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Immunohistochemical Study Of Nestin and Fibroblast Growth Factor 9 (FGF9) in Rabbit Kidney During Postnatal Development

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ABSTRACT

Immunohistochemical study of nestin and fibroblast growth factor (FGF9) in rabbit kidney during postnatal development are still a matter of debate. In our investigation, the expression of nestin and FGF9 was studied in neonatal and adult rabbit kidney using the following ages of 2 days,7 days, 14 days and 30 days old rabbit using immunohistochemistry. Nestin in neonatal rabbit was not detected in immature glomeruli while in mature glomeruli it was observed only in glomerular basement membrane. In adult it was expressed only in podocytes. FGF9 in neonatal rabbit was appeared moderate in immature and surrounding undifferentiated tubules while the reactivity was increased in mature glomeruli as well as in the surrounding proximal convoluted tubules (PCT) and collecting tubules (CT). In adult it was increased in glomeruli than the surrounding PCT and CT. So, this study shows that the expression of nestin is more important in adult than neonatal rabbit in maintaining the structural integrity of the podocytes while FGF9 is critical for virtually all renal linage at early and later stages of development. The present findings were discussed with previous publications.

1. INTRODUCTION

The metanephric (permanent) kidney of higher vertebrates is a complex organ comprising thousands of nephrons that are connected by a branched collecting duct system. The nephrons represent the functional units of the kidney. They filter the blood through a basement membrane and drain the filtrate via tubules and collecting ducts to the bladder. During embryonic development, the metanephric kidney develops from two different tissues, both of which are derived from the intermediate mesoderm, the ureteric bud and the metanephric mesenchyme (Costantini and Kopan, 2010).

Developmentally, the kidney is derived from two primordial structures: The ureteric bud and the metanephric mesenchyme (Saxen and Sariola, 1987). The epithelial cells in the collecting duct are derived from ureteric bud, and the remainder of the nephron derives from metanephric mesenchyme, which differentiates into more than 26 distinct cell types (Al-Awqati and Oliver, 2002). Determining whether nestin marks particular progenitor cells during kidney development could be important for understanding renal development and the damage–repair process in the diseased kidney. In addition, because nestin is a cytoskeletal intermediate filament protein, which contains binding domains for microtubule and microfilament actin, it also may be involved in maintenance of the integrity of the cytoskeletal structure of renal cells, including the glomerular podocyte (Pavenstadt et al., 2003). Disorganization or abnormal expression of podocyte cytoskeleton proteins has been suggested to cause podocyte foot process effacement and proteinuria (Schiffer et ai., 2001; Mundel and Shankland, 2002).

Nestin is a cytoskeleton-associated class VI intermediate filament protein (Lendahl et al., 1990). The functional significance of nestin expression has not been fully elucidated (Michalczyk and Ziman, 2005) Recent studies demonstrate that nestin can interact with the three major components of cytoskeleton (microfilaments, intermediate filaments, and microtubules), suggesting an important role in coordinating changes in cell

dynamics, including cell division (Herrmann and Aebi, 2000).

Members of the Fibroblast Growth Factor (FGF) family of secreted signaling molecules are also known to play a role in kidney organogenesis. The absence of either FGF7 or FGF10 (Qiao et al., 1999; Ohuchi et al., 2000), or the receptor they activate (FGFR2-IIIb) (Revest et al., 2001; Zhao et al., 2004), the number of collecting duct branches is reduced. This in turn results in a decrease in the number of nephrons that form. Another FGF family member, Fgf9, is expressed in the developing kidney (Crossley and Martin, 1995; Mahmood et al., 1995), but its function in kidney development has not been assessed.

The expression patterns of FGFs showed that all of them from FGF1 to FGF10 except for FGF6 are expressed in the developing rat kidney, suggesting an important role in kidney development (Cancilla et al. 2001). It has been reported that the renal mesenchyme secretes one or more FGF family members, e.g. FGF2 and/or FGF7, in order to regulate ureteric bud growth (Drummond et al. 1998). FGF expression in the epithelial cells of the branching ureteric bud epithelium, S-shaped bodies, proximal tubule epithelium and parietal epithelium of the glomerulus (Drummond et al. 1998). Furthermore, FGF2 is one of the limited number of factors that induce ureteric bud cells to undergo morphogenesis (Sakurai *et al.* 1997a), and it can mimic some effects of established inductor tissues for metanephrogenic mesenchyme. Very little is known about localization of the nestin and FGF9 in the neonatal and adult rabbit kidney during the postnatal development and not studied well, at least recently So the current study was conducted to through some highlights on the immunohistochemical study of nestin and FGF9 during rabbit kidney development.

2. MATERIAL AND METHODS

2.1. Immunohistochemistry (ICH).

Kidney samples were collected from rabbits at the ages (2days, 7days, 14 days and 30days old rabbit) and fixed in 4% paraformaldehyde in PBS at pH 7.4 for 2 days at 4°C. Paraffin blocks were prepared from these samples, cut at the desired thickness (3-5µm) and mounted on positively charged gelatin coated slides. The slides were then dried in an incubator at 45°C for a few hours and used for immunohistochemical localization of nestin and FGF9 in the developing kidney. The samples were stained for immunohistochemistry at medical research institute, Alexandria University, Alexandria, Egypt.

Immunostaining of paraffin-embedded kidney tissue was done as the followings:

1. Inactivation of endogenous peroxidase by H2O2 then the antigen retrieval methods that is vary according to the antibody used.
2. Then the sections were blocked by PBS containing 5% bovine serum albumin for an hour, and then incubated with primary antibody at 4 ◦C overnight and for an hour at room temperature.
3. The sections were washed by PBS for 5 min and incubated with biotinylated secondary antibody for 30 min at room temperature. The secondary antibody was detected with Vectastain ABC kit ((Vector Laboratories, USA). and the color was developed using DAB (Sigma–Aldrich).
4. Finally, the sections were counterstained with hematoxylin, washed by distilled water, air-dried and mounted with Entellan (Merck) and photographed by light microscopy.
5. In control experiments, reabsorption of the antibody with its respective antigen or elimination of the primary antibody was performed, which abolished all staining.
6. Table 1 summarized the primary antibody, their source, their final dilution, secondary antibody used and the antigen retrieval methods.

Table 1: The antibodies used antigen retrieval methods and incubation periods, and working dilutions.

Primary AB	Source	Dilution of 1ry AB	Secondary AB	Dilution of 2ry AB	Antigen retrieval
Nestin, mouse	Covance, Uk	1:4000	Anti- mouse	1:100	CB (citrate buffer) at 105°C for 20 min
Fibroblast Growth Factor (FGF9), mouse	Abcam, UK	1:200	Anti-mouse	1:100	Heat mediate Ph 9 at 105°C for 20 min

3. RESULTS

3.1. At age 2days old rabbit.

During early rabbit postnatal life, at 2 days old, the development of nephrons in nephrogenic zone continued at this stage. The subcapsular zone had different stages of nephron development; comma-shaped, S-shaped and vesicles-like nephrons in nephrogenic zone. The immunostaining of nestin was very week or not found in immature glomeruli and it also very week in surrounding mesenchymal tissues and capsule (Fig. 1A). The mature glomeruli which formed from tuft of capillaries showed increased reactivity of nestin especially in glomerular basement membrane (Fig. 1B). In the medulla, the nestin showed mild reactivity in loop of Henle, CT and surrounding mesenchymal tissues (Fig. 1C).

Figure 1D showing the immunostaining of FGF9 in renal cortex with increased reactivity in immature glomeruli and surrounding undifferentiated tubules. While mature glomeruli showed intense reactivity, moderate in surrounding proximal convoluted tubules (PCT) which lined by high cuboidal epithelium with narrow lumen and distal convoluted tubules (DCT) (Fig. 1E). In medulla the FGF9 showed moderate reactivity in collecting tubules and loop of Henle (Fig. 1F).

3.2. At age 7day old.

The renal cortex showed mature renal corpuscle surrounded by parietal epithelium and bowman's space which became developed than the previous age. Nestin immunostaining showing increased reactivity in mature glomerulus in glomerular basement membrane than the younger age while no reactivity in surrounding PCT and DCT (Fig.2A). In the medulla nestin reactivity was mild in collecting tubules and moderate in loop of Henle (Fig. 2B). Figure 2C showing the FGF9 immunostaining was increased in mature glomeruli than the younger age while PCT showed moderate reactivity. In the medulla the reactivity of FGF9 increased in collecting tubules and surrounding loop of Henle (Fig. 2D).

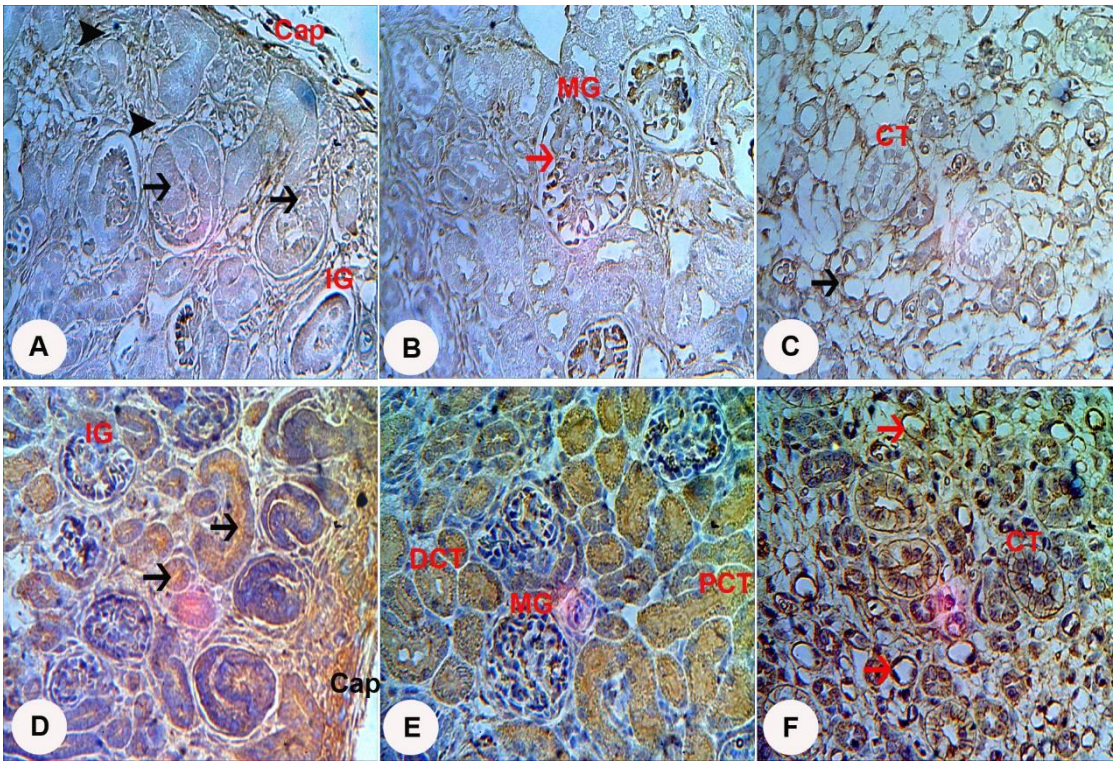

Figure 1: Photomicrographs of rabbit kidney at 2days old. A; showing sub capsular zone with different stages of nephron development (arrows) and showed nestin immunostaining with mild reactivity in immature glomeruli (IG), mild in capsule (Cap) and mesenchymal tissues (head arrow). B; illustrating nestin immune staining increased in mature glomeruli (MG) especially in glomerular basement membrane (arrow). C; showed the medulla by mild reactivity with nestin in loop of Henle (arrow) and collecting tubules (CT). D; illustrates FGF9 with moderate reactivity in IG, surrounding undifferentiated tubules (arrows) and Cap. E; showing increased reactivity of FGF9 in MG and moderate in PCT. F; showed the medulla with FGF9 reactivity in CT and loop of Henle (arrow). X40 from A-F.

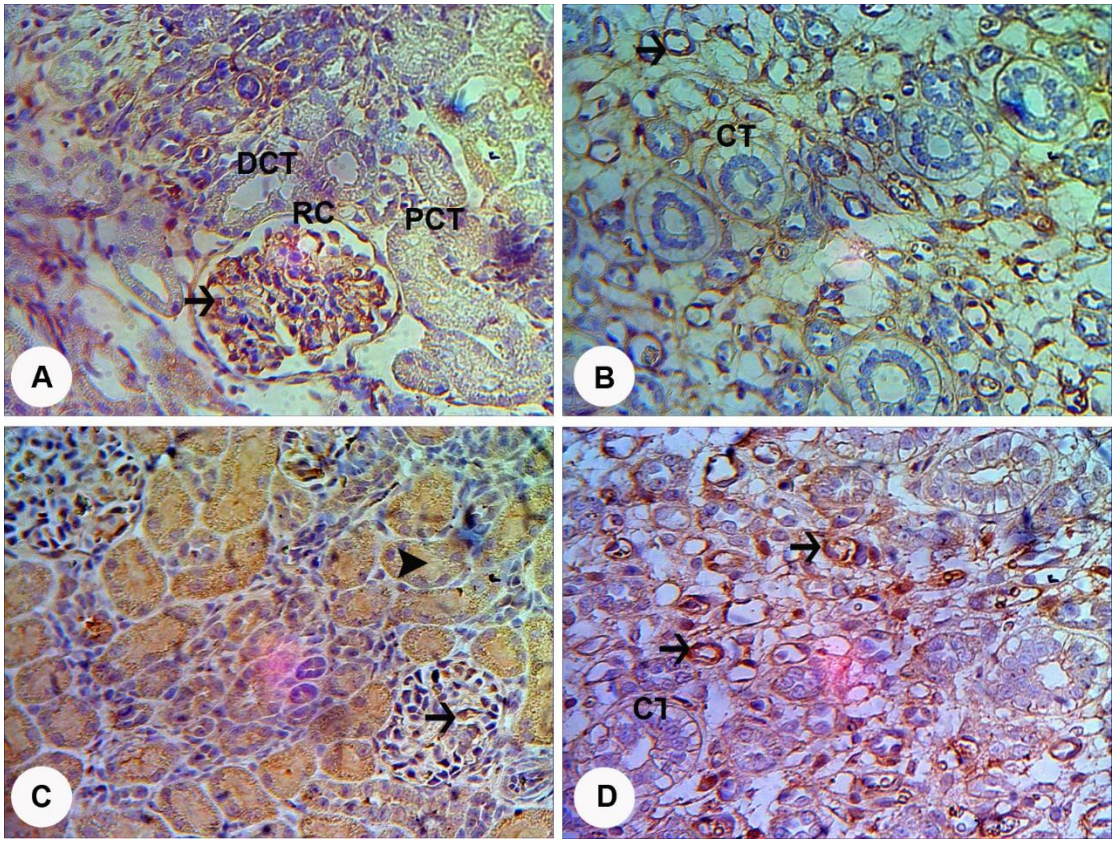

Figure 2: Photomicrographs of rabbit kidney at 7days old. A; showing nestin immunostaining in renal cortex with intense reaction in RC especially in glomerulus in glomerular basement membrane (arrow) while no reactivity in PCT and DCT. B; nestin immunostaining in medulla with mild reactivity in CT and moderate in loop of Henle (arrow).C; showing FGF9 immunostaining with intense reaction in mature glomeruli (arrow) and moderate in PCT. D; The medulla showed FGF9 with increased reactivity in CT and loop of Henle (arrow). X40 from A-D.

3.3. At age 14 days old.

In this age the kidney reaches to maturity where all immature form of nephron was disappeared from the subcapsular zone. The nestin immunostaining was more intense in mature glomeruli especially in podocytes cells than the previous age and no reactivity appeared in PCT and DCT. The distal convoluted tubules which lined by low cuboidal epithelium with wide lumen was showed macula densa cell in contact with renal corpuscle, macula densa cell was consider one component of juxtaglomerular apparatus (Fig. 3A). The medulla showed nestin with intense reactivity in loop of Henle and surrounding blood capillaries (Fig. 3B).

Figure 3 C showed FGF9 immunostaining in renal cortex with intense reaction in mature glomeruli and moderate in surrounding PCT cytoplasm. The medulla showed moderate reaction in collecting duct and intense in loop of Henle (Fig. 3D).

3. 4. The age of 30 days old (adult kidney).

The adult kidney showed well developed renal corpuscles with wide bowman's space which lined by squamous epithelium. The nestin immunostaining was more intense in adult glomeruli than neonatal one. The nestin showed also moderate reactivity in interstitial myofibroblasts cells around renal tubules (Fig. 4A). The nestin immunostaining in adult kidney medulla more increased in loop of Henle and surrounding blood capillaries while no reactivity in collecting tubules (Fig.4B).

Figure 4C showed FGF9 in adult renal cortex intense in glomeruli, moderate in PCT, blood vessels and mild in DCT. While in medulla it was showed moderate reactivity in collecting tubules and surrounding loop of Henle (Fig. 4D).

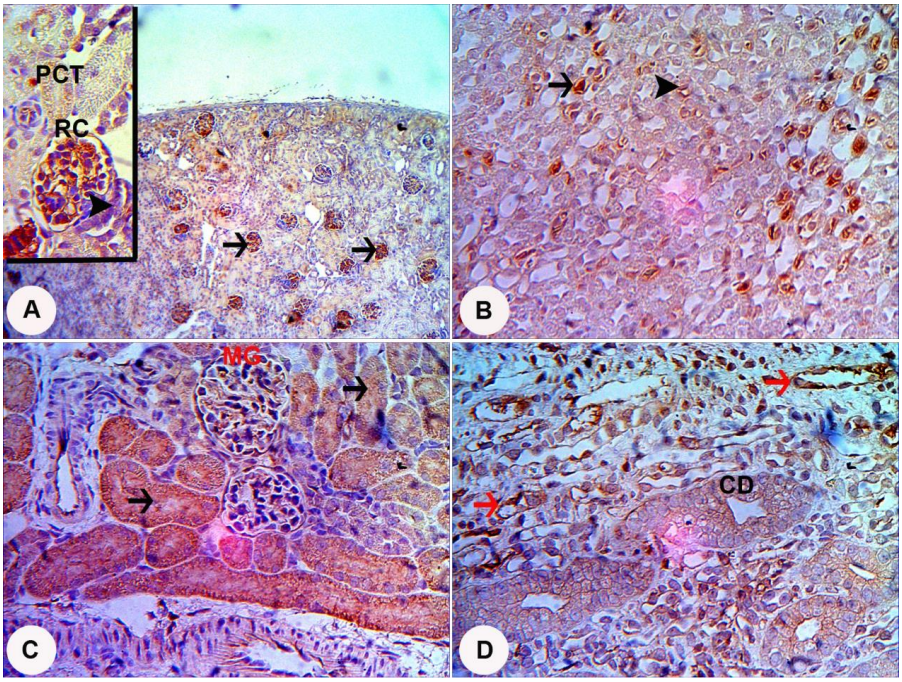

Figure 3: Photomicrographs of rabbit kidney at 14days old. A; showing nestin immunostaining in renal cortex with intense reaction in renal corpuscles (arrows) only. The inset shows mature RC with intense reaction in glomeruli only while no reactivity in PCT and DCT, the macula densa (head arrow) in wall of DCT. B; showed medulla with intense reactivity of nestin in loop of Henle (head arrow) and blood capillaries (arrow). C; illustrating FGF9 immunostaining that was intense in MG and moderate in PCT. D; showed the medulla with FGF9 immunostaining with moderate reactivity in collecting duct (CD) and intense in loop of Henle. X40 in inset, B, C, D, and X10 in A.

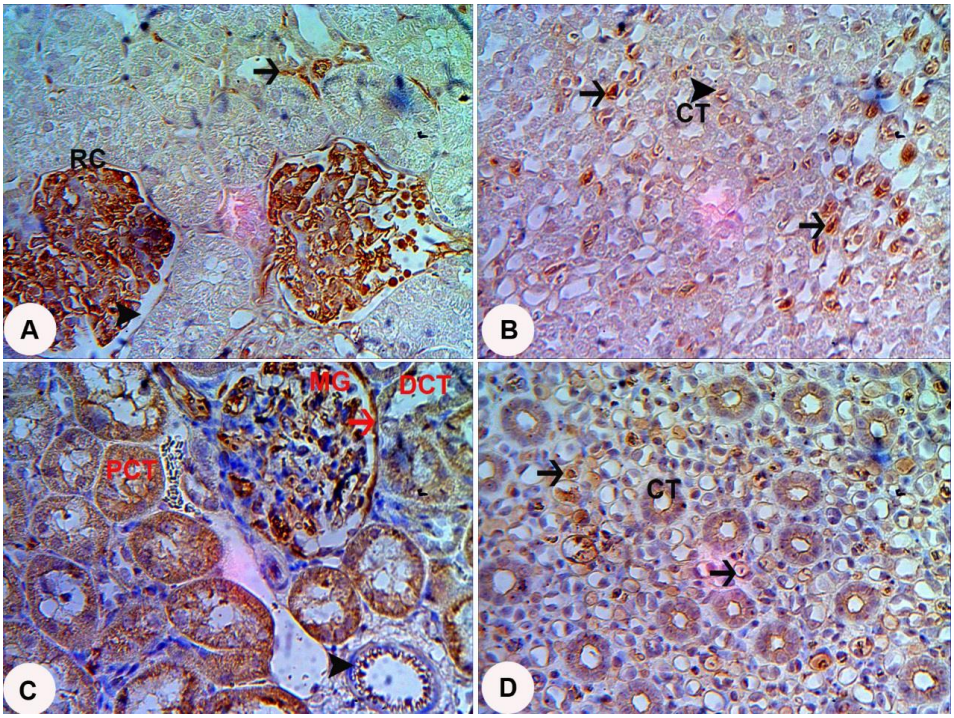

Figure 4: Photomicrographs of rabbit kidney at 30 days old. A; showing nestin immunostaining in renal cortex with intense reaction in RC which lined by wide bowman's space (head arrow) and moderate reactivity myofibroblasts cells (arrow). B; the medulla showed nestin immunostaining in blood capillaries (arrows) and loop of Henle (head arrow) while no reactivity in CT. C; showing FGF9 immunostaining that was intense in MG and parietal epithelium (arrow) while moderate in PCT, DCT and blood vessels. D; illustrating the medulla with FGF9 which showed moderate reactivity in CT and loop of Henle (arrows). X40 from A-D.

4. DISCUSSION

In this study we showed the immunohistochemical localization of nestin and FGF9 in rabbit kidney during postnatal development. At neonatal age where the kidney showed different stages of development which the immature nephrons located in subcapsular zone while mature ones located deeper in medulla. These results are coincided with the observation of (leeson and Baxter 1957) who reported the same result in rabbit fetuses at the same age. They stated that there was a distinct zoning of maturity of glomeruli. Linda et al (2011) reported in human fetal kidney that the subsequent generation of nephrons formed in the nephrogenic zone are added to the underlying developing cortex in ill-defined layers. Although nephrogenesis is not completed at birth, the newborn kidney remains functionally primitive with respect to the capacity to concentrate urine, control acid-base balance, and reabsorb filtered nutrients.

The kidney of rabbit become mature at the end of 14 days where all immature nephron disappeared from subcapsular zone of the cortex. And adult kidney showed the renal corpuscles appeared spherical in shape with evident Bowman's spaces and parietal epithelium, well developed PCT and DCT also seen. This comes in accordance with the result of Quaggin and Kreidberg (2008) in mouse. this gross structure is important for the functionality of the metanephric kidney as it establishes an osmotic gradient between the cortex and medulla that drives the extraction of water from the urine.

The immunostaining reaction of nestin was showed in neonatal was not detected in immature glomeruli or very week reaction. In contrast, in mature glomeruli was observed only in glomerular basement membrane while in adult was expressed only in podocytes while in medulla increased in loop of Henle and blood capillaries in adult than younger ages. Similar result was reported by Mokry and Nemecek, (1998) in rat. They stated that in immature glomeruli, nestin is expressed in the progenitors of glomerular endothelial cells. Nestin is also transiently expressed by epithelial cells of immature proximal tubules in the newborn kidney. In contrast, in the mature adult kidney, podocytes are the only cells that exhibit persistent nestin expression (Chen et al., 2006).

Zou et al (2006) reported in adult rat kidney that nestin was strongly expressed by tubular and interstitial cells, in addition to podocytes nestin-positive tubular cells and tubulointerstitial cells were localized mainly at the outer medulla. In the adult kidney, nestin expression is restricted to differentiated podocytes, suggesting that nestin could play an important role in maintaining the structural integrity of the podocytes. Because nestin is a cytoskeletal intermediate filament protein, which contains binding domains for microtubule and microfilament actin, it also may be involved in maintenance of the integrity of the cytoskeletal structure of renal cells, including the glomerular podocyte (Pavenstadt et al., 2003). The abundant and well-organized cytoskeletal proteins within podocyte also are believed to be critical for counterbalancing the mechanical stretch and stress on these cells, thereby preventing outward ballooning of the vessel and preserving the normal architecture of the glomerular tuft (Mundel and Shankland., 2002).

The present study revealed that the FGF9 is first expressed in young age in immature and mature glomeruli with surrounding tubules while in adult the reactivity increased in glomeruli than the surrounding tubules, the medulla showed intense reactivity in adult collecting tubules and loop of Henle than younger ages. This result coincides with the results of Uta et al (2005) in mouse. human fetal kidney demonstrated FGF9 expression in the epithelial cells of the branching ureteric bud epithelium, S-shaped bodies, proximal tubule epithelium and parietal epithelium of the glomerulus (Drummond et al. 1998). FGF9 signaling is essential for normal kidney development, although the earliest events in kidney morphogenesis, including ureteric bud formation and at least some collecting duct branching, occur in its absence. the complete loss of Fgf9 function during kidney development results in a failure of nephron formation prior to the S-shaped body stage.

Cauchi et al (1996) reported that FGF-1 and FGF-9 proteins are localized in glomeruli, proximal tubules, distal tubules, and collecting ducts of adult rat kidneys. In adult rat kidneys, FGF9 has been localized in medullary interstitial cells (Ichimura et al., 1996). Van et al (1998) found immunoreactivity for FGFR-9 in the tunica media of arteries and arterioles of adult rat kidney specifically localized to smooth muscle of arteries and arterioles, suggesting that they may control FGF definitive signaling through FGFR-1 in these cells. The decreased vascular smooth muscle contractility and low blood pressure of FGF-2 knockout mice (Zhou et al., 1998) suggest that FGF action through FGFR-1 is involved in maintenance of vascular tone.

5. Conclusion

The present study was carried out to illustrate the immunohistochemical study of nestin and FGF9 on rabbit kidney during postnatal development. these studies show that nestin play an important role in adult than the neonatal as maintaining the structural integrity of the podocytes while FGF9 is critical for virtually all renal linage at early and later stages of development. The adult form of histological structure of rabbit kidney is reached postnatally by the end of the second week.

6. Acknowledgement

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