and the sympathetic nervous system. It is also synthesized in a variety of other tissues, including the kidneys [1, 2]. The roles of ANP in the kidney include increasing glomerular filtration rate, inhibiting renal tubular reabsorption of sodium and chloride, as well as redistributing blood flow to the renal outer medullar region which can be of beneficial value in the treatment of acute renal failure [3, 4]. In addition, it has been found that the mechanical actions of ANP are mediated by the A-type natriuretic peptide receptor (NPR-A), a single

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transmembrane sequence receptor linked to guanylate cyclase (GCase). Subsequently, the activation of guanylate cyclase generates cGMP from GTP, in which cGMP is the common mediator of vascular relaxation. This contributes to negative inotropic and chronotropic effects in the kidney, as well as natriuresis and inhibition of water and salt intake [5–8]. Moreover, ANP has some biological activities on LLC-PK1 renal tubular kidney cells. These activities can include the attenuation of ANG II-induced hypertrophy of renal tubular cells [9], the induction of a significant increase in cyclic GMP (cGMP) formation in LLC-PK1 cells [10], and the reduction of cyclosporine toxicity in renal tubular cells [11]. However, the whole gene expression profiling of ANP-stimulated LLC-PK1 cells and their differential gene functions have yet to be reported. Although some renal actions of ANP in renal tubular kidney cells have been elucidated, the effects of ANP on the gene expression profiling of renal tubular cells, including gene functions, signal transduction pathways, and mechanism of renal hypertrophy have not been clearly understood.

To study the biological effect of ANP on gene expression profile of the cellular response of kidney cells, namely renal tubular epithelial cells (LLC-PK1), we used the high throughput technique of DNA microarray and bioinformatics tools to explore the differentially expressed genes and to search the gene ontology that provided the categorical classifications of cellular component, molecular function and biological process. Interestingly, the biological functions of four differentially expressed genes involved in the alleviation of renal failure progression and some related effects. Moreover, we also proposed the hypothetical pathway of ANP signal transduction in LLC-PK1 cell's response. We believe that our finding of differentially expressed genes in response to ANP stimulation and the regulation of genes in renal system may become useful information for diagnostic and therapeutic applications.

## Materials and methods

### *Materials*

LLC-PK1 cells (ATCC CL101, *Sus scrofa*, pig renal tubular epithelial kidney cells) were obtained from ATCC (Rockville, MD, USA). Medium 199 was obtained from HyClone (Logan, UT, USA). D-PBS and Trypsin-EDTA were purchased from ATLANTA biologicals (Norcross, GA, USA). Fetal bovine serum, antibiotic-antimycotic, and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). ANP and sodium bicarbonate were purchased from Sigma (St. Louis, MO, USA). RNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA), and cyanine 3- and 5-labeled CTP (10.0 mM) were purchased from Perkin-Elemer/NEN Life Science (Boston, MA, USA). The RNA 6000 Nano LabChip Kit, Low RNA Input Fluorescent Linear Amplification Kit, Human 1A Oligo Microarray Kit (V2), in situ Hybridization Kit Plus, and the Stabilization and Drying Solution were purchased from Agilent Technologies (Palo Alto, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

### *Cell culture*

LLC-PK1 cells were cultured in Medium 199 supplemented with 1.5 g/l sodium bicarbonate, 3% fetal bovine serum, and 1% antibiotic-antimycotic in a humidified incubator with 5% $CO_2$ and 95% air at 37 °C [9–11]. Upon confluence, the cells were detached by treatment with 0.05% trypsin and 0.53 mM EDTA. During subculture, the medium was replaced every 2–3 days.

### *ANP treatment*

To perform cell attachment, the cells were seeded at $2 \times 10^6$ cells in a 10 cm tissue culture dish (NUNC$^{TM}$, Roskilde, Denmark) for 24 h. The cells were then washed with PBS and were treated with vehicle (D-PBS, 0.01%) and ANP ($10^{-7}$ mol/l) for 24 h [10]. The number of independent paired samples of cultured cells that were treated with ANP either present or absent was done in triplicate for each paired sample.

### *RNA preparation and quantitative measurement*

RNA was extracted by a modified method using TRIzol combined with RNeasy Mini Kit. Briefly, the total RNA was extracted with 1 ml of TRIzol reagent per $1 \times 10^6$ cells or 10 cm cell culture dish following the manufacturer's instructions. The

TRIzol samples were added with 0.2 ml of chloroform, vigorously shaken for 15 s, and incubated for 2–3 min at 15–30 °C. The aqueous phase was separated by centrifugation at 12,000 × g for 15 min at 2–8 °C. The supernatant was used as the input material for the RNeasy Mini Kit, and the total RNA was isolated as indicated in the manufacturer's instructions. The total RNA was quantified by a UV spectrophotometer and RNA quality was evaluated by capillary electrophoresis on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip Kit.

*RNA amplification and labeling*

Targets of cRNA were amplified and fluorescently labeled from 0.5 μg total RNA in each reaction using the Agilent Low RNA Input Fluorescent Linear Amplification kit following the instructions in the user's manual. For each sample pair, the control samples were labeled with Cy3 and the treated samples were labeled with Cy5. After purification using Qiagen's RNeasy mini-spin columns, the quantification, quality and size distribution of the labeled cRNA targets were then determined by ultraviolet (UV) spectrophotometry and RNA 6000 Nano LabChip Assay.

*Microarray hybridization*

Hybridization was performed following the Agilent oligonucleotide microarray hybridization user's manual and Agilent in situ Hybridization Kit Plus. Briefly, 2 μg of labeled cRNA per channel was mixed with 50 μl 10 × control targets and nuclease-free water to come up with a final volume of 240 μl. Each sample tube was added with 10 μl of 25 × fragmentation buffer, and was incubated at 60 °C in a water bath for 30 min in the dark. Afterward, the reaction was terminated by the addition of 250 μl of 2 × hybridization buffer. A volume of 500 μl of hybridization mix was applied to Agilent's Human 1A Oligonucleotide Microarray which contains 20,173 (60 mer) oligonucleotide probes spanning conserved exons across the transcripts of 18,716 targeted full-length genes, and hybridized in a hybridization rotation oven at 60 °C for 17 h. The slides which were disassembled in 6 × SSPE and 0.005% *N*-Lauroylsarcosine were washed with 6 × SSPE, 0.005% *N*-Lauroylsarcosine for 1 min at room temperature, then with 0.06 × SSPE, 0.005% *N*-Lauroylsarcosine for 1 min and with Stabilization and Drying Solution for 30 s.

*Data analysis and bioinformatics*

The microarray chip was scanned using an Agilent G2565BA Microarray Scanner System, and the Agilent Feature Extraction software 7.5 used defaults for all parameters including a parameterized error model to compute the significance (*p*-values) of log ratios. The image quantities of interest produced by the image analysis methods were the (R, G) fluorescence intensity pairs for each gene on each array probe, where R = red for Cy5 and G = green for Cy3. An 'MA-plot' was used to represent the normalized (R, G) data, where $M = \log R/G$ and $A = \log\sqrt{(R \times G)}$ [12, 13].

For the bioinformatics tools to search gene ontology, we used the combination of databases to gain information on gene name and symbol, subcellular location, family and superfamily classification, chromosome map location, similar gene, molecular function, biochemical function-related protein and references. The gene search programs were used the following sequential order of databases: NCBI (http://www.ncbi.nlm.nih.gov) Ensembl (http://www.ensembl.org) GeneCards (http://www.genecards.org) and TIGR (http://www.tigr.org). For the database search of porcine gene matching, the TIGR Pig (*Sus scrofa*) Gene Index (SsGI) database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=pig) supplemented with NCBI and Swiss-Prot/TrEMBL database was used for identifying the porcine homolog of human gene. In addition, the category classification of gene expression was done by in-house Bulk Gene Search System for Java (BGSSJ) program that is a searching system accomplished by open database connectivity, UniGene database and Gene Ontology knowledgebase, and is available at http://www.servx8.sinica.edu.tw/bgss-cgi-bin/gene.pl or http://www.bgssj.sourceforge.net. On the other hand, the protein search program used the Swiss-Prot/TrEMBL (http://www.expasy.ch/sprot), Proteome (http://www.proteom.. com/databases/HumanPD/reports) and PubMed (http://www.ncbi.nlm.nih.gov/PubMed). Moreover, the combining pathway databases of BioCarta (http://www.biocarta.com), KEGG (http://www. genome.

ad.jp/kegg/pathways.html) and the PubMed literature were used to search the correlated signaling pathways and mechanisms of renal response in LLC-PK1 cells.

### *Real-time quantitative PCR*

Specific oligonucleotide primer pairs were designed using the analysis Beacon designer 4.00 (Premier Biosoft International) and were then used for RT-qPCR. The sequences of the primers obtained from TIGR *Sus scrofa* Gene Index (SsCI) (http://www.tigr.org) and Swiss-Prot/TrEMBL (-http://www.expasy.ch/sprot) were: (1) ATP1B1 (TC221443, 106 bp): forward 5′-AGGCGTATGGTGAGAACATTGG-3′ and reverse 5′-GGCTAGTGGGAAAGAGCTTGTG-3′; (2) H3F3A (TC205667, 110 bp): forward 5′-TCTGAAGTCCAGAGGGCTAAGC-3′ and reverse 5′-CTAGCAGCTTGAAAGGCGTTCC-3′; (3) ITGB1 (TC220012, 134 bp): forward 5′-GCTGGTGTGGTTGCTGGAATTG-3′ and reverse 5′-CC CGTGTCCCATTTGGCATTC-3′; (4) RHO (TC234249, 197 bp): forward 5′-CTGTGGTCCTTGGTGGTCCTG-3′ and reverse 5′-TCAATCCCGCACGAGCACTG-3′. The specificity of each primer pair was tested using a common reference RNA (Stratagene) to perform RT-PCR reaction, followed by DNA 500 chip run on Bioanalyzer 2100 (Agilent Technologies) to check the size of the PCR product. Primer pairs of production predicted product size and no other side products were chosen to conduct the following SYBR reaction.

RT-qPCR was performed on the LightCycler instrument 1.5 (Roche) using the LightCycler® FastStart DNA Master$^{PLUS}$ SYBR Green I kit (Roche Applied Science). The LightCycler software was used to calculate the threshold cycle (Cp), defined as the fractional cycle number at which the fluorescence reached the baseline and the fold expression of the target gene relative to $\beta$-actin in each sample.

## Results

### *Gene expression analysis of ANP-stimulated LLC-PK1 cells*

The microarray results showed different fluorescence intensities between Cy3 (untreated LLC-PK1 cells) and Cy5 (ANP-treated LLC-PK1 cells), corresponding to the differential expression level of thousands of genes, and represented the different Cy3 and Cy5 signal intensities with a $p$ value of less than 0.01 ($p < 0.01$) that were considered to be significantly different (Figure 1). An analysis of gene expression changes with human oligonucleotide microarray revealed the total number of 807 differentially expressed genes in ANP-stimulated LLC-PK1 cells, containing 483 up-regulated genes and 324 down-regulated genes. In addition, we used the bioinformatics tools combining NCBI, Ensembl, GeneCards and TIGR database to access the gene annotations and description. Due to the human oligonucleotide microarray used, we used the TIGR-SsGI database supplemented with NCBI and Swiss-Prot/TrEMBL database to search the porcine homolog of human genes and obtained approximately 148 up-regulated genes and 108 down-regulated genes. Then, we used the in-house BGSSJ program to search the gene ontology of the above gene numbers, which were classified the gene category, according to cellular component, molecular function and biological process. The summary of the category classification of differentially expressed genes in ANP-stimulated LLC-PK1 cells is shown in Figure 2. The total numbers of 129 up-regulated genes and 98 down-regulated genes were classified into each category with different ratios. Meanwhile, the remaining numbers of 19 up-regulated genes and 10 down-regulated genes could not be classified by this program because they may be the new genes and none of categorical information. Some of genes could be classified into more than one category, depending to their cellular component, molecular function and biological process. The expressed genes were found to be located in the subcellular region rather than organelle, extracellular region, protein complex and extracellular matrix, respectively. It may indicate the signal transduction pathway occurring from extracellular region to intracellular region because some of genes served as receptors and/or transporters and have mediated the signaling genes to another region. So, it is possible to be found in both regions. Otherwise, they possessed the molecular functions in different ratios of binding, catalytic activity, signal transduction activity, transcription regulator activity, structural molecule activity, transporter activity, enzyme regulator activity,

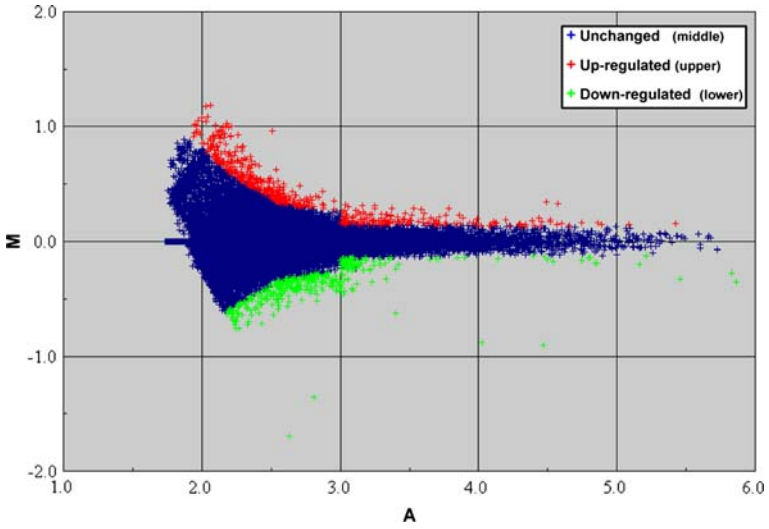


*Figure 1.* M-A plot of microarray data representing the differentially expressed probes of ANP-stimulated LLC-PK1 cells. A representative probe of comparative experiments between untreated mRNA labeled with Cy3 and ANP-treated mRNA labeled with Cy5, where M is the common log ratio of two dyes and A is the average logarithmic fluorescence intensities of both channels. The gene expression pattern shows approximately 842 significant differentially expressed probes ($p < 0.01$). Blue + (middle) represents any data point whose log ratio is not significantly different from 0; red + (upper) and green + (lower) represent data points whose log ratios are greater or less than 0, respectively.

motor activity, translation regulator activity and antioxidant activity. Moreover, the expressed genes involved some biological processes, such as physiological process, cellular process, regulation of biological process, development and behavior, leading to the understanding the related mechanisms of renal protective system and other actions of ANP. Thus, this category classification provided more useful information of gene ontology in response to ANP stimulation. Furthermore we suggest that all of the differentially expressed genes may be regulated by various gene networks and possess related molecular functions involving biological processes in the regulation of renal response.

*RT-qPCR validation of array analysis*

The ANP-stimulated genes chosen for RT-qPCR analysis were selected from a mean degree of higher expression level with significant $p < 0.001$. The selected genes were validated by RT-qPCR analysis to confirm the result of gene expression level with microarray data and the result of gene expression level of four expressed genes was agreed with the DNA microarray expression data (Table 1). There were $Na^+/K^+$ transporting ATPase (ATP1B1) [14], H3 histone (H3F3A) [15], integrin beta-1 subunit (ITGB1) [16] and rhodopsin (RHO) [17]. They were up-regulated genes in ANP-stimulated LLC-PK1 cells and have been found to have the correlation with the renal protective system. In addition, the classification of these genes by the BGSSJ program showed multiple locations in cell component, multiple functions in binding, catalytic activity, signal transducer activity and transporter activity, and multiple biological processes at the physiologic, cellular, developmental and regulation levels (Figure 3).

*Correlation of differentially expressed genes with ANP signaling pathways*

Combining the pathway databases of BioCarta, KEGG and the PubMed literatures, we built the hypothetical model of ANP-signal transduction pathways, in which the differentially expressed genes of ATP1B1, H3F3A, ITGB1 and RHO are correlated in this pathway by alleviating the hypertrophy in ANP-stimulated LLC-PK1 cells (Figure 4). Our hypothetical model suggests that ANP from the outer membrane can pass through the inner membrane via the NPR-A

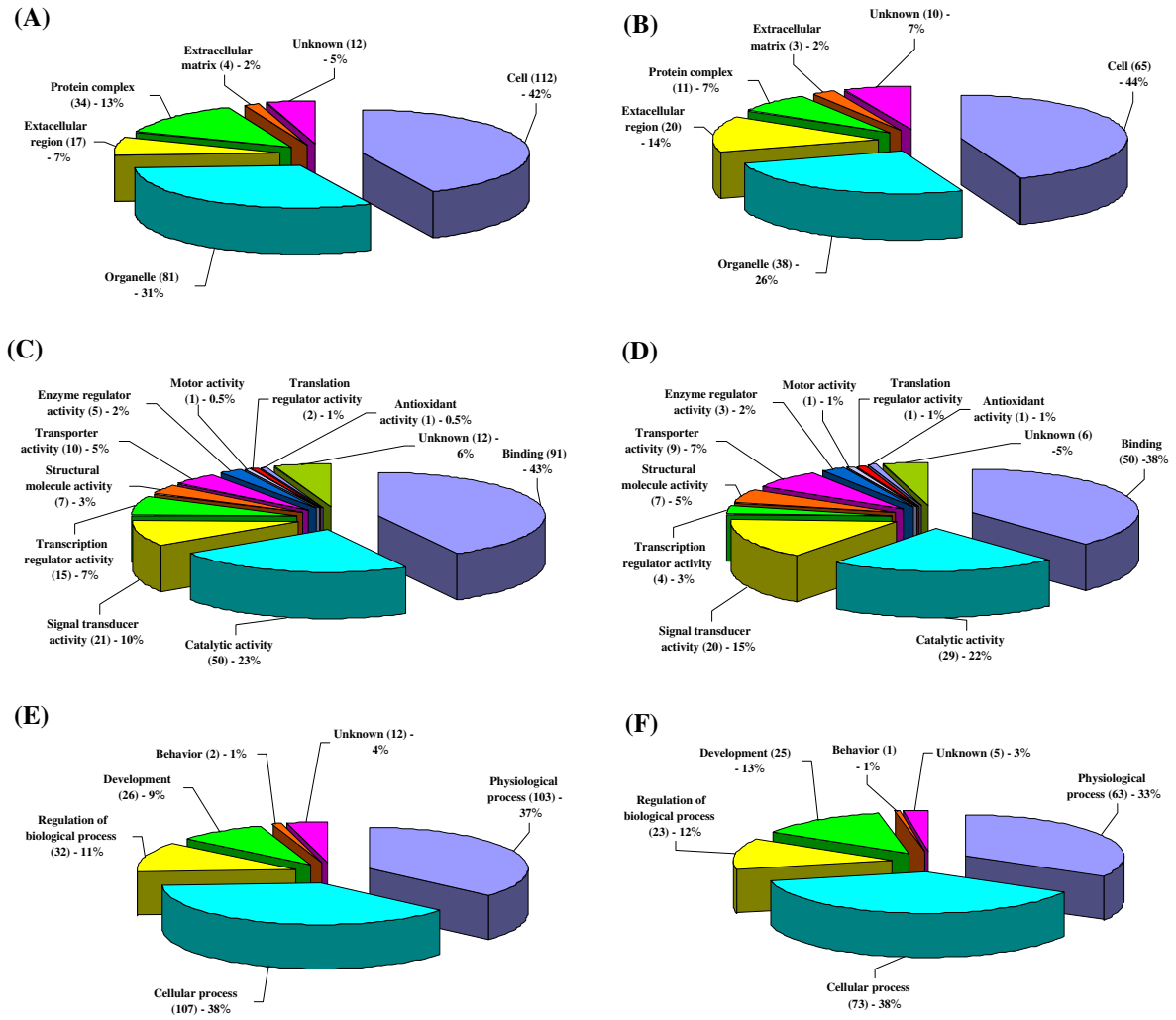


*Figure 2.* Classification of up-regulated and down-regulated genes by using the in-house BGSSJ program. The up-regulated and down-regulated genes are classified according to cellular component (A, B), molecular function (C, D), and biological process (E, F), respectively. The following numbers are the classified genes and percentage of genes in each category.

receptor, and interact with other associated genes in many related pathways, such as guanylyl cyclase (GCase), cyclic GMP, FAK, MAPK, PKC/PKG, and Ras/Rac [18–22]. This leads to the stimulation of ANP-responded genes encoding proteins (ATP1B1, H3F3A, ITGB1, and RHO). The signal transduction pathway is closely related to biological processes which can lead to changes in physiological and biological functions. Thus, these up-regulated genes are related to the regulatory mechanisms of renal hypertrophy as well as other mechanisms, in which these genes can potentially alleviate the progression of renal failure.

## Discussion

In this study, the effect of ANP on the gene expression change in the renal system was carried out in the LLC-PK1 cells from the pig epithelial kidney cells because LLC-PK1 cells principally exhibit the NPR-A receptor [10, 23] and the only limitation to the use of the model is its rapid onset, while the positive features of this pig model include its ‘‘acceptance’’ or etiologic relevance to clinical renal failure. It has a neurohumoral and functional profile similar to most human disease failures, with relatively low cost and simple preparation, ability to reliably or reversibly manipulate

*Table 1.* Comparison of quantitative genes of ANP-stimulated LLC-PK1 cells by microarray and real-time quantitative PCR (RT-qPCR) analysis.

| Gene name | Accession no. | | Description | | *p* Value | Ratio | |
|---|---|---|---|---|---|---|---|
| | Human | Porcine | Human | Porcine | | Microarray | Q-PCR |
| ATP1B1 | NM_001677 | TC221443 | ATPase, $Na^+/K^+$ transporting, beta 1 polypeptide | ATNB (P05027) Sodium/potassium-transporting ATPase beta-1 chain (sodium/potassium-dependent ATPase beta-1 subunit) | 2.57E–04 | 1.54 | 1.58 |
| H3F3A | NM_002107 | TC205667 | H3 histone, family 3A | Q71DI3 (Q71DI3) Histone H3 (Histone gene complex 1) (Histone H3/o) | 9.24E–04 | 1.47 | 1.46 |
| ITGB1 | NM_033666 | TC220012 | Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 including MDF2, MSK12) | Q9GLP0 (Q9GLP0) Integrin beta-1 subunit | 8.69E–05 | 1.59 | 1.69 |
| RHO | NM_000539 | TC234249 | Rhodopsin (opsin 2, rod pigment) (retinitis pigmentosa 4, autosomal dominant | OPSD (O18766) Rhodopsin | 4.55E–04 | 1.51 | 1.77 |

The gene accession numbers of human (*Homo sapiens*) and porcine (*Sus scrofa*) were obtained from NCBI and TIGR SsCI database. The gene description was obtained from Swiss-Prot/TrEMBL.

the degree of failure with pacing rate, and a large amount of published multi species [24]. Following the microarray analysis, the human array was used to analyze the gene expression in the porcine cells with two main reasons. First, the porcine microarray is not much widely available and may have the variability due to the new coming array. Second, the cross-species hybridization between human and porcine could generate highly reproducible data of expressed genes and the same number of genes could be expressed at higher levels in the pig kidney as in the human kidney [25–29]. Otherwise, the high relevance of cross-species comparison of gene expression between human and porcine tissue suggests that the pig tissue is an ideal donor for kidney transplantation to human recipients. Thus, this system approach using the human array for gene expression analysis in pigs is very useful and important to understand the gene regulation in the porcine organs for further therapeutic application, especially in kidney transplantation. By these reasons, we used the human microarray to investigate the effect of ANP on the gene expression change in LLC-PK1 cells that was able to correlate with the homologous human genes and led to understanding the correlated response of gene regulation in human renal system. On the other hand, the single ANP concentration and 24 h post-treatment condition in LLC-PK1 cells were used to study the gene expression level because this condition could access the $G_1$ phase of the cell cycle which might be a critical period in the evolution of renal hypertrophy [10].

After microarray analysis, we used RT-qPCR to validate the gene expression changes and the expression level of ANP-responsive genes agreed with the DNA microarray data. Meanwhile, four significantly expressed genes of ATP1B1, ITGB1, H3F3A and RHO were found to be up-regulated in ANP-stimulated LLC-PK1 cells and highly effective to the regulation of renal hypertrophy. ATP1B1 encoding $Na^+$, $K^+$-transporting ATPase beta-1 chain was one of the up-regulated genes in the ANP-stimulated LLC-PK1 cells. The gene-encoded protein, which belongs to the family of $Na^+$, $K^+$, and $H^+$, $K^+$ ATPase beta chain proteins and the subfamily of $Na^+$, $K^+$-ATPase, is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of $Na^+$ and $K^+$ across the plasma

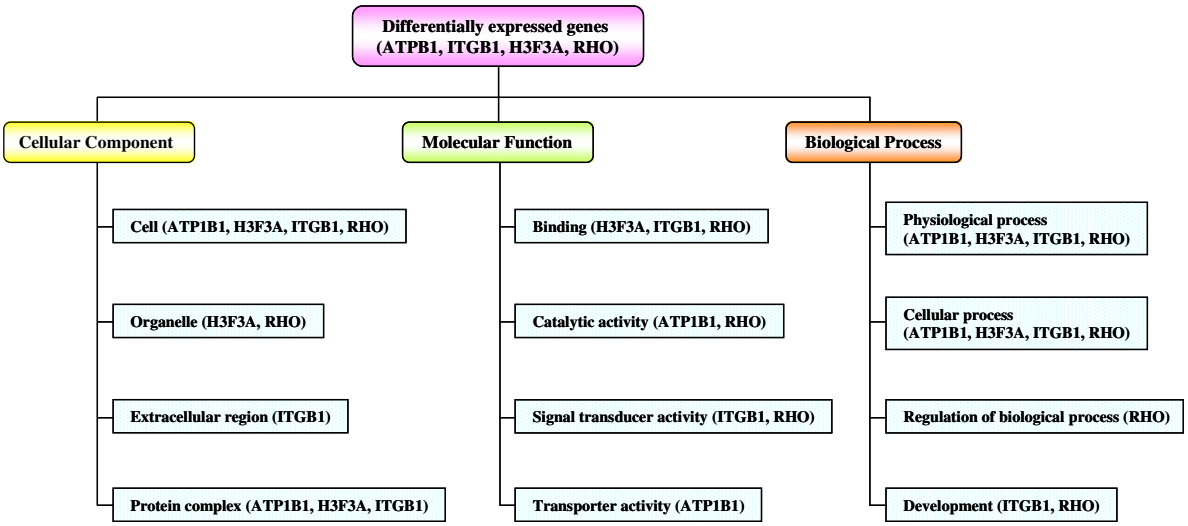


*Figure 3.* Classification of four significantly expressed genes (ATP1B1, H3F3A, ITGB1, and RHO) in ANP-stimulated LLC-PK1 cells by using the in-house BGSSJ program.

membrane [30, 31]. In addition, ANP could directly modulate primarily active sodium transport in the proximal tubule, and this effect is mediated by the activation of the NPR-A/guanylate cyclase/cGMP pathway that involves reversible activation/deactivation of renal tubular $Na^+$, $K^+$-ATPase [32, 33]. Thus, the up-regulated ATP1B1 in ANP-stimulated LLC-PK1 cells can reduce $Na^+$ resorption by excreting $Na^+$ outside the membrane, and thereby leading to natriuresis. It is also related to the downstream signaling pathways that can increase muscle contraction and reduce the hypertrophy, therefore ameliorating renal failure.

H3F3A encoding histone H3.3 or H3.3A belongs to the replacement histone gene family. It has several features that distinguish the H3F3A gene from the main histone gene types [34, 35]. The regulatory functions of the H3F3A gene and its expression are involved in cell proliferation and apoptosis, and could be a possible candidate gene for the rippling muscle disease [35, 36]. Thus, we suggest that the up-regulated H3F3A gene may be a mediator in the cell signaling pathway that leads to the decrease in hypertrophy.

ITGB1 encoding integrin beta-1 subunit was the up-regulated gene in the ANP-stimulated LLC-PK1 cells. Integrins were co-localized and associated with $Na^+$, $K^+$-ATPase, in which the close proximity of ATPase ion pumps to chondrocyte mechanoreceptor complexes could facilitate rapid homeostatic responses to the ionic perturbations brought about by the activation of mechanically gated cation channels. Integrins efficiently regulate the intracellular milieu of chondrocytes [37]. Otherwise, the biological function of integrins was shown to mediate a variety of signaling molecules including the activation of $Na^+/H^+$ antiporter [38], focal adhesion kinase (FAK) [39], Src kinases [40], small G-proteins (Ras and Rho) [41, 42], and ERK/JNK kinases [43, 44]. The up-regulated ITG1B1 gene in the ANP-stimulated LLC-PK1 cells could give the advantage in terms of the promotion of ANP therapy of renal failure and the stimulation of mediated cell adhesion and recognition in a variety of processes including hemostasis, immune response, motility and invasiveness, cell growth, and cell survival [45–47].

RHO encoding rhodopsin was the up-regulated gene in the ANP-stimulated LLC-PK1 cells. In general, rhodopsin acts as the dim-light photoreceptor of the rod cell and it mutations or differentiation cause a blinding retinal degenerative process, as known retinitis pigmentosa (RP), and lead to a constitutive activation of the phototransduction cascade in the absence of light [48–50]. RHO is a prototypical member of the superfamily of G protein-coupled receptors (GPCRs) [51–53] and served the molecular function in the regulation of guanylyl cyclases (GCase) and signaling of cyclic GMP by activating a cGMP to inhibit the

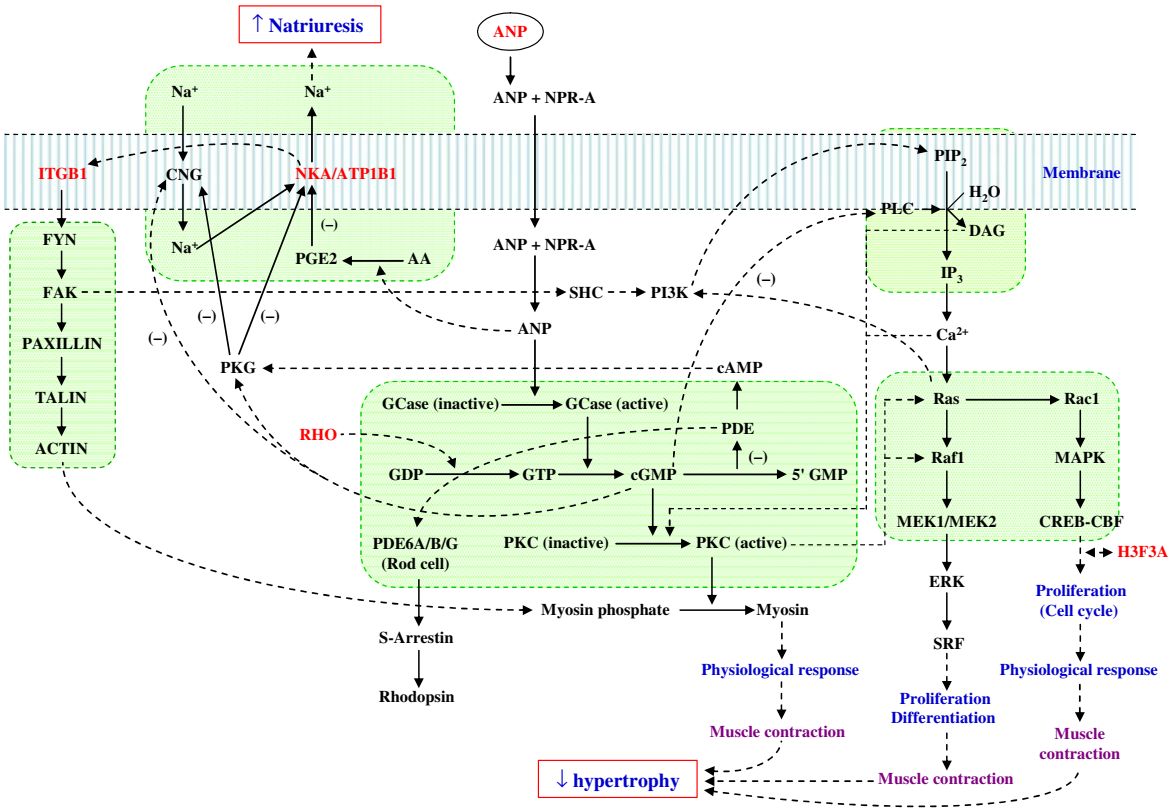


*Figure 4*. Hypothetical model of ANP signal transduction pathways and associated pathways in response to the ANP stimulation of LLC-PK1 cells. Four differentially expressed genes encoding proteins (ATP1B1, H3F3A, ITGB1, and RHO) are correlated with these pathways. Symbols: + , activation; − , inactivation. Abbreviation: AA, Arachidonic acid; ANP, Atrial natriuretic acid; CNG, Cyclicnucleotide-gated cation channel; DG, Diacylglycerol; GCase, Guanylyl cyclase; GMP, Guanine monophosphate; GTP, Guanine triphosphate; $IP_3$, Inositol triphosphate; NKA, $Na^+$,$K^+$-ATPase; NPR-A, Natriuretic peptide receptor A; PDE, Phosphodiesterase; PGE2, Prostaglandin E2; $PIP_2$, Phosphoinositol diphosphate; PKC, Protein kinase C; PKG, Protein kinase G; PLC, Phosphalipase C. The green-colored area represents the major signaling pathways.

specific PDE [54, 55]. Although the relationship of up-regulated RHO gene in ANP-stimulated LLC-PK1 cells and renal hypertrophy is not clear, the correlated functions of RHO in the downstream signaling pathways of GCase and cGMP may be involved in the regulation of hypertrophic process.

Furthermore, it is interesting to note that the biological functions of $Na^+$, $K^+$-ATPase transporting beta 1-encoded ATPB1, integrin-encoded ITGB1 and rhodopsin-encoded RHO involved in the numbers of ANP signal transduction pathways, particularly at the region of the cell membrane layers. Meanwhile, $Na^+$, $K^+$-ATPase transporting beta 1-encoded ATPB1 and integrin-encoded ITGB1 could induce the expression of the downstream mitogen-activated protein kinase (MAPK) signaling pathway [56]. Thus, we believe that ANP can effectively stimulate the signaling pathways via GCase, cGMP, GPK, MAPK and $Na^+$, $K^+$-ATPase, which turn on the expressed genes synthesizing related proteins for biological actions of renal failure suppression and other related actions (Figure 4). However, this is the first hypothetical model on the signal transduction pathway of ANP stimulation in LLC-PK1 cells. This model is based upon our finding of four significantly expressed genes from microarray and RT-qPCR results which are linked to ANP's actions in correcting renal hypertrophy. Moreover, the expressed genes may provide useful information on ANP mechanism, and thus provide better understanding of the gene expression level in the regulation of renal hypertrophy. The expressed genes may be used for further studies regarding the diagnostics and therapeutics of renal failure and other related diseases.

In conclusion, we used the DNA microarray to explore the global analysis of the gene expression in ANP-stimulated LLC-PK1 cells. The classification and validation of differentially expressed genes led to an exploration of valuable data in the ANP acting on the renal system. The gene lists of differentially expressed genes may be useful in the development of molecular diagnostic products or for research comparison with other therapeutics. In addition, four up-regulated genes served as candidate markers in the renal tubular cells for alleviation of renal failure progression, particularly renal hypertrophy, as well as other related effects. However, we will conduct further studies on the effects of ANP on the biological and molecular mechanisms of human renal tubular kidney cells and also examine other biological characterizations of ANP-responsive gene markers in diagnostic and therapeutic applications.

## Electronic supplementary material

The online version of this article (doi: 10.1007/s11373-007-9152-8) contains supplementary material, which is available to authorized users.

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