



Epithelial–mesenchymal Transition of Glomerular Podocytes: Implications in Proteinuria

¹Rajkishor Nishad, ²Krishnamurthy Nakuluri, ³Manga Motrapu, ⁴Anil K Pasupulati

ABSTRACT

The kidneys play an essential role in filtration of blood plasma, regulation of water, electrolyte, and acid/base balance of the body, and thus maintain overall homeostasis. The glomerular filtration barrier serves as a size, shape, and charge barrier to ensue glomerular permselectivity, so that kidneys excrete almost protein-free urine. Podocytes are glomerular visceral epithelial cells and significantly contribute to the glomerular permeability owing to their unique structure and specialized function. Nevertheless, podocytes are susceptible to various insults, including altered metabolites, aberrant signaling molecules, and mutations to critical proteins that otherwise ensue normal function. Podocyte injury is a predominant indicator of several glomerular diseases that are manifested by proteinuria. Epithelial–mesenchymal transition (EMT) is considered as one of the responses of podocytes to the noxious stimuli, which consequently results in podocyte depletion and proteinuria. This review discusses the importance of podocytes in normal renal filtration and details the molecular and cellular events that lead to EMT of podocytes vis-à-vis impaired glomerular filtration.

Keywords: Epithelial–mesenchymal transition, Glomerulus, Kidney, Nephron, Podocytes, Proteinuria.

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KIDNEYS ENSUE EXCRETION OF PROTEIN-FREE URINE

Over a million nephrons in each kidney work in concert to regulate water and acid/base balance of the body and to ensue excretion of protein-free urine. Thus, kidneys become vital organs to ensure homeostasis of the body. The two essential segments of a nephron are glomerulus and renal tubule. The glomerulus is essential for filtering water and small molecules from plasma. The tubular

system ensues both selective reabsorption of glomerular filtrate and selective secretion of ions into glomerular filtrate. Therefore, both glomerulus and renal tubule work in concert and dictate the final composition of urine. The potential of kidney to excrete almost protein-free ultrafiltrated urine gets compromised during disease conditions, and as a result varying amounts of plasma proteins get excreted in urine. Albuminuria is an index of adverse renal outcome, which can be assessed by measuring albumin levels in urine, collected for 24 hours. According to the American Diabetic Association, microalbuminuria describes levels of urine albumin ranging from 30 to 300 mg/24 hours; and macroalbuminuria describes a urinary albumin excretion of ≥ 300 mg/24 hours. The condition of macroalbuminuria often progresses to overt proteinuria and even further to end-stage renal disease (ESRD), warranting renal transplant therapy.

Appearance of protein in the urine indicates a structural and/or functional artifact, particularly in the glomerular region.¹ The glomerular filtration barrier (GFB) of the kidney serves as a size, shape, and charge selective molecular sieve. The three critical components that constitute GFB are: (a) Fenestrated endothelium of glomerular blood vessels; (b) basement membrane that covers the blood vessels; and (c) the podocytes that provide epithelial coverage to basement membrane (Figs 1A and B). Though, all the three components contribute to the integrity of GFB, there is much debate on the critical role of each component toward size, shape, and charge-dependent permselectivity of GFB. It was proposed that endothelial dysfunction is a causal factor in the pathogenesis of proteinuria.² Thickening of glomerular basement membrane (GBM) by excess deposition of collagen and altered charge selectivity implicates the pathogenesis of proteinuria.^{3,4} The third and final barrier that restricts entry of proteins from circulation into the urine is the podocytes, also known as visceral epithelial cells. There is increasing evidence for the crucial role of podocytes in this glomerular filtration process.⁵

PODOCYTES ARE UNIQUE CELLS WITH SPECIALIZED PROPERTIES

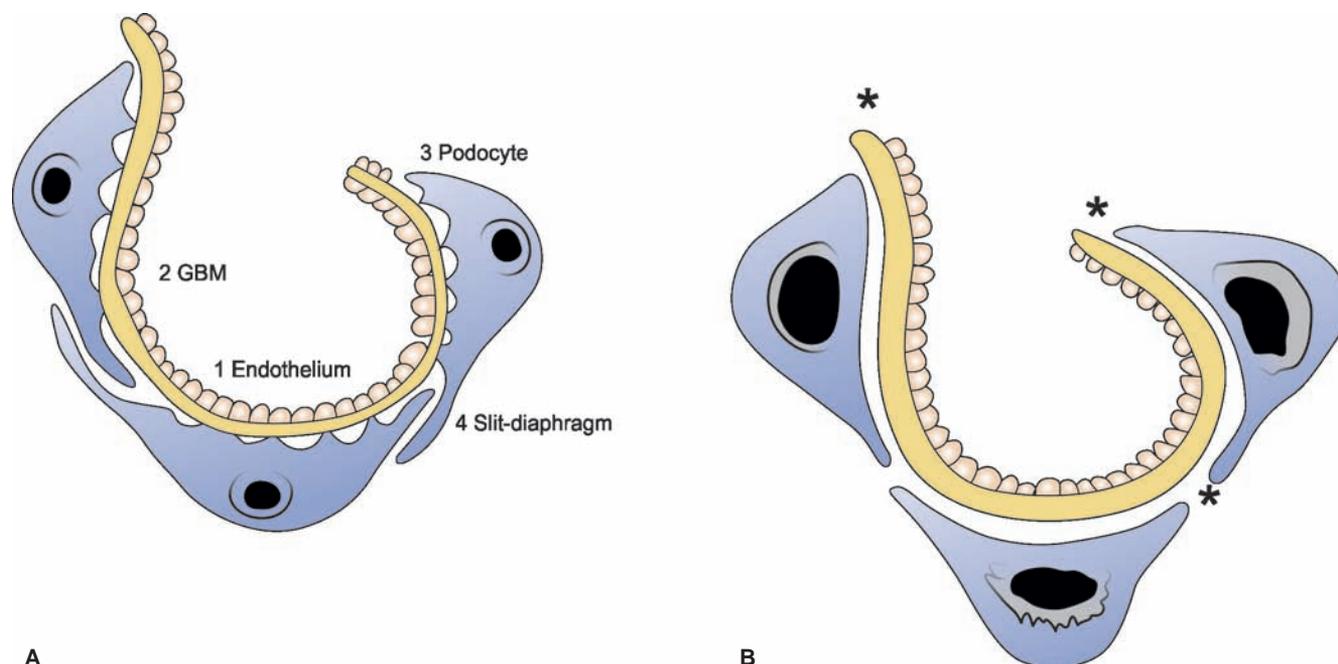
Podocytes are the major cell type of glomerulus and account for about 30% of all glomerular cells. Podocytes are highly branched epithelial cells and cover the urinary

^{1,2}PhD Student, ³Project Assistant, ⁴Assistant Professor

¹⁻⁴Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana, India

Corresponding Author: Anil K Pasupulati, Assistant Professor, Department of Biochemistry, School of Life Sciences University of Hyderabad, Hyderabad, Telangana, India, Phone: +914023134519, e-mail: pasupulati.anilkumar@gmail.com





Figs 1A and B: Components of GFB. (A) The essential components of GFB are: (a) endothelium; (b) glomerular basement membrane; (c) podocytes; and (d) slit-diaphragm. (B) The major alterations of GFB during proteinuria conditions are indicated as * (asterisk), which include thickening of glomerular basement membrane, effacement of podocyte foot process, and damage to slit-diaphragm

side of the GBM. The important functions of podocytes are to (1) synthesize the components of GBM, including collagen; (2) provide structural support for the glomerular vasculature and filtration; (3) serve as a contractile apparatus together with basement membrane to counteract the expansive forces of the glomerular vasculature, (4) offer glomerular permselectivity, and (5) secrete several survival factors [vascular endothelial growth factor (VEGF) and angiopoietin] for endothelial cells. Therefore, podocytes are considered to be crucial in the regulation of glomerular architecture and function. Zimmerman⁶ made the very first description of podocyte way back in 1929; he described podocytes as a heavily branched cell type within the glomerulus. The unique architecture of podocytes includes a voluminous cell body, from which long cellular processes extend, comprising primary and secondary processes. Secondary processes end in large number of foot processes, which constitute the main functional unit of the podocytes. The primary processes are composed of microtubules and vimentin filaments, while the foot processes are made of filamentous actin (F-actin). Foot processes play a critical role in cell/cell contact between podocytes, attachment of podocytes to the GBM and adjusting the shape of podocytes in response to the glomerular pressure.

The neighboring foot processes of podocytes are connected with an adherens junction, namely slit-diaphragm (SD), which represents the only cell/cell contact between podocytes. Though the SD is freely permeable to water and small solutes, it restricts the passage of large mol-

ecules, particularly proteins. Several proteins (Nephrin, Podocin, TRPC6 CD2AP, P-cadherin, etc.) constitute the SD and enable it to act as a charge, size, and shape selective glomerular barrier. The filtration slits of SD are 40 nm wide and smaller than the size of albumin, a predominant plasma protein. The apical membrane of podocyte, located above the SD, is negatively charged due to the presence of the glycocalyxin. Negatively charged glycocalyxin repels serum albumin and also helps maintain adjacent foot processes separate from each other. The basal membrane of podocytes, located below the SD, is associated with anchorage of podocytes to GBM, which is mediated by integrin family proteins.

A key feature that distinguishes podocytes from other renal cells is that they are terminally differentiated cells, postmitotic, quiescent in nature, and do not proliferate in response to injury or noxious stimuli. Whereas glomerular endothelial cells and mesangial cells readily proliferate in response to injury caused by an array of insults,^{7,8} it is postulated that with a loss of critical proportion of the podocyte population in response to the injury or mutations, the remaining cells are unable to compensate for the glomerular filtration.⁹ The range of podocyte injuries that can be caused by altered milieu, mutations in podocyte proteins, and also due to extrinsic stressors are collectively known as podocytopathy. Genetic podocytopathy [nephrotic syndrome (NS)] refers to the injury to the podocytes owing to mutations in key genes that encode proteins localized to SD. On the contrary, reactive podocytopathy refers to the podocyte injury triggered by

alterations in podocyte microenvironment during metabolic disorders (diabetes and obesity) or external agents (human immunodeficiency virus, immunoglobulin [Ig] A immune complexes, etc.). Nevertheless, podocytopathies manifest in considerable amount of proteinuria and, if left untreated, may progress to ESRD.

RESPONSE OF PODOCYTES TO INJURY DURING GLOMERULAR DISEASES

There are several diseases whose pathophysiological manifestation involves podocyte injury, which includes diabetic nephropathy (DN), membranous nephropathy, NS, and focal segmental glomerulosclerosis. One of the predominant ultrastructural manifestations of podocyte injury is effacement, characterized by flattening of podocyte foot process (Fig. 1). It leads to distortions of SD architecture and impairment of its permselectivity. Podocytes respond to the injury by secreting excess extracellular matrix (ECM) proteins, such as collagen IV, laminin, and fibronectin, leading to thickening of GBM. Altered composition of basement membrane leads to enhanced permeability. Reactive oxygen species secreted by injured podocytes damage the integrity of ECM, which increases the permeability to plasma proteins. Matrix metalloproteases secreted by injured podocytes selectively degrade target ECM proteins. Alternatively, podocytes, upon injury, fail to secrete sufficiently enough growth factors (e.g., VEGF) to promote the growth of glomerular endothelial cells. Endotheliosis, secondary to podocyte injury, limits the function of GFB.¹⁰ Podocyte injury, therefore, results in concomitant morphological changes, such as effacement, thickening of GBM and endotheliosis. These events manifest in reduced function of GFB vis-à-vis proteinuria. Apart from the events discussed above, podocytes respond to injury by undergoing apoptosis, autophagy, anoikis and necrosis, or detachment from GBM. These events account for decreased podocyte number (podocytopenia). Neighboring podocytes respond to podocyte loss by undergoing hypertrophy. Compensatory hypertrophy of podocytes is an adaptation to cover the GBM exposed due to podocyte loss. Nevertheless, it leaves the GBM denuded and eventually results in loss of epithelial coverage to the GBM.¹¹

Studies in both patients with DN and animal models of diabetes mellitus revealed that proteinuria is associated with decreased density and altered morphology of the podocytes.^{12,13} Decrease in podocyte number predicts progressive decline in renal function and proteinuria in Pima Indians with diabetes mellitus.¹⁴ Among several modes of podocyte depletion mentioned above, apoptosis was proposed as the major mode of podocyte loss, based on experiments pursued in transforming growth factor

(TGF)- β 1 transgenic mice, CD2AP^{-/-} mice and puromycin aminonucleoside treated rats.¹⁵⁻¹⁷ These studies argue that ~90% of the podocytes detected in urine are apoptotic.¹² An interesting finding is that podocytes that are shed in urine are viable and can be cultured. It is not readily explainable how podocytes from urine could be viable and can be cultured, if majority of the podocytes have undergone apoptosis.^{18,19} Recovery of podocytes from urine strongly suggests that injured podocytes detach from GBM with intact renewable machinery of cells. Podocytes are anchored to GBM with the help of ECM proteins including laminin, integrins, dystroglycan, and collagen and alterations in cell-matrix adherence leading to podocyte detachment. Evidence for this mechanism is provided by data showing elevated expression of antiadhesive proteins and integrin receptors in DN.¹⁹⁻²² Moreover, it is evidenced from diabetic rats that podocytes detach from GBM into urinary space.²¹ Podocyte detachment could be explained by two possible mechanisms: Alterations in expression of ECM components; and enervated cell-cell and cell-ECM interactions. Since reduction in podocyte density and appearance of podocytes in urine is an early pathological feature in patients with diabetes and animal models of diabetes,²³⁻²⁵ podocyte depletion could be considered as a hallmark of human and experimental DN and NS.

It was proposed that podocytes, in response to injury, are capable of undergoing a phenotypic switch to attain an embryonic form by shedding their specialized epithelial characteristics and by acquiring mesenchymal features. This phenotypic switch is known as epithelial-mesenchymal transition (EMT). It is conceivable that podocytes, after undergoing EMT, abandon their complex morphological architecture and relinquish their highly specialized functions, impairing GFB integrity, and altogether leading to the onset of proteinuria. Although it is debatable whether EMT contributes to decreased podocyte density in diabetic kidney disease, the presence of significant number of viable urinary podocytes in both experimental models of DN and in patients with DN suggest that podocyte dropout might be caused by decreased podocyte adhesion, which is a potential consequence of EMT.^{19,26}

EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition is a tightly controlled cellular event during which an epithelial cell that otherwise interacts with basement membrane undergoes phenotypic switch and attains characteristics of mesenchymal cells, including enhanced migratory capacity, invasiveness, resistance to apoptosis, and increased production of ECM. Although EMT is considered as a pathological event in case of nephropathy, tissue fibrosis, and cancer metastasis, it is a fundamental process that occurs during several stages of development, such as mesoderm forma-

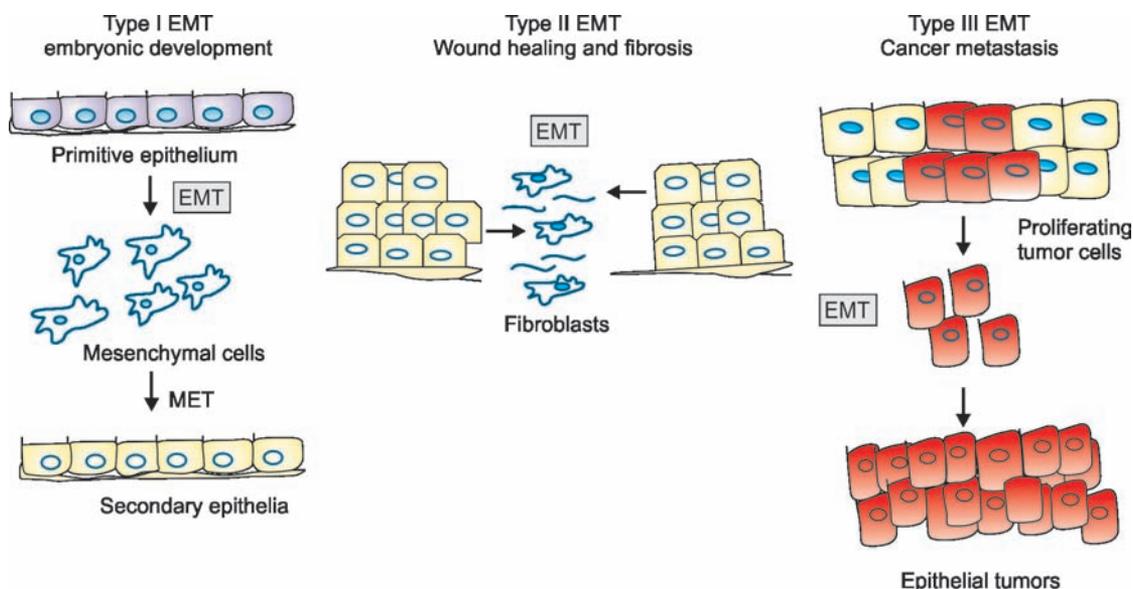


Fig. 2: Three types of Epithelial–mesenchymal transition (EMT). Type I EMT is associated with embryonic development, such as implantation and embryonic gastrulation. The primitive epithelium undergoes EMT to give rise to primary mesenchyme, which in turn undergoes MET to form secondary epithelia. Type II EMT is associated with inflammation and wound healing. If the primary inflammatory insult is not attenuated, type II EMT results in fibrosis. Type III EMT is associated with metastasis. Proliferating tumor cells in secondary epithelia undergo EMT that ensue their metastasis to form epithelial tumors

tion from embryonic epithelium and delamination of the neural crest.

Epithelial–mesenchymal transition has been categorized into three different subtypes, based on functional consequences (Fig. 2). Epithelial–mesenchymal transition, i.e., associated with implantation, embryogenesis, and organ development are categorized as type I. Nevertheless, mesenchymal cells, known as primary mesenchyme, that arise as a result of type I EMT, have the potential to subsequently undergo a mesenchymal–epithelial transition (MET) to generate secondary epithelium. Secondary epithelia may further differentiate and undergo subsequent EMT to generate the cells of various lineages including astrocytes, adipocytes, chondrocytes, and osteoblasts. Epithelial–mesenchymal transition, i.e., associated with tissue regeneration and wound healing is of type II. Type II EMT is a repair-associated event that generates fibroblasts in order to reconstruct damaged tissue following a trauma and/or inflammation (Fig. 2). However, aberrations in type II EMT may eventually lead to fibrosis and organ destruction. Type III EMT occurs in neoplastic cells that may invade, metastasize, and ensue in tumor outgrowth. Further insights about the types of EMTs are detailed elsewhere.²⁷

PODCYTE UNDERGOES EMT

It is considered that phenotypic switch between healthy and diseased podocytes partially resembles type II EMT only.²⁸ Podocytes possess epithelial features, such as apical-basal cell polarity and tight junctions. Podocytes are anchorage dependent with a low invasive capacity.²⁸

Epithelial markers expressed by podocytes include E- and P-cadherin, WT-1, and ZO-1 (Flow Chart 1 and Fig. 3). The predominant manifestations of podocyte EMT are loss of their epithelial polarity, rearrangement of actin cytoskeleton, and injury to SD. Podocytes undergo cadherin switch from E- and P-cadherin to N-cadherin during EMT. On the contrary, podocytes express vimentin and intermediate filaments; and also possess high migration capacity. These later innate features of podocytes resemble that of mesenchymal cells and, therefore, podocytes are considered as specialized or atypical epithelial cells. List of acquired markers and attenuated markers during different types of EMT are provided in Flow Chart 1.

Elevated expression of fibroblast-specific protein-1 (FSP-1) in podocytes exposed to hyperglycemic conditions ensues EMT of podocytes.²⁹ Expression of increased collagen by injured podocytes also indicates that podocytes undergo EMT.³⁰ Although podocytes express vimentin, following an exposure to TGF- β 1, a potent inducer of EMT, a further increase in vimentin was observed.³¹ Injured podocytes express multiple transcriptional factors that ensue EMT, which include ZEB2, SNAIL, and SLUG. It was also reported that podocyte dedifferentiation is associated with attenuation of epithelial markers, such as P-cadherin and ZO-1. Despite the fact that podocytes undergo EMT upon noxious stimuli that are prevalent during various pathophysiological conditions, it has been proposed that the EMT of podocytes satisfies criteria of type II EMT partially.²⁸ The unique nature of differentiated podocyte further distinguishes them from the three types of EMT. Differentiated podocytes are spindle-

Flow Chart 1: List of acquired and attenuated markers during EMT. Various mesenchymal markers are upregulated and epithelial markers are downregulated during different types of EMT. The EMT of podocytes resembles type II EMT

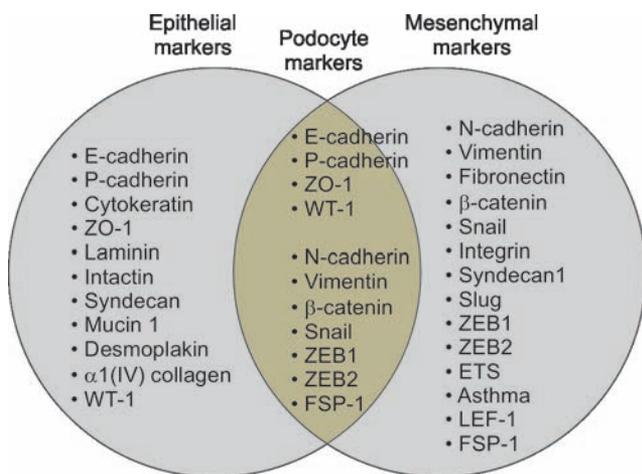
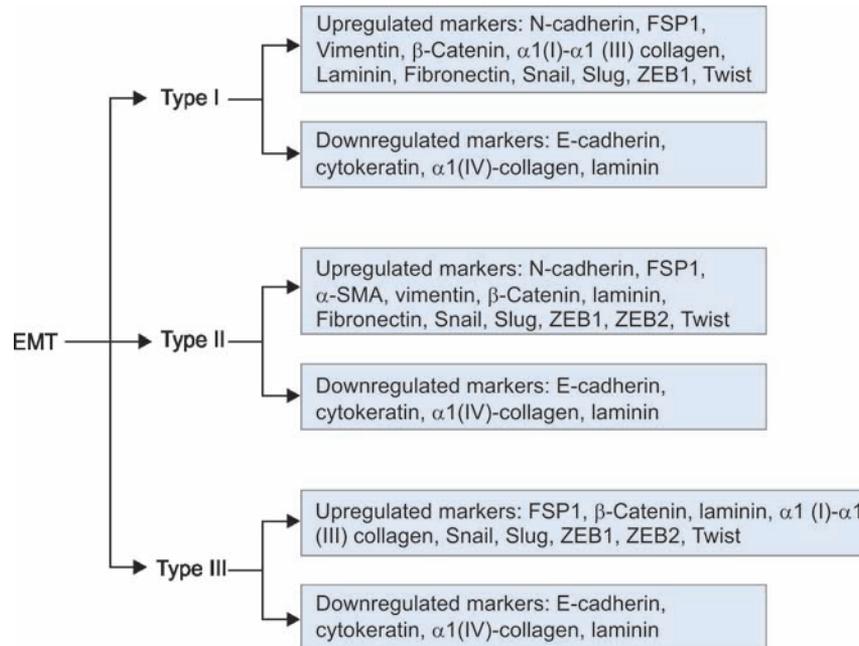


Fig. 3: Epithelial and mesenchymal features of the podocyte. Predominant characteristics of epithelial and mesenchymal cells are listed. The podocytes possess some of the features that are both epithelial and mesenchymal

shaped and arborized. Upon subjected to various insults, podocytes lose their apical-based polarity, and there is a compromise in their morphological features and function, as evidenced by redistribution of stress fibers occurs, and also in permselectivity. Features, such as redistribution of stress fibers and loss of polarity are unique to podocyte EMT and have not been seen in the other three types of EMT. Lists of epithelial and mesenchymal markers present in podocytes are provided in Figure 3.

FACTORS THAT INDUCE PODOCYTE EMT

Although TGF-β1 is a well-known inducer of type III EMT, the primary evidence for the role of TGF-β1 in podocyte

EMT was provided by a study from Li et al.³⁰ The TGF-β1 treatment attenuated the expression of P-cadherin, ZO-1, and nephrin, while promoting the acquisition of mesenchymal markers, such as FSP-1 and Desmin. Nephrin, P-cadherin, and ZO-1 are constituents of SD; decreased expression of these proteins is expected to impair the integrity of the SD leading to foot process effacement and altered podocyte permselectivity to serum proteins. Increased transcriptional activity of Snail implicated in TGF-β1 triggered EMT of podocytes, whereas ectopic expression of Snail suppressed P-cadherin and nephrin. It is also reported that treatment with TGF-β1 resulted in retraction and shortening of podocyte foot processes and contraction of the cell body. Besides these morphological changes, TGF-β1 induced dedifferentiation of podocytes with increased motility. Following treatment with TGF-β1, attenuation of podocyte epithelial markers and adhesive proteins and acquisition of mesenchymal markers was observed.³¹ The TGF-β1 suppresses α3 integrin expression in nephrotic rats.³² These *in vivo* observations in nephrotic rats are supported by a study from cultured podocytes, wherein cells exposed to TGF-β1 showed loss of α3-β1 integrin expression. Since α3 integrin subunit regulates podocyte interaction with GBM, it is speculated that loss of α3-integrin expression by TGF-β1 ensues podocyte detachment and depletion.³³ In summary, the multiple effect of TGF-β1 on podocytes includes reduced expression of adhesion proteins (particularly integrins), attenuation of epithelial markers and acquisition of mesenchymal markers, and suppression of proteins that control the integrity of SD.³¹



Human pituitary growth hormone (GH) is another molecule, whose effect on podocyte EMT is well documented. In the setting of diabetes, particularly in type I DM, insulin deficiency is associated with reduced insulin-like growth factor (IGF-1) production by liver. Decreased IGF-1 secretion in concert with impaired IGF-1 action, due to increased secretion of hepatic IGF binding protein 1, elicits a negative feedback response to induce GH secretion by the pituitary gland.³⁴ Podocytes express GH receptor (GHR) and respond to GH by activation of canonical JAK-STAT signaling.³⁵ Elevated GH levels manifest in podocyte injury and proteinuria. Supraphysiological levels of GH in transgenic animals are associated with degenerative changes in the kidney.^{34,36} A direct relationship has been observed between elevated GH levels and renal macroalbuminuria and glomerulosclerosis. On the contrary, GH deficiency or ablation of GHR activation conferred protective effect in diabetic conditions. In our study, we observed that GH induces ZEB2, a transcription factor that mediates EMT. It was also shown that GH-induced ZEB2 suppresses SD proteins and consequently increases podocyte permeability to albumin.³⁷ Furthermore, we have also demonstrated that administration of GH to rats induced podocyte EMT vis-à-vis decreased podocyte count, and increased proteinuria.³⁸

Advanced glycation end-products (AGEs) that are derived from glucose via nonenzymatic reactions are also implicated in the pathogenesis of DN. Carboxymethyllysine (CML) is one of the predominant AGEs that accumulate in glomerular region of diabetic patients and animal models of diabetes. In our recent study, we demonstrated that CML induced the expression of ZEB2 in podocytes via activation of nuclear factor (NF)- κ B signaling cascade. The CML treatment induced promoter activity of NF- κ B and ZEB2 suppressed promoter activity of E-cadherin and increased podocyte permeability to albumin. Attenuation of NF- κ B cascade prevented CML-dependent ZEB2 expression. It is interesting to note that shRNA-mediated knockdown of ZEB2 expression abrogated both CML-mediated invasiveness and permeability of podocytes.³⁹ Elevated CML levels are concurrent with increased expression of ZEB2 in glomeruli and proteinuria.

Glomerulus is composed of microvasculature, which is exposed to high volume and continuous perfusion. The kidney's demand for oxygen is very high. The low oxygen conditions that prevail during pathological conditions, such as diabetes and stroke induce alpha subunit of hypoxia-inducible factor-1 α (HIF-1 α) in the kidneys. In our recent study, we demonstrated that HIF-1 α induced ZEB2 expression, both by directly interacting with ZEB2 promoter and by increasing expression of a ZEB2-natural

antisense transcript. Elevated expression of ZEB2 resulted in suppression of E- and P-cadherin, which is implicated in podocyte EMT and proteinuria observed in hypoxic conditions.⁴⁰

NEPHROTIC SYNDROME VS EMT OF PODOCYTES

Other than various insults that arise as a consequence of metabolic and physiological alterations, genetic defects are also associated with podocyte EMT, for instance, mutations in genes encoding podocyte SD proteins. In particular, single gene defects causing NS tend to cause irreversible damage to podocytes. Most of the genetic defects that cause NS occur in proteins that constitute SD or in proteins that are critical to the podocyte biology, such as nephrin, podocin, CD2AP, or alpha-actinin. The extracellular domain of nephrin protein forms a protein scaffold with the help of cysteine residues present in its eight IgG-like motifs.⁴¹ This scaffold of nephrin interacts with other proteins in order to maintain the integrity of SD, whereas intracellular domain of nephrin interacts with actin cytoskeleton of the podocyte foot process.⁴² Loss of extracellular domain of nephrin due to mutations results in aberrations in SD architecture.⁴³ Podocin is a raft-associated podocyte foot process protein, which is essential for nephrin transport to the podocyte membrane.⁴⁴ It is considered that podocin interacts with nephrin and CD2AP. CD2AP is an adaptor molecule, which possesses actin-binding sites. Nephrin, CD2AP, and podocin are capable of interacting with each other, thus contribute to the integrity of SD, and dynamic actin assembly.^{45,46} Mutation in these SD proteins is associated with distortion of SD assembly and cause proteinuria.

OTHER MODES OF PODOCYTE DEPLETION

Although existing paradigm argues that podocytes are terminally differentiated and are unable to proliferate, it was suggested that they might proliferate in case of crescentic glomerulonephritis. Nevertheless, cells found in crescentic glomerulonephritis did not express predominant podocyte markers including WT-1.⁴⁷ Large body of evidence suggests that parietal epithelial cells and progenitors of podocytes are the major cell type in crescents.⁴⁸ This led to a new perception that podocytes undergo dedifferentiation during injury in order to engage in the cell cycle. All the evidence reiterates that mature podocytes are terminally differentiated, whereas parietal epithelial cells undergo proliferation and likely to contribute for the glomerular disease, such as crescentic and collapsing forms of glomerulopathy. In case of progressive glomerular diseases, podocyte loss cannot be matched by proliferation of

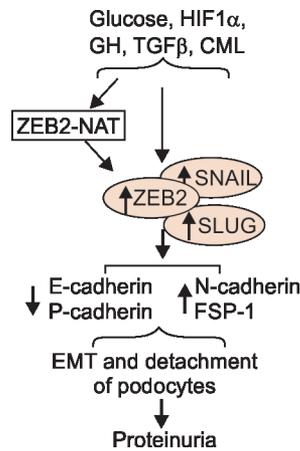


Fig. 4: Mechanism of podocyte EMT; TGF- β 1, HIF-1 α , GH, and CML induce expression of transcriptional factors, such as ZEB2, Snail, and Slug. These transcriptional factors suppress expression of epithelial markers and SD proteins while inducing expression of mesenchymal markers. A series of events lead to increased motility and consequent loss of podocytes. Together, these events manifest in proteinuria

parietal epithelial cells, which leads to overall podocyte depletion, with consequent glomerulosclerosis.

Another mode of podocyte injury that manifests in podocyte depletion is hypertrophy. The podocyte loss leaves a denuded area on GBM, which is covered by hypertrophy of neighboring podocytes, as podocytes are unable to proliferate. Podocyte hypertrophy involves various stages as evidenced in aged rats: Initial non-stressed podocyte hypertrophy (without proteinuria), followed by adaptive hypertrophy, and decompensated podocyte hypertrophy are characterized by reduced nephrin expression and proteinuria.⁴⁹ These events finally culminate in podocyte depletion. Several other modes of podocyte depletion have been described. Necrosis of podocytes occurs in membranous nephropathy. Anoikis, mitotic catastrophe, and apoptosis of podocytes have been documented in various glomerular diseases.⁵⁰

CONCLUSION

Our understanding of podocyte biology has increased significantly in the past two decades. Nephrologists recognized the importance of podocytes in the glomerular filtration. Accumulated evidence highlights the novel mediators of podocyte injury, particularly which contributes to podocyte detachment by transforming these cells from epithelial form to more invasive and motile phenotype. Dearth of sufficient number of podocytes compromises glomerular permselectivity, which manifests in increased permeability to protein across GFB. Thus, the depletion of podocytes accounts for proteinuria observed in majority of glomerular diseases. Moreover, EMT is considered to be one of the predominant molecular mechanisms to explain podocytopenia in DN. The TGF- β 1,

HIF-1 α , GH, and CML are some of the well-studied agents that trigger podocyte EMT, whereas ZEB2, Snail, and Slug are the key transcription factors that transduce EMT (Fig. 4). Our understanding of podocyte EMT with insights from cultured podocytes and animal models may enable us to target EMT as a therapeutic option.

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