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Developmental and Pluripotent Genes in Rat Adult and Neonatal Kidney

Sumreen Begum, Atta-ur-Rahman and Asmat Salim*

Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

ABSTRACT

Investigating the role of genes in kidney with respect to their specific mechanism in renal development is imperative to regeneration. The purpose of this study is to analyze systematic identification of genes with respect to nephron lineage and pluripotency during postnatal renal development and at adult stage of rat kidney. In the current study, the mRNA levels of nephron genes were analyzed by reverse transcriptase PCR in neonatal and adult rat kidney tissues. These genes were divided into three categories. Group 1: renal multipotent progenitor genes; Group 2: self-renewal and pluripotency genes; Group 3 pluripotent state regulator genes. In neonates, renal progenitor genes *Wt1*, *Pax2*, *Cad6*, *Six2* and *HNF1β* were significantly expressed with concomitant expression of pluripotency genes. *Nanog* was highly expressed as compared to *Oct4* and *Sox2* at neonatal stage. *Aicda, Glis1, Tbx3* and *Dppa5* revealed higher expression among the regulatory genes in neonates, while *Lin28, Klf4* and *Stat3* were found with no significant difference as compared to adult stage. The nephron specific and pluripotency genes co-expressed in neonatal kidney and highly influenced by the *Aicda, Glis1, Tbx3*, and *Dppa5*. Further, *Lin 28, Klf4*, and Stat3 are required to maintain their expression states during and after development of rat kidney. Hence, over-expression of these genes may provoke the reprogramming of the adult injured tissue for the mechanism of regeneration.

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SB conducted experiments, analyzed the data and wrote the first draft. AR and AS conceived the idea. AS finalized the draft in the current form.

Key words
Pluripotent, Gene expression, Renal regeneration, Development, Nephronal

INTRODUCTION

The current understanding of regeneration and repair mechanism is still lacking including in kidney, which is one of the vital organ of the body. It is evidenced in other species that regeneration process follows orchestration of events of the development. Kidney has been investigated for long to explore the possible mechanisms of repair and development. Kidney develops from intermediate mesoderm (IM) and has three phases of its maturation; which include pronephros, mesonephros and metanephros which constitute initial non-functional part, intermediate form, and complete / mature mammalian adult kidney, respectively (Li and Wingert, 2003). A sequential developmental program initiated from metanephric mesenchyme (MM) and condenses to form progenitor populations of cap or committed mesenchyme (CM) in presence of growth factors around ureteric bud (UB) (Pleniceanu *et al*., 2010). It leads to mesenchyme to, epithelial transition (MET) with silencing of major transcription factors while acquisition of the epithelial markers. A renal vesicle (RV) was formed subsequently, and each RV develops into one nephron. The first evidence of epithelialization is the expression of adhesive proteins Cadherin 4 and then Cadherin 6 (Hendry *et al*., 2011; Miller-Hodges and Hohenstein, 2012).

The relationship of cell proliferation and apoptosis is important in normal kidney development. A recent study showed that with the maturation of mouse kidney, cell proliferation activity decreases. *ZFX* gene is important for regulation of growth, proliferation and differentiation in tissue cells, while the *Bcl2* and *BAX* genes regulate apoptosis. *ZFX* gene may participate in the developmental process of the kidney through the balance of cell proliferation and apoptosis regulation (Wang *et al*., 2021).

Precise information regarding the mechanism of differentiation of precursor cells to lineage specific cells is

* Corresponding author: asmat.salim@iccs.edu
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Abbreviations

Pax2, Paired box gene 2; Wt1, Wilm's tumor suppressor gene1; Six2, Sine oculis homeobox homolog 2; Cad6, Cadherin-6; HNF1β, Hepatic nuclear factor 1 beta; Oct4, Octamer-binding protein 4; Sox2, SRY (Sex determining region Y)-box 2); Nanog, Homeobox transcription factor Nanog; Lin28, Lin-28 homolog A (C. elegans); Klf4, Kruppel-like factor 4 (Gut); Aicda, Activation-induced cytidine deaminase; Dppa5, Developmental pluripotency associated 5; Tbx3, T-Box 3; Glis1, GLIS family zinc finger 1; Stat3, Signal transducer and activator 3.

useful in specifying target cells for cellular repair. The precursor cells identified in adult renal papilla, Bowman's capsule and tubular compartment express *Pax2, CD133* and *CD24* (Li and Wingert, 2003). These tubular cells can differentiate into renal epithelial-like cells *in vitro* (Sallustio *et al*., 2010). It has been demonstrated that precursor population expressing *CD133*$^{+}$, *CD24*$^{+}$, and *CD106*$^{-}$ engrafted into tubules. Similar cells can serve as regenerating population in tubules of acute and chronic tubular damage (Angelotti *et al*., 2012). The data, however, is still conflicting in determining the exact cell source for nephron repair. Multipotent renal progenitor cells (MRPCs) and mouse kidney progenitor cells (MKPCs) express early nephrogenesis genes and are involved in this repair. They rescue renal damage in murine kidneys (Li and Wingert, 2013; Gupta *et al*., 2006; Lee *et al*., 2010). Kidney derived cells identified and isolated from transgenic rats, showed expression of *Pax2* and *Oct4* (Liu *et al*., 2016).

Wt1, Pax2, Cas6, Six2, and *HNF1β* are important regulators of normal kidney development (Miller-Hodges and Hohenstein, 2012). *Oct4, Sox2,* and *Nanog* are pluripotency genes and autoregulate each other and share a substantial overlap in their downstream target genes (Ng *et al*., 2008). *Glis1*, *Klf4* belong to the same family. The exact and systematic role of the other pluripotency regulatory genes such *as Lin28, AID, Dppa5, Tbx3,* and *Stat3* is still not fractioned in the rat kidney.

In the present study, we have analyzed the systematic expression pattern of genes that are known to play significant roles in pluripotency, and nephronal lineage development. These genes are important for development of neonatal kidney, renal lineage differentiation, regulation of self-renewal and pluripotency of embryonic stem cells, and for reprogramming from mesenchymal to epithelial transition. Recognition of the expression pattern of these genes during development and at adult stage would be useful in designing novel strategies for improved renal differentiation of mature cells through stem or somatic cells in regeneration for the damaged kidney tissue.

Combination of these genes can be tested for either reprogramming of mature cell types for the generation of more potent induced pluripotent cells or differentiation of stem cells to directed nephronal lineage.

MATERIALS AND METHODS

Animals

All the animals were used according to the international guidelines for the care and experimental use of laboratory animals with the approval from the local ethical committee. Sprague Dawley (SD) rats (N=4/group) weighing 200-250 gm, and 1 day old pups were used for the isolation of kidneys. The potential quantitative errors in the relative estimates to *GAPDH* were reduced by repeating the tissue samples.

Sample collection

Adult rat kidneys (ARK) and neonatal rat kidneys (NRK) were isolated from adult rat and one day old rat pups, respectively. Kidneys were cut in small pieces and blood was removed by rinsing with saline. Tissues were quickly transferred to RNA Later solution (Qiagen, Germany), and stored at -20°C until further use.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from the sample was isolated using Trizol. Samples were processed in a ratio of 1mL Trizol reagent per ≤ 20 mg tissue. After dissociation, homogenization and sonication, sample mixtures were incubated for 15 min at room temperature (RT). Chloroform (200 µL) was added to the mixture and incubated for 15 min at RT. Samples were phase separated by centrifugation (8000-11000 x g). Aqueous phase was collected for RNA extraction. Ice cold isopropyl alcohol (750 µL) was added and mixture was centrifuged (6000 x g at 4°C; 10 min) to get RNA pellet. The pellet was washed in 1 mL of 75% ethanol and centrifuged (6500 x g at 4°C; 10 min). The pellet was air-dried (~ 30 min) and dissolved in 30-40 µL of nuclease-free water. Total RNA yield and purity (A_{260} / A_{280} ratio) was determined by diluting samples (1:200) and measuring absorbance at 260nm in a UV visible spectrophotometer.

Total RNA (1µg) from rat kidney tissues was calculated and used for cDNA synthesis via Revert Aid™ First Strand cDNA Synthesis kit (Fermentas, Life Sciences, Germany) according to the manufacturer's instructions. All samples were stored at -80°C or used directly for amplification by PCR.

Kidney developmental genes and core and downstream pluripotency genes were selected for the analysis. Accession numbers of selected genes, primer sequences, product sizes and annealing temperatures are listed in Table I. Online mRNA servers, http://primer3.wi.mit.edu and http://www.ncbi.nlm.nih.gov/tools/primer-blast were used for primer designing. The primers were synthesized by Integrated DNA Technologies (IDT), USA.

cDNA (1µg) was amplified by using GoTaq Flexi DNA polymerase kit (Promega, USA) for gene expression analysis. The protocol was followed according to manufacturer's instructions. The thermal cycle comprised initial denaturation at 95 °C for 2 min, and 35 cycles each of denaturation at 95 °C for 1 min, annealing at 55-64 °C for 1 min, and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min.

Table I. Genes and their primer sequences, accession numbers, expected product sizes and annealing temperatures.

Genes	Accession # (NCBI)	Primer sequence (5'-3')	Product size (bp)	Annealing temp (°C)
Group 1: Kidney specific developmental genes				
Wt1	NM_031534	F GCCTTCACCTTGCACTTCTC R GACCGTGCTGTATCCTTGGT	186	58
Pax2	NM_001106361.1	F AGGAGGTGGAGGTTTGCATCTGG R ACTTCATCAAGCCCAGGGGTCAG	216	64
Cad6	NM_012927	R CCGTGAGGGGTTCTCCGTTGT F AGTAAGGGGCGTGGCCAACCT	232	64
Six2	NM_053759	F TTGATTCTGGGGTTCTTTGC R CAAGCCTGGGTGTTTTTGTT	183	58
HNF1β	NM_013103	F GACACTCCTCCCATCCTCAA R ACATCAACCACCTCCCTCTG	167	58
Group 2: Core pluripotency genes				
Oct4	NM_001009178.2	F GAGGGATGTGGTTCGAGTGT R CCAGAGCAGTGACAGGAACA	248	58
Nanog	NM_001100781	F CCCAAGCTAAAGCTGTCTGG R ATCTGCTGGAGGCTGAGGTA	167	55
Sox2	NM_001109181.1	F AAGGGTTCTTGCTGGGTTTT R GCCCTAAACAAGACCACGAA	160	58
Group 3: Pluripotency regulatory genes				
Lin28	NM_001109269	F CCCAGTGTCACCCTGTCTTT R CCTGTAACAGCCACTCAGCA	164	58
Klf4	NM_053713	F CACACTGCCAGGAGAGAGTT R CAGTCTCAGACCCCATCTGT	164	58
Aicda	NM_001100779.1	F CAAGACCATGGCAAGGAAAT R TCCCGTGGGTCTTTTAAGTG	189	55
Tbx3	NM_181638.1	F CAGAGCCAACGACATTCTGA R CCTCATGGACTGCAGAGTGA	205	55
Glis1	XM_003749991.1	F AGACTACAGCGTGTCCAAGG R ATTCGCATACGTAGCCTGAG	235	58
Dppa5	XM_001059859	F GGATCTCGAATGCCTCACAT R CAGTTCCAGGGTCTTCATGG	213	58
Stat3	NM_012747	F CAGCCAAACTCCCAGATCAT R GGCGGACAGAACATAGGTGT	231	57
GAPDH was used as internal standard		F GAAAAGCTGTGGCGTGATGG R GTAGGCCATGAGGTCCACCA	414	60

PCR sample with *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) expression and reverse transcriptase negative (-RT) sample served as positive and negative controls, respectively.

The amplified PCR products were subjected to electrophoresis using 1-2 % agarose gel in 1X TBE buffer (89mM Tris base, 89mM boric acid, 50mM EDTA) using horizontal gel casting unit. PCR products (10µL) were loaded into each well while one well was reserved for loading molecular weight ladder / marker (HyperLadder™ 50 or 100bp).

The DNA bands were visualized under ultraviolet (UV) light in a gel documentation system (Alpha Innotech, Alpha EAse FC imaging system, FluorChem™, USA) and photographed for semi-quantification. The intensity of bands was quantified using FluorChem TM AlphaEase FC software. The integrated density value (IDV) of each band was calculated, normalized with the corresponding *GAPDH* band density and relative gene expression was compared with the control groups. The repeating quantitative estimation was carried out for more rigorous estimations.

Statistical analysis

Results were illustrated as means with their standard errors (means ± SEM). Statistical analyses were performed using Shapiro-Wilk (Student's t test) by Sigma Plot 12. Probability values less than 0.05 ($p < 0.05$) were considered to be statistically significant for each experimental finding.

RESULTS

Expression of kidney specific developmental genes

Specific kidney developmental genes showed significantly higher mRNA expression levels of *Wt1* (*$p<0.05$), *Cad6, Pax2, Six2,* and *Hnf1β* (**$p<0.01$) in neonatal rat kidney as compared to that in case of adult kidney (Fig. 1). *Pax2* expression was not detected in adult kidney tissue.

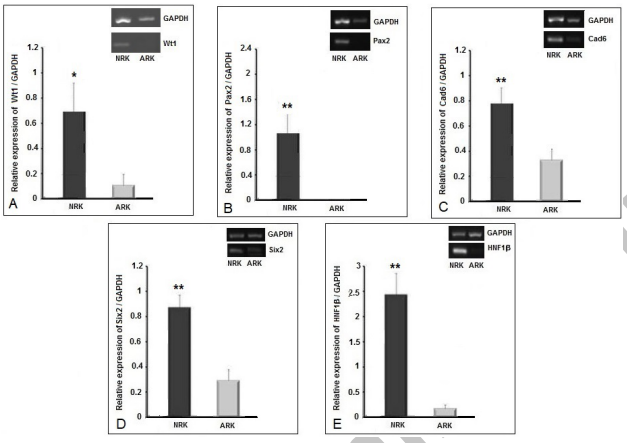

Fig. 1. mRNA expression levels of kidney developmental genes in neonatal and adult rat kidney. One dimensional agarose gel image showing gene expression levels by RT-PCR for (A) *Wt1*, (B) *Pax2*, (C) *Cad6*, (D) *Six2*, and (E) *HNF1β*. Each gel image is accompanied by bar diagrams showing representative densitometry analyses of these genes. Y-axis shows the relative expression of each gene with respect to *GAPDH* expression. Data is presented as means ± standard error of means (SEM) where n = 3. For statistical analysis, a Shapiro-Wilk Normality Test was performed and all comparisons were made with Student's *t*-test. 95 percent two-tailed confidence interval for differences of means was considered significant (p-value<0.05).

Core pluripotency genes

Core pluripotency genes showed significantly higher mRNA expression levels in case of *Oct4, Sox2* (*$p<0.05$) and *Nanog* (**$p<0.01$) in neonatal rat kidney as compared to that in case of adult kidney (Fig. 2). *Nanog* was highly expressed as compared to *Oct4* and *Sox2* at neonatal stage. *Oct4* expression was not detected in adult kidney tissue.

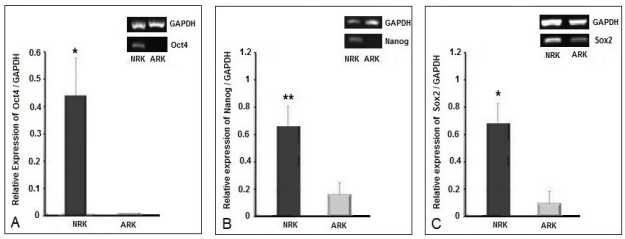

Fig. 2. mRNA expression levels of core pluripotency genes in neonatal and adult rat kidney. One dimensional agarose gel image showing gene expression levels by RT-PCR for (A) *Oct4* (B) *Nanog* and (C) *Sox2*. Each gel image is accompanied by bar diagrams showing representative densitometry analyses of these genes. Y-axis shows the relative expression of each gene with respect to *GAPDH* expression. Data is presented as means ± standard error of means (SEM) where n = 3. For statistical analysis, a Shapiro-Wilk Normality Test was performed and all comparisons were made with Student's *t*-tests. 95 percent two-tailed confidence interval for differences of means was considered significant (p-value<0.05).

Pluripotency regulatory genes

Pluripotency regulatory genes showed significantly higher mRNA expression levels in case of *Aicda* (*$p<0.05$), *Glis1, Dppa5 and Tbx3* (**$p<0.01$) in neonatal rat kidneys as compared to that in case of adult kidney. Difference in the expression levels of *Lin28, Klf4* and *Stat3* genes was however, not significant (Fig. 3). *Glis1* expression was not detected in adult kidney tissue.

DISCUSSION

Expression levels of specific genes play a critical role in the regulatory mechanisms associated with nephrogenesis during kidney development (Hendry *et al*., 2011). The varying patterns of unique set of genes during developmental and mature stages can provide insight for regeneration mechanisms of injured kidney. With this objective, the present study was designed to determine and compare transcriptional levels of certain genes that maintain rat kidney development at neonatal stage and in adult stage. It can provide novel insights into the gene expression patterns and networks active in immature and fully differentiated cells in rat kidney. Secondary objective of this study was to deduce converging points of the two cellular phenotypes.

Kidney is a very complex organ for tissue engineering as its function is dependent on the correct spatial conformation of many different cell types. During

normal development, two cell type i.e. MM ureteric bud (UB) can build the complete structure (Miller-Hodges and Hohenstein, 2012). DNA-binding factors, which are essential for the IM and the renal epithelial lineage, could provide the locus and tissue specificity for histone methylation and chromatin remodeling and thus establish a kidney-specific fate during development (Dressler, 2008).

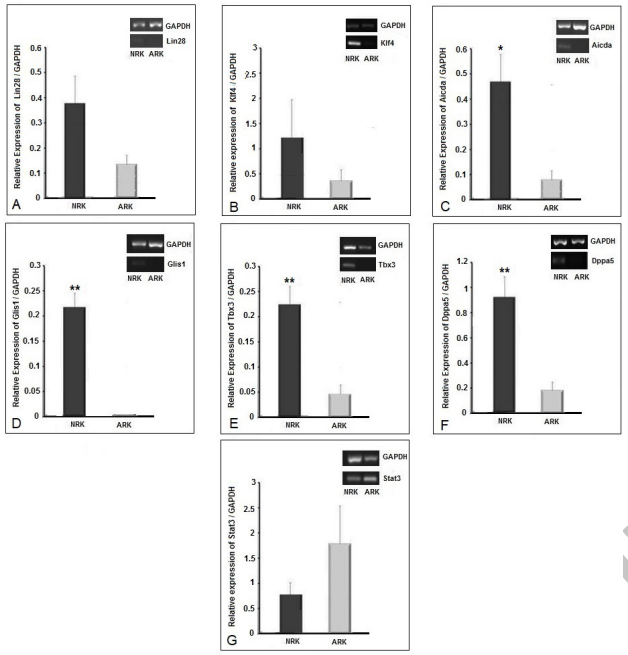

Fig. 3. mRNA expression levels of pluripotency regulatory genes in neonatal and adult rat kidney. One dimensional agarose gel image showing gene expression levels by RT-PCR for (A) *Lin28*, (B) *Klf4*, (C) *Aicda*, (D) *Glis1*, (E) *Tbx3*, (F) *Dppa5* and (G) *Stat3*. Each gel image is accompanied by bar diagrams showing representative densitometry analyses of these genes. Y-axis shows the relative expression of each gene with respect to *GAPDH* expression. Data is presented as means ± standard error of means (SEM) where n = 3. For statistical analysis, a Shapiro-Wilk Normality Test was performed and all comparisons were made with Student's *t*-test. 95 percent two-tailed confidence interval for differences of means was considered significant (p-value <0.05).

We observed that the group of genes specifically involved in nephrogenesis was up-regulated in neonatal kidney. These results confirm that the continuation of postnatal nephron development is in conjunction with the expression of progenitor genes of nephrons including *Wt1, Pax2, and Six2*. *Wt1* and *Pax2* are considered as key cap mesenchyme (CM) genes, which act as transcription factors (TF) in the formation of a pronephric field from animal cap ectoderm (Hendry *et al*., 2011; Yamamura *et al*., 2021). *Wt1* is a critical regulator of MM that plays a role even before ureteric bud (UB) formation and controls transitions between the mesenchymal and epithelial states of cells in a tissue dependent manner (Miller-Hodges and Hohenstein, 2012). *Pax2* is expressed in ureteric bud (UB) and in the induced mesenchyme. The inductive signals from WNT pathway is interpreted by *Pax2* which results in aggregation, polarization, and proliferation of epithelial cells (Dressler, 2009). Complete repression or down-regulation of *Pax2* has been observed in adult rat kidney as the differentiation of nephron proceeds (Imgrund *et al*., 1999). *Six2* maintains undifferentiated, self-renewing and pluripotent state of nephron's stem or progenitor cells (Murphy *et al*., 2012) promoting MM in kidney development (Kobayashi *et al*., 2008). Cadherin 6 is involved in adhesion activity and maintains orderly structure of cells at developmental stage, involved in tissue integrity and morphogenesis, specifically renal proximal tubular epithelium (Gumbiner, 2005). Down-regulation of these core nephron specific developmental genes was observed in adult rat kidney as compared to that of one day postnatal pups. This demonstrates complete epithelialization for unique functionality of nephron at adult stage, which may not require these genes. *HNF1β* gene expression at neonatal stage confirms its role in normal proximo-distal segmentation pattern and podocyte formation but not in epithelialization of normal kidney organogenesis (Paces-Fessy *et al*., 2012). This may be due to increased segmentation process, still going in postnatal stage, which is associated with the function of *HNF1β*. Malfunctioning, absence or mutation of these genes are involved in kidney diseases which are being utilized for modeling kidney tissues *in vitro* (Rooney *et al*., 2021).

Pluripotency and stemness are believed to be associated with high *Oct-3/4, Nanog* and *Sox-2* expression. Pluripotent cells are master cells. They are able to make cells from all three basic layers, so they can potentially produce any cell or tissue of the body that needs to repair itself. They are also able to self-renew, hence they can perpetually create more copies of themselves. *Oct4* considered as the master regulator of pluripotency and differentiation (Paces-Fessy *et al*., 2012) is involved in regeneration by promoting cellular de-differentiation (Skvarca *et al*., 2019). Its expression influences several genes including *Sox2, FGF4, Rex1*. *Nanog* and *Sox2* also maintain pluripotent state and *Sox2* is also associated with multipotent and unipotent stem cells (Zhao *et al*., 2012). Similar to embryonic stem cells (ESCs), high *Sox*2 expression eventually became the measure of pluripotency in any cell. According to Ng *et al*. (2008), *Oct4, Sox2,* and *Nanog* auto-regulate each other. *Oct4* may serve as pluripotency determinant in reprogramming. Neonatal rat kidney has shown the presence of possible

pluripotent cells, as significant levels of core pluripotency genes including *Oct4, Nanog* and *Sox2* and other maintenance genes *Aicda, Esg1/ Dppa5, Glis1* and *Tbx3*, were detected. These results revealed that pluripotent genes are not repressed in neonatal kidney at this stage; their repression may be a gradual process, which is regulated by the above mentioned maintenance genes. Moreover, perfect interplay of these expression levels is necessary in this stage of kidney development. We observed that the expression levels of *Lin28, Klf4,* and *Stat3* were not increased in adult rat kidney. *Stat3* represent higher expression ratio within neonatal and adult kidney groups showing its importance in both states as it is involved in many pathways. However, its expression was not significantly different in neonatal and adult kidney groups. Elevated expression levels of *AID/Aicda* in neonatal kidney support its essential role as epigenetic modifier of pluripotency network. It regulates transcription of many genes by deaminating methylated cytidine (5-methylcytosine) (Popp *et al*., 2010; De Carvalho *et al*., 2010). *Tbx3* sustains pluripotency by direct binding and activation of the Oct4 promoter (Han *et al*., 2010). *Glis1* and *Esg1* have multiple roles in stage specific differential gene expression in development. *Stat3* and *Klf4* displayed >50% of gene expression in neonatal kidney. Several cellular functions have been attributed to these genes even at mature cellular stage. Hence, these are the genetic switches, which are needed to maintain pluripotency as well as transformation of pluripotent state into adult states. These molecular switches can help in elucidating the regeneration mechanisms to repair kidney tissues.

The pattern of gene expression analyzed in this study suggested that nephron specific and pluripotency genes co-expressed in neonatal and in adult kidney to some extent. The reprogramming phase from MET is significantly reduced in adult rat kidney with the suppression of pluripotency and renal progenitor or neonatal genes. Over-expression of neonatal genes may provoke the reprogramming of the adult tissue. Understanding gene regulation in the developmental stages can help to develop ways not only to generate early kidney progenitor cells from other cell types for kidney repair but they can be maintained through certain protein expression before being transplanted *in vivo* to start their differentiation as cellular therapeutic approach.

CONCLUSIONS

Co-expression of pluripotency genes and nephron specific genes are required for complete orchestration of events for the complete development of the rat kidney. During developmental stage, a transition state persists with advanced levels of expression of nephron specific genes, *Wt1, Pax2, Cdh6, Six2* and *HNF1β*. However, pluripotency genes, *Oct4, Nanog,* and *Sox2* also persist suggesting a dual role in self-maintenance and facilitating lineage specificity after induction of renal developmental genes. Regulators of pluripotency genes, *Aicda, Glis1, Tbx3* and *Dppa5* were expressed in the kidney with concomitant expression of specific kidney developmental genes, showing their unidentified role in nephrons. It can be suggested that renal regeneration potential may be due to the simultaneous expression of pluripotency and developmental genes for nephron lineage by mechanism opposing developmental stage i.e. epithelial to mesenchymal transition. Co-expression of nephron specific and pluripotency genes can initiate the repair and regeneration of the damaged kidney tissue while over-expression of neonatal specific genes may provoke the reprogramming of the adult injured tissue by co-existence of *Aicda, Esg1/ Dppa5, Glis1* and *Tbx3* gene expression.

Statement of conflict of interest

The authors have declared no conflict of interest.

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