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REVIEW ARTICLE

Cell biology of mesangial cells: the third cell that maintains the glomerular capillary

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Abstract The renal glomerulus consists of glomerular endothelial cells, podocytes, and mesangial cells, which cooperate with each other for glomerular filtration. We have produced monoclonal antibodies against glomerular cells in order to identify different types of glomerular cells. Among these antibodies, the E30 clone specifically recognizes the Thy1.1 molecule expressed on mesangial cells. An injection of this antibody into rats resulted in mesangial cell-specific injury within 15 min, and induced mesangial proliferative glomerulonephritis in a reproducible manner. We examined the role of mesangial cells in glomerular function using several experimental tools, including an E30-induced nephritis model, mesangial cell culture, and the deletion of specific genes. Herein, we describe the characterization of E30-induced nephritis, formation of the glomerular capillary network, mesangial matrix turnover, and intercellular signaling between glomerular cells. New molecules that are involved in a wide variety of mesangial cell functions are also introduced.

Keywords Thy1.1 nephritis · Extracellular matrix · Cell adhesion · Actin cytoskeleton · Cell signaling

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Introduction

The glomerular capillary network maintains high blood pressure and its functions contribute to effective glomerular filtration, one unit of which consists of fenestrated glomerular endothelial cells, the intervening glomerular basement membrane (GBM), and podocytes. Numerous foot processes of podocytes are attached to the lamina rara externa of the adjacent GBM, which is divided between podocytes and other types of glomerular cells, i.e., endothelial cells and mesangial cells. Podocytes play an important role in blood filtration and produce urine through the slit diaphragm, which connects with foot processes from neighboring cells (Kurihara et al. 1992; Kriz et al. 1994).

Mesangial cells, the third type of cell in the glomerular tuft, were first identified by Zimmerman (1933), and confirmed using electron microscopy more than 30 years later (Yamada 1955; Latta et al. 1960; Farquhar and Palade 1962). However, an additional two decades were required before the functional meaning of mesangial cells was elucidated by Sakai and Kriz (1987). Mesangial cells are situated between glomerular capillary loops and are completely embedded in the mesangial matrix. They form a supporting framework that maintains the structural integrity of the glomerular tuft, including its capillaries. Moreover, the GBM and contractile mesangial cells together establish a biomechanical unit capable of developing wall tension in glomerular capillaries and changing the geometry of glomerular capillaries following mesangial contraction or relaxation. Mesangial cells possess many receptors for vasoactive agents, such as angiotensin II and endothelin. Therefore, mesangial cells significantly affect glomerular hemodynamics by altering glomerular vascular resistance (Ausiello et al. 1980; Savin 1986; Singhal et al. 1986).

Thy1.1 nephritis as a model for mesangial proliferative glomerulonephritis

In an attempt to elucidate the mechanisms responsible for morphological changes in glomerular cells, including podocytes, endothelial cells, and mesangial cells, under physiological and pathological conditions, we produced monoclonal antibodies that specifically recognize the different types of glomerular cells (Kurihara et al. 1998). The monoclonal antibody technique is a powerful tool for targeting a new molecule, the localization of which may be predicted. One of these antibodies, called E30, was found to specifically recognize mesangial cells in the rat glomerulus. An immunoblot analysis with E30 showed that a single band of 27 kDa was detectable in a glomerular lysate. By employing immunogold staining with E30, gold particles were detected on the cell surface of mesangial cells in normal rat glomeruli. An injection of E30 into rats induced massive proteinuria. An E30 antigen was strongly expressed in the brain, thymus, and renal glomerulus. Based on its distribution and molecular weight, we predicted that the antigen of E30 may be Thy-1. We then cloned rat brain Thy-1 and expressed it in COS cells. Thy-1 molecules expressed on the cell surface of COS cells were successfully recognized by E30. Cell morphology was changed after Thy-1 gene transfection. Furthermore, most of the transfected cells had long projections and large flattened cell bodies (Shinosaki et al. 2002a).

Thy-1 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein containing a single immunoglobulin-like domain. The mature form of Thy-1 does not cross the lipid bilayer, but is anchored to the extracellular leaflet of the plasma membrane by a covalent linkage to phosphatidylinositol. This molecule is involved in cell proliferation, apoptosis, T cell activation, neurite outgrowth, tumor suppression, fibrosis, and wound healing (Rege and Hagood 2006). In order to mediate these diverse effects, Thy-1 affects intracellular signaling cascades through src family protein kinases and modulates cellular adhesion and migration. Thy-1 is situated in non-caveolar lipid raft microdomains (Jacobson et al. 2007). Thy1-null mice are viable and have a neurologically normal appearance, but display excessive GABAergic inhibition in the dentate gyrus and regional inhibition of long-term potentiation (Nosten-Bertrand et al. 1996). Although the Thy1 molecule is a major constituent of the neural cell membrane in all species studied, its function in mesangial cells currently remains unknown. One of the two allotypes, Thy1.1 is expressed in rat tissues. The expression of the Thy1 molecule has also been reported in human mesangial cells, but not in mouse mesangial cells (Sengoelge et al. 2002). Therefore, Thy1 is a specific marker for rat and human mesangial cells.

Here, we describe the nephritis model induced by the E30 antibody. When E30 (100 microgram/rat) is injected into rats, antibodies bind to the cell surface of mesangial cells and induce complement-dependent mesangial cell injury (so-called mesangiolysis, Morita and Churg 1983) within 15 min (Fig. 1). Thy-1 molecules recognized by E30 are located on the cell processes, and cell–cell interactions between mesangial cells, suggesting that this molecule plays a role in cell–cell and cell–matrix interactions. Immunoblot data show that E30 antigen levels are reduced markedly 1 day after the E30 injection due to mesangial cell death. Light microscopy (LM) examinations have revealed mesangiolysis and vacuolization of the mesangial region within 1 day. Enlarged glomerular capillary loops are apparent on day 1, suggesting that the maintenance of the complex capillary network is due to the tensile force derived from mesangial cells. Many balloon-like glomeruli, which are formed by the fusion of glomerular capillaries, are observed on day 3, by which time the proliferation of mesangial cells is already observed in some glomeruli. A marked increase in mesangial cell numbers is observed on day 5 (Fig. 2). Proliferating mesangial cells, which are not stained by E30, show a round-shaped morphology, suggesting that the Thy-1 molecule is expressed on differentiated mesangial cells, but not on proliferating cells. The surface of proliferating mesangial cells is smooth and these cells have a well-developed rough endoplasmic reticulum (rER) and Golgi apparatus. On the other hand, on day 14 after the E30 injection, mesangial cells have many cell processes, while the rER is still developing (Fig. 3). Moreover, the findings of the double injection experiment demonstrate that the second injection on day 5 does not induce any effects, whereas additive effects are observed on day 8 or 14. Most glomeruli return to a normal state after 1 month, while accumulated collagen fibers remain in the mesangial matrix. The overall process of Thy1.1 nephritis induced by the novel monoclonal antibody, E30, is reproducible in repeated experiments as an animal model for human mesangial proliferative glomerulonephritis. Thus, the E30 nephritis model offers an opportunity to analyze the wound healing process after mesangial injury.

Glomerular capillary repair process after mesangial cell injury

Mesangial cell injury induced by an E30 injection is followed by severe damage to the glomerular capillary network, and a subsequent repair process including endothelial regeneration (Iruela-Arispe et al. 1995; Masuda et al. 2001; Wada et al. 2002; Kang et al. 2002). Two different types of angiogenesis have been demonstrated: (1) the sprouting of capillaries from preexisting vessels,

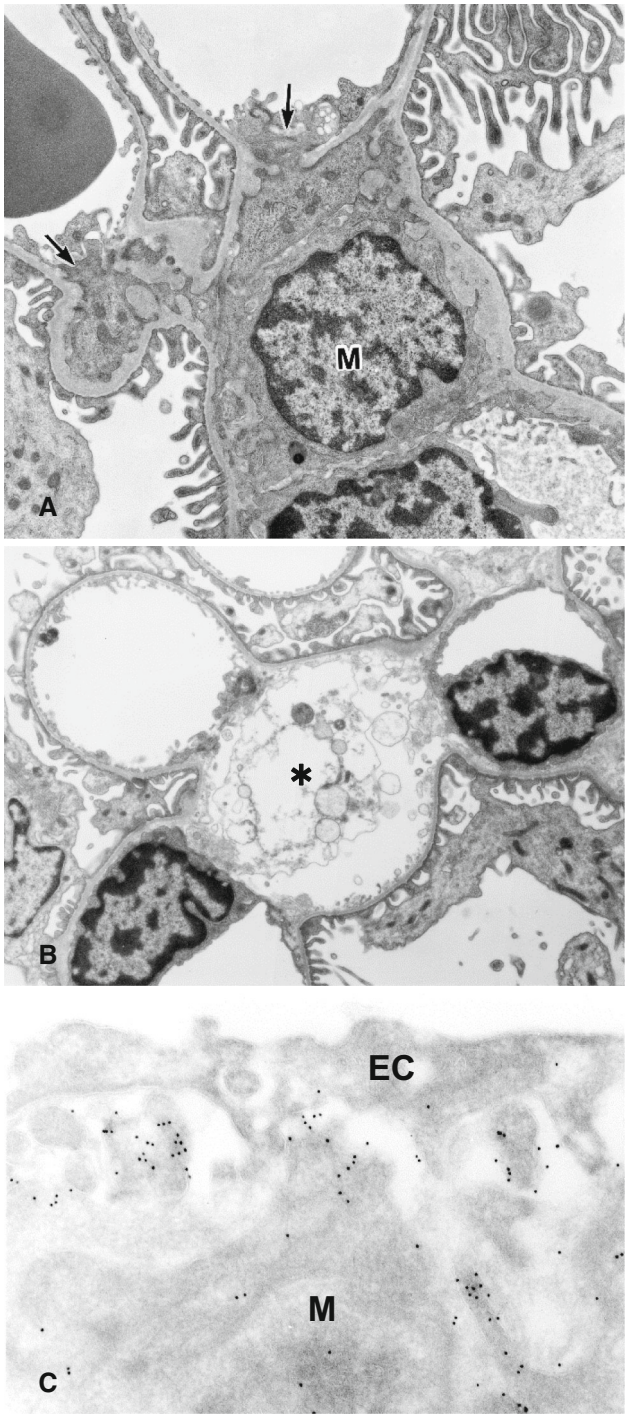

Fig. 1a–c Mesangial cell injury after an injection of E30. **a** A mesangial cell (M) is completely embedded in the mesangial matrix. The mesangial cell elongates cell processes toward endothelial cells and these eventually make contact with each other (*arrows*). After an E30 injection into rats, antibodies rapidly bind to the cell surface of mesangial cells. **b** Only mesangial cells are destroyed due to complement-dependent cell injury (*asterisk*). **c** Thy-1 molecules recognized by E30 are located on the cell surface of the mesangial cell (M), particularly cell processes and cell–cell interactions between mesangial cells. *EC* Endothelial cell

and (2) non-sprouting angiogenesis or intussusception (Risau 1997). Sprouting angiogenesis implies the proteolytic degradation of the basement membrane and proliferation and migration of endothelial cells, followed by tube formation with the final connection of the blind ending sprouts to another blood vessel. Intussusception causes the growth of capillaries through the insertion of tissue pillars, or columns of cell components and/or interstitial tissue inside the vessel lumen and its subsequent partitioning, a vessel splitting into multiple vessels (Burri and Tarek 1990; Djonov et al. 2000; Burri et al. 2004). Increasing evidence shows that this process participates in the various physiological and pathological conditions of neovascularization (Bartel and Lametschwandtner 2000; Patan et al. 1993, 2001).

In order to clarify the pattern of angiogenesis during the glomerular repair process, we analyzed the anti-Thy-1.1 nephritis model with some morphological techniques, including LM, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) with corrosion casting (Notoya et al. 2003). The corrosion casting technique is a useful method for observing three-dimensional (3D) vascular structures with SEM (Lametschwandtner et al. 1990). SEM observations of corrosion casts revealed marked changes in the glomerular capillaries of the glomeruli after the E30 injection. Capillary density was the lowest on day 5 after the E30 injection, and the complex glomerular capillary network was reconstructed by day 14, resulting in an almost normal architecture. A change in glomerular capillaries was also confirmed by the immunofluorescence staining of endothelial cells using our newly established monoclonal antibody against rat ICAM-2 (clone D12), which is expressed constitutively on the endothelial cell surface. On days 8 and 14 (the repair phase), when revascularization was extensively observed in the glomerulus, small holes with a diameter of approximately 1–2 μm and small capillary loops were apparent in glomerular casts. These structures have been detected in the vasculature of various tissues, including normal postnatal glomeruli (Patan et al. 1992) and are regarded as a hallmark to facilitate the identification of intussusceptive capillary growth. In addition to these small holes, we observed holes of various sizes on the vascular casts. With increases in diameter, the holes took on the appearance of small capillary loops. A recent study on a model of tissue repair showed that loop formation is involved in intussusceptive capillary growth (Patan et al. 2001). Capillaries undergoing splitting similar to our findings have also been detected in fetal and postnatal glomeruli (Osathanondh and Potter 1966; Zamboni and De Martino 1968). Thus, the repair process may be a recapitulation of the developmental process of glomerular capillaries. The inclusion of

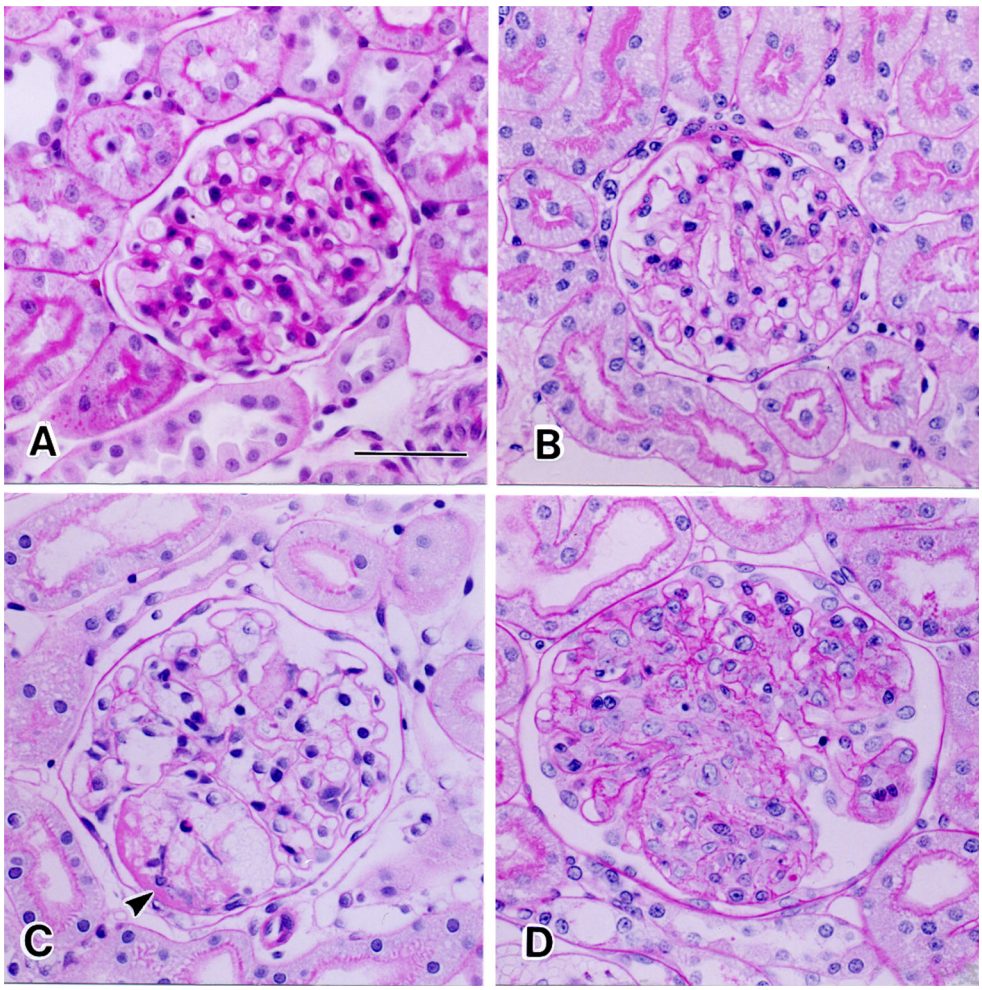

Fig. 2a–d Morphological change in the glomerulus after an injection of E30. **a** In the normal glomerulus, mesangial cells are located between the capillary loops and embedded in the mesangial matrix. **b** Complement-dependent mesangiolysis induced by an E30 injection results in an enlarged capillary loop on day 1. **c** The *balloon-like* glomerulus (*arrow*), which is formed by the fusion of glomerular capillaries, is observed on day 3. (**d**) The proliferation of mesangial cells peaks on day 5. PAS-Hematoxylin stain. *Bar* 100 μm

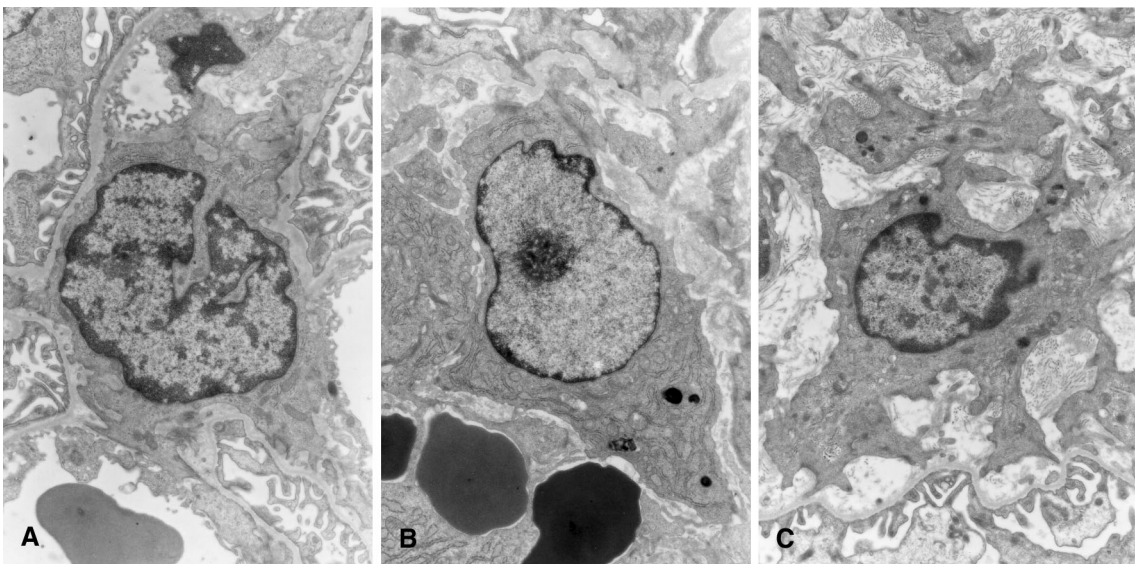

Fig. 3a–c Morphological changes in mesangial cells under nephritic conditions. **a**, **b** The proliferating mesangial cell on day 5 (**b**), the surface of which is smooth, has a more well-developed rough endoplasmic reticulum (rER) and Golgi apparatus than those of a normal cell (**a**). On the other hand, on day 14 after the E30 injection, the mesangial cell has many cell processes, although its rER is still developing. Note the accumulation of collagen fibers in the mesangial matrix (**c**)

mesangial cytoplasmic extensions in the tissue pillar suggests that mesangial cells are involved in the initial step of intussusception. During the course of the disease, angiopoietins (angiopoietin-1 and angiopoietin-2) play a role in the process of capillary repair after mesangiolysis. Our findings indicate that intussusceptive capillary growth contributes to revascularization following glomerular injury.

Intussusceptive angiogenesis has been examined in the developing lung, in which opposing endothelial cells in the capillary wall make contact and form a tissue pillar through the reorganization of cells, including membrane fusion. The tissue pillar core is then formed by the cytoplasmic extensions of myofibroblasts or pericytes (Burri and Tarek 1990; Bartel and Lametschwandtner 2000). In the case of glomeruli, a similar pathway has been reported through the repair process of injured glomeruli, except that mesangial cells are involved instead of myofibroblasts or pericytes. We speculate that capillary growth may proceed as follows. Endothelial cells reorganize and form a transcapillary pillar structure. Mesangial cell projections then invade through the core portion of the pillar, come into contact with the basement membrane, and become tethered to it. The contraction of the mesangial cell process making contact with the GBM results in the formation of a new capillary (Fig. 4).

Our findings on the recovery of the glomerular capillary loop indicate that the mesangial cell process plays a role in intussusceptive capillary growth as a tissue pillar. We considered the contractile force involved in the mesangial cell process in the tissue pillar to be important for new capillary formation through the final step of intussusceptive angiogenesis. Myofibroblasts expressing α-smooth muscle actin (αSMA) exert a contractile force to pull the surrounding extracellular matrices, whereas fibroblasts that express only non-muscle actin do not (Hinz et al. 2002). Furthermore, impaired vascular contractility has been detected in αSMA-null mice, in which α-skeletal muscle actin is ectopically expressed in vascular smooth muscle cells (Schildmeyer et al. 2000). Therefore, we focused on αSMA, which is expressed only in the mesangial cells of human and experimental glomerulonephritis, and is regarded as a marker for phenotypic changes in mesangial cells in diseased kidneys (Johnson et al. 1991). On day 5 after the E30 injection, prominent signals for αSMA were observed in the mesangial area, and the signal then gradually decreased from day 8 to day 30 (Fig. 5). On day 5, αSMA-positive mesangial cells were found throughout the expanded mesangial area, but mainly in the peripheral region of the expanded mesangial area, with no or faint signals in the central region on day 8. In order to investigate the detailed localization of αSMA, we performed immunoelectron microscopy with a specific antibody to αSMA. On day 5, immunoreactivity for αSMA was recognized along whole portions of the cell periphery, especially at cytoplasmic processes, in most mesangial cells. On day 8, the peripheral mesangial cells labeled with αSMA antibodies were situated close to the GBM or endothelial cells, and central mesangial cells (αSMA-negative) were separate from the GBM. The localization of αSMA within peripheral mesangial cells was restricted to the cytoplasmic processes radiating toward the GBM, to which they attached with their tips. In order to clarify the contribution of mesangial cell processes to tissue pillar formation, serial sections labeled with the anti-αSMA antibody were analyzed, and revealed that αSMA-containing cell processes penetrated the capillary canal via the tissue pillar involved in intussusceptive capillary growth at the recovery phase of Thy-1.1 nephritis. These findings suggest that some of the αSMA-containing mesangial cell processes contribute to the formation of a tissue pillar in the Thy-1.1 nephritic glomerulus (Ichimura et al. 2006).

Besides αSMA, drebrin, a 120-kDa actin-binding protein (Peitsch et al. 1999), is localized in the cytoplasmic processes of proliferating mesangial cells in Thy-1.1 nephritic glomeruli. The increase observed in the expression of drebrin in the injured glomeruli of E30-treated rats from day 3 to 14 suggests that mesangial cell processes are actively formed during the restorative stage of Thy-1.1 nephritis. Drebrin is also known to directly interact with profilin, another actin-binding protein, the expression of which is increased in Thy-1 nephritis in a similar manner (Mammoto et al. 1998; Tamura et al. 2000). Our findings indicate that drebrin is extensively colocalized with the actin isoforms expressed in mesangial cells. Drebrin remains in all cell processes after the disappearance of αSMA in mesangial cells.

Based on our findings, the αSMA-containing processes of mesangial cells are suitable for effectively pulling the GBM, and these processes contribute to re-folding of the GBM and the normalization of an expanded glomerular volume during the restorative remodeling of Thy-1.1 nephritic glomeruli. Moreover, drebrin is involved in the formation and maintenance of mesangial cell processes in glomerular remodeling.

As previously described, the cellular mechanisms underlying intussusceptive angiogenesis have been studied extensively in the developing lung. We also found that αSMA and drebrin were expressed temporarily in septal interstitial cells in the developing lung (Yamada et al. 2005), suggesting that they both contribute to the formation of cell processes, which is involved in the capillary network organization of endothelial cells in the lungs and kidneys.

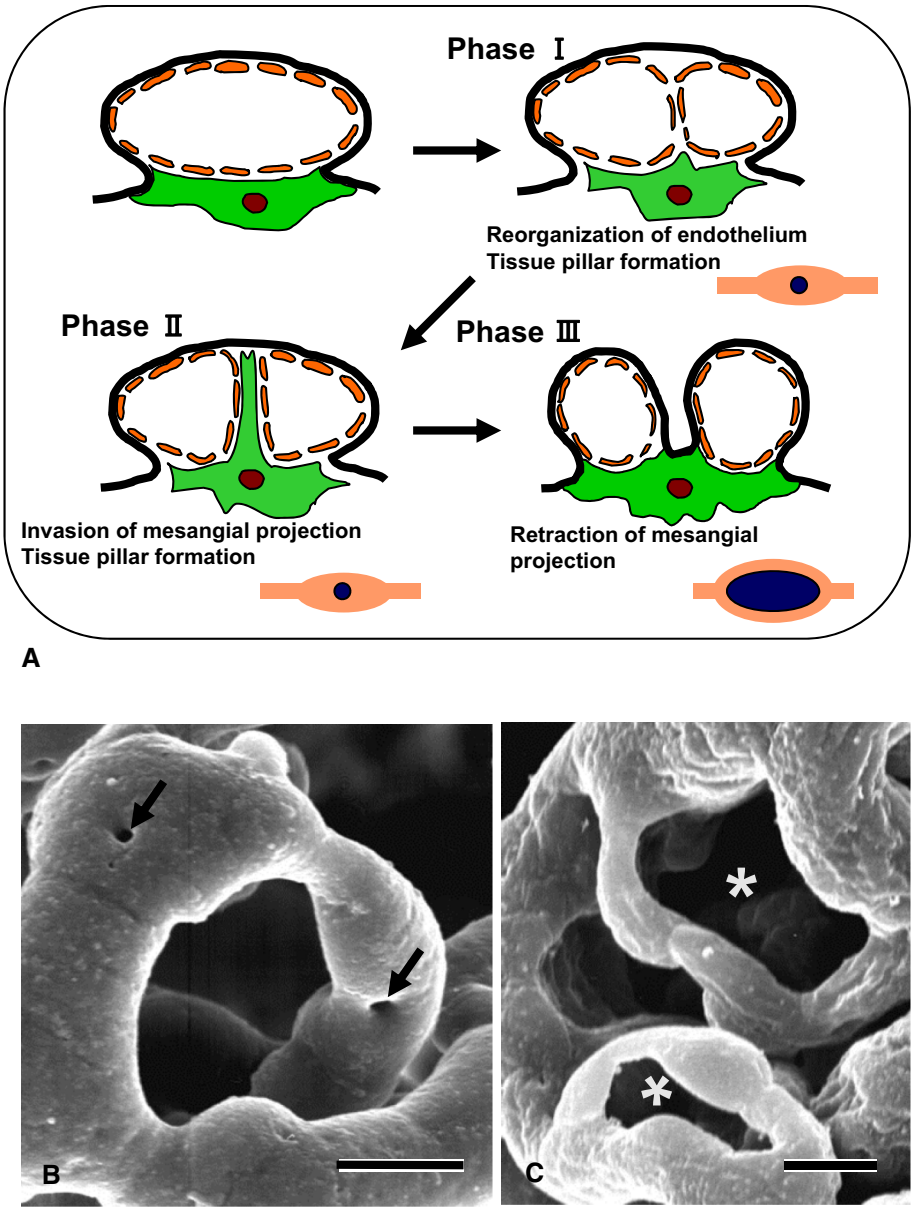

Fig. 4a–c Reorganization of the glomerular capillary loop after mesangial cell injury. Based on our studies, the reconstruction process of glomerular capillaries after mesangial cell injury is assumed to be as follows. **a** Opposing endothelial cells in the capillary wall make contact and form a tissue pillar through the reorganization of cells (*Phase I*). Mesangial cell projections invade through the core portion of the pillar, come into contact with the basement membrane, and become tethered to it (*Phase II*). The contraction of the mesangial cell extension contacting the glomerular basement membrane (GBM) results in the formation of a new capillary (*Phase III*). **b** *Small holes* with diameters of approximately 1 μm indicating the tissue pillar are often observed in the casts of glomerular capillaries on day 8. **c** The loop structures indicating new capillary formation (intussusceptive capillary growth) are observed on day 14. *Bars* 5 μm

Interaction between the GBM and mesangial cells

Mesangial cells and the GBM appear to be mechanically connected throughout the mesangial region. The glomerular capillary is incompletely surrounded by the GBM, which is lacking at the endothelial cell–mesangial cell interface, i.e., the mesangial angle. The GBM of opposing mesangial angles is interconnected by the single cell process of a mesangial cell (Fig. 6a, b). Consequently, the space in the juxtacapillary region of the mesangium is completely bridged. All these cell processes are filled with actin bundles that run in a tangential direction to the circular circumference of the capillaries. The distending forces of the glomerular capillary wall may be balanced at the mesangial angles and at the mesangium–GBM interface by a complex anchoring system that connects the GBM to mesangial cell processes (Sakai and Kriz 1987; Kriz et al. 1990; Lemley et al. 1992).

At this site, mesangial cells directly adhere to endothelial cells. We very recently found that afadin, a multi-domain F-actin binding protein, is located at the cell–cell contact between mesangial and endothelial cells (Tsurumi et al. 2016). Its localization and association with the actin cytoskeleton are regulated by platelet-derived growth factor (PDGF). Afadin also localizes with beta-catenin at the intercellular junction between neighboring mesangial cells, although N-cadherin is the intercellular junction molecule between two mesangial cells (Nameta et al. 2009). While

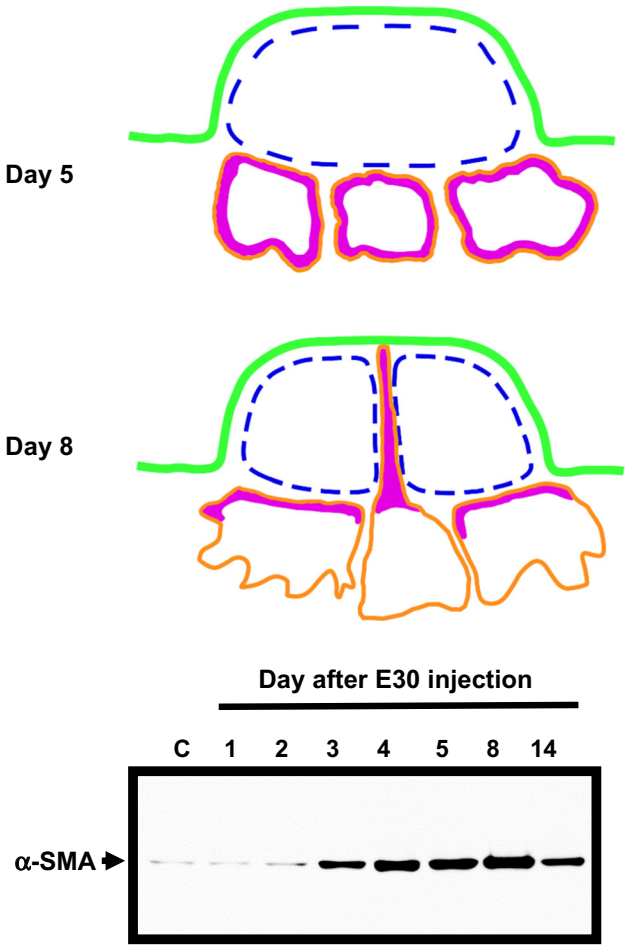

Fig. 5 Expression of alpha-smooth muscle actin in mesangial cells. Normal mesangial cells do not express alpha-smooth muscle actin (alpha-SMA). After mesangiolysis, proliferating mesangial cells express alpha-SMA as shown in an immunoblot analysis (*bottom*). On day 5 after the E30 injection, alpha-SMA is located beneath the mesangial plasma membrane in all mesangial cells (*top*). On the other hand, alpha-SMA-positive cells are restricted at the subendothelial region on day 8 (*middle*). In these cells, alpha-SMA is concentrated at the cell periphery facing the GBM. The cell process including alpha-SMA functions as a tissue pillar involved in intussusceptive capillary growth

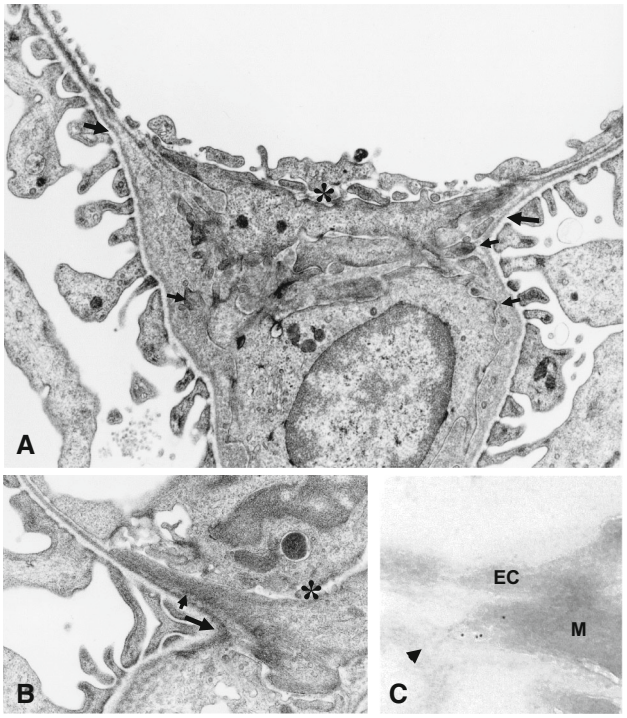

Fig. 6a–c Interaction between mesangial cell processes and GBM. In the juxtacapillary region (*asterisk*), mesangial cells cover the glomerular capillary, which lacks the GBM. At that site, mesangial cells make direct contact with endothelial cells (**a**). Long mesangial cell processes extend between opposite mesangial angles (*arrows*), where they are fixed to the GBM (**a**, **b**). Mesangial cell processes contain actin bundles (*short arrows*). Gold particles for EPLIN are concentrated at the tip of mesangial cell processes that make contact with the GBM (*arrowhead*) (**c**). *M* Mesangial cell, *EC* endothelial cell

afadin interacts with nectins at the adherens junction (Takai and Nakanishi 2003), we have not yet found any nectin molecule specifically expressed in mesangial cells. The expression of afadin at mesangial intercellular junctions was found to be markedly decreased in mesangial proliferative nephritis in rats and humans. The RNA interference-mediated depletion of afadin in cultured mesangial cells resulted in delayed directional cell migration. Furthermore, reorientation of the Golgi complex at the forward edge of wound healing was disturbed in afadin-depleted cells, suggesting aberrant migratory polarity in proliferative glomerulonephritis. These findings indicate that afadin, a cell adhesion molecule, plays a role in cell–cell contact and migratory polarity.

The kinds of molecules contributing to adhesion between the GBM and mesangial cell processes at the mesangial angle currently remain unclear. We recently demonstrated the strong expression of EPLIN (epithelial protein lost in neoplasm) in glomerular mesangial cells (Tsurumi et al. 2014). EPLIN (also known as Lima-1) was originally identified as a genetic product that is down-regulated or lost in some cancer cells (Maul and Chang 1999). Two isoforms of EPLIN, alpha and beta, are generated from a single gene and differ only at the 5′ ends (Chen et al. 2000a). Both isoforms contain a centrally located LIM (LIN11-ISL1-MEC3) domain that may allow homodimerization, as well as N- and C-terminal actin-binding sites that flank the LIM domain (Schmeichel and Beckerle 1997). EPLIN is located at the cortical cytoskeleton and serves to cross-link and bundle actin filaments in order to form stable filament structures such as stress fibers (Maul et al. 2003). EPLIN mediates the interaction between the cadherin-catenin complex and F-actin at the apical cell–cell junction of epithelial cells (Abe and Takeichi 2008; Taguchi et al. 2011). The focal

adhesion protein PINCH-1 was recently shown to associate with EPLIN at integrin adhesion sites (Karaköse et al. 2015). Therefore, EPLIN plays a role in the organization of the actin cytoskeleton at the cell–cell or cell–matrix interaction.

EPLIN transcript levels are particularly high in the kidney. EPLIN localizes in the long cell processes at the mesangial angle, at which they attach to the GBM (Fig. 6c). In cultured mesangial cells, EPLIN localizes in peripheral actin bundles at focal adhesions and forms a protein complex with paxillin. The MEK-ERK (extracellular signal-regulated kinase) cascade regulates the EPLIN-paxillin interaction and induces the translocation of EPLIN from focal adhesion sites to peripheral ruffles. Glomerular reconstruction after mesangial injury is controlled by several growth factors. PDGF and its receptor were shown to be up-regulated in a rat model of mesangial proliferative glomerulonephritis (Iida et al. 1991). PDGF-mediated mesangial cell migration is also an essential process in glomerular development (Betsholtz and Raines 1997; Lindahl et al. 1998). However, the mechanisms that regulate the morphology and migratory phenotype of mesangial cells have not yet been elucidated in detail. The deletion of EPLIN in mesangial cells enhances PDGF-induced focal adhesion disassembly and cell migration. Furthermore, the expression of EPLIN is decreased in mesangial proliferative nephritis in rodents and humans in vivo. Reductions in the expression of EPLIN in some cancer cells results in enhanced motility and transformation due to the instability of the actin filament organization and rapid filament turnover (Zhang et al. 2013). These findings indicate that the altered expression of EPLIN is involved in the phenotypic changes that occur in mesangial cells in glomerulonephritis.

The interaction between the cell and components of the extracellular matrix (ECM) is mediated by specific cell surface receptors, of which integrins are the most abundant. Integrins constitute a family of hetero dimeric transmembrane glycoproteins consisting of non-covalently associated alpha and beta subunits, both of which bind to ECM ligands and are linked to cytoskeletal elements in the cytoplasm (Hynes 2002). Beta1 series integrins often mediate cell–matrix interactions and provide a mechanical or physical linkage. The dominant alpha chains expressed by human and rat mesangial cells are alpha1, alpha3, alpha5, alpha8, and alphav (Gauer et al. 1997). These mesangial cell surface receptors have the ability to bind to and interact with a number of ligands. As receptors, they also transmit information from the ECM context into the cell and are involved in ECM-controlled signaling events that modulate multiple cellular functions. Besides integrins, the expression of CD44, a cell-surface receptor for hyaluronan, is increased in proliferating, but not normal mesangial cells (Nikolic-Paterson et al. 1996).

Tensin, an actin-binding protein, is associated with the ECM receptor, integrin (Lo et al. 1994; Chuang et al. 1995; Chen et al. 2000b). This protein contains a functional Src homology 2 (SH2) domain (Davis et al. 1991), which is able to interact with certain tyrosine phosphorylated proteins. It binds to the barbed ends of actin filaments and is also able to crosslink with these filaments. Takahara et al. (2004) found that tensin is selectively located in glomerular mesangial cells. Tensin is mostly localized in mesangial cell processes that have well-developed microfilament bundles and are in contact with the perimesangial GBM. The most prominent expression of tensin has been detected in juxtacapillary tongue-like processes that interconnect with the GBM at the two opposing mesangial angles of a single capillary.

The GBM plays a pivotal role in maintaining glomerular tuft and filtration functions. Therefore, alterations in GBM components result in abnormalities in the glomerular structure and renal dysfunction. We previously reported that a reduction in anionic charge sites on the GBM was associated with massive proteinuria in the chronic nephritic stage of anti-Thy1.1 glomerulonephritis (Shinosaki et al. 2002b). Another group demonstrated the importance of the GBM as a charge barrier. Laminin beta-2 is a component of the GBM. In laminin beta-2-deficient mice, proteinuria has been directly attributed to the altered composition of the GBM. In these mice, the disorganization of the GBM occurs because the distribution of the anionic charge sites is irregular and extends beyond the lamina densa. As a result, increased amounts of ferritin and albumin pass through the GBM (Jarad et al. 2006). These findings suggest that the remodeling of matrix components in the GBM is attributed to the progression of glomerular sieving defects.

In the developing kidney, temporal changes in laminin isoform expression occur as the formation of glomeruli proceeds. The earliest precursor of the GBM contains laminin-111 (alpha1beta1gamma1). In contrast, the GBM contains laminin-521 (alpha5beta2gamma1) at later developmental stages and in adulthood. Although laminin alpha1 is absent from the mature GBM, it is present in the glomerular mesangial matrix, an amorphous matrix made of mesangial cells that is one of the few prominent sites in which laminins are present outside of a definitive basement membrane (Miner et al. 1997). Mice lacking laminin alpha5 exhibit avascular glomeruli associated with the breakdown of the GBM during glomerulogenesis (Miner and Li 2000). This defect has been correlated with the failure of the developmental switch in laminin alpha chain deposition in which alpha5 replaces alpha1 in the GBM at the capillary loop stage. The G domain of laminin alpha5 is essential for adhesion between mesangial cells and the GBM and plays a key role in capillary loop formation during glomerular development (Kikkawa et al. 2003).

Mesangial cells and the mesangial matrix

Mesangial cells generate and embed in their own ECM. The mesangial matrix is composed of type IV and V collagen, laminin, fibronectin, heparan sulfate, and chondroitin sulfate proteoglycans. Furthermore, small amounts of the proteoglycans decorin and biglycan are found in the mesangial matrix. The composition and amount of the mesangial matrix are tightly controlled under physiological conditions, but may be markedly altered by disease (Couchman et al. 1994; Fogo 1999). In general, cytokines regulate the various matrix components emanating from mesangial cells similar to other tissues.

In addition to matrix production, the ECM degradation system also plays a key role in the development of irreversible renal disease (Schnaper 1995; Lenz et al. 2000). ECM degradation in the kidneys is regulated mainly by matrix metalloproteinases (MMPs) and a plasmin cascade (Baricos et al. 1995). MMPs are a large family of zinc-dependent peptidases that include interstitial collagenase, stromelysins, gelatinases, and membrane-type MMPs. Tissue inhibitor of metalloproteinases (TIMPs) is a major regulator of MMP activity (Visse and Nagase 2003). In general, reductions in enzymatic activities for MMPs result in ECM accumulation in kidney tissue. However, the enhanced enzymatic activity of MMP-2 causes GBM damage or a phenotypic change in mesangial cells (Turck et al. 1996).

Laminin alpha1 (LAMA1) is found transiently in the developing GBM, is absent from the mature GBM, but is present in the mesangial matrix in adult kidneys. We recently investigated how the absence of LAMA1 in the mesangial matrix affects the function of mesangial cells (Ning et al. 2014). Since *Lama1* gene disruption results in lethality at an early embryonic stage, we used a conditional knockout mouse model with the deletion of *Lama1* in the epiblast lineage ($Lama1^{CKO}$). GBM thickening, mesangial expansion, and increased mesangial cell proliferation were observed in 6-month-old $Lama1^{CKO}$ mice. As the mice aged, mesangial expansion increased due to glomerulosclerosis, which was accompanied by progressive proteinuria. These pathological conditions are similar to those observed in diabetic nephropathy; however, we did not observe significant differences in podocyte numbers or foot process effacement between $Lama1^{CKO}$ and control mice. Although proteinuria was detectable in $Lama1^{CKO}$ mice, it was at a lower level than that in most kidney diseases. Since there is no or little LAMA1 in the GBM, podocytes are not expected to have abnormalities. Proteinuria in the later stages may have been due to mesangial cell defects.

Our findings demonstrated that the deletion of LAMA1 results in age-dependent mesangial expansion, which develops into focal glomerulosclerosis, and led us to hypothesize that LAMA1 may be an endogenous negative regulator that specifically suppresses mesangial cell activation.

TGF-β molecules are multifunctional cytokines that regulate the production of the ECM. TGF-β is a key regulator of ECM turnover in many events; wound healing, immune functions, cell proliferation, and cell differentiation (Mozes et al. 1999). TGF-β is secreted as a latent form, which is stored in the ECM. It stimulates the expression of ECM proteins, including collagens, laminin, and fibronectin, while suppressing the synthesis of ECM protease inhibitors (Schnaper et al. 1996). TGF-beta is recognized as a causative factor of tissue fibrosis because TGF-beta 1 stimulates the production of the ECM in mesangial cells, tubular epithelial cells, and renal fibroblasts in the kidneys (Border and Noble 1997). Although we have not detected increases in TGF-β1 mRNA expression in $Lama1^{CKO}$ mesangial cells, the increase in Smad2 phosphorylation induced by TGF-β1 was evident. The Smad pathway is known to mediate the functions of TGF-β in renal fibrogenesis and subsequent accumulation of the ECM in the mesangial matrix (Schnaper et al. 2003). Inhibitors of TβRI kinase have been shown to block Smad2 phosphorylation in control and $Lama1^{CKO}$ mesangial cells. These findings indicate that LAMA1 suppresses mesangial cell activation by inhibiting the TGF-β/Smad pathway.

Laminins not only function as structural components, but also bind cell surface receptors, including integrins and α-dystroglycan (Ryan et al. 1996). We demonstrated that a treatment with LM-111 abrogated the increase in TGF-β1–induced Smad2 phosphorylation in cultured $Lama1^{CKO}$ mesangial cells. Furthermore, the restoration of LAMA1 expression in $Lama1^{CKO}$ mesangial cells by transfection with the LAMA1 expression vector inhibited increases in mesangial cell proliferation and TGF-β1-induced type IV collagen expression. Therefore, we speculate that LM-111 synthesized by mesangial cells inhibits TGF-β1 signaling. TGF-β1 signals through its type I and type II serine/threonine kinase receptors, and these pathways are tightly controlled by multiple positive and negative regulator proteins (Dai and Liu 2004). The negative regulation of TGF-β signaling is accomplished by the rapid attenuation or even inhibition of TβRI/II and/or Smad activities (Di Guglielmo et al. 2003; Feng and Derynck 2005). Integrin signaling is involved in the negative regulation of TGF-β signaling at the cell–matrix junction because adhesion reduces TGF-β-induced Smad2 phosphorylation. Laminins regulate cellular behavior through interactions with cell surface receptors, including integrins, syndecans, and α-dystroglycan (Miner and Yurchenco 2004). In addition, the Biacore analysis and solid-phase binding assays showed that the active form of TGF-β binds to LM-111. This finding suggests that LAMA1 plays a direct role in the

inhibition of TGF-β signaling by binding to active TGF-β1 in order to prevent it from engaging with its receptors or by interacting with integrin signaling, which is involved in the negative regulation of TGF-β signaling.

Taken together, our results suggest that LAMA1 plays a role in the negative regulation of TGF-β/Smad signaling. This may be important for maintaining mesangial cell populations and mesangial matrix deposition. Our findings demonstrate that the deletion of LAMA1 results in age-dependent mesangial expansion, which develops into focal glomerulosclerosis with age.

Cell signaling between glomerular cells

The renal glomerulus consists of glomerular endothelial cells, podocytes, and mesangial cells, which cooperate with each other for effective glomerular filtration. During glomerulogenesis, the fate of mesangial cells is closely linked with that of endothelial cells. The intercellular signaling system between mesangial cells and endothelial cells has been demonstrated in genetic knockout studies on PDGF-B and its receptor PDGFR-beta. The elimination of PDGF-B gene products in glomerular endothelial cells leads to the failure of mesangial cell maturation in the glomerulus and complicated capillary loop formation, resulting in a single balloon-like capillary loop (Levéen et al. 1994). Similar findings have been obtained with the deletion of the PDGFR-beta gene in mesangial cells (Soriano 1994).

Intercellular signaling between glomerular endothelial cells and podocytes has been established using podocyte-specific VEGF knockout mice (Eremina et al. 2003). The function of glomerular endothelial cells is severely damaged by the deletion of VEGF in podocytes.

We recently described the CD47-SIRP-alpha system for intercellular signaling between mesangial cells and podocytes in normal and diseased kidneys (Kurihara et al. 2010). SIRPα is a type I transmembrane glycoprotein that has three immunoglobulin-like domains in the extracellular region and two SH2-binding motifs in the cytoplasm. This molecule functions as a scaffold to many proteins, particularly SHP2-tyrosine phosphatase to the plasma membrane (Matozaki et al. 2009). The cytoplasmic domain of SIRPα was found to be strongly phosphorylated in normal glomeruli, and its phosphorylation was decreased markedly upon podocyte injury in vivo. The slit diaphragm components, nephrin and Neph1, assemble a signaling complex in a tyrosine phosphorylation-dependent manner, and regulate the unique actin cytoskeleton of podocytes (Harita et al. 2008). The possibility that SIRPα interacts with slit diaphragm proteins has also been investigated, and the findings obtained revealed that SIRPα is concentrated at the slit diaphragm region of podocytes. In the glomeruli of CNS patients carrying mutations in *NPHS1*, in which slit diaphragm formation is disrupted, the expression of SIRPα as well as Neph1 and nephrin was significantly decreased, indicating that SIRPα is closely associated with the nephrin complex. SIRPα formed hetero-oligomers with nephrin in cultured cells and glomeruli. Thus, SIRPα interacts with nephrin at the slit diaphragm, and its phosphorylation is dynamically regulated in proteinuric states (Kajiho et al. 2012). More recently, the role of the expression of SIRPα in podocytes was examined using knockin mice (C57BL/6 background) expressing mutant SIRPα, which lacks a cytoplasmic region and loses its SHP-2 binding ability (Takahashi et al. 2013). Electron microscopic examinations showed morphological changes in the podocytes (irregular major processes and flattened foot processes) of SIRPα-mutant mice. In addition, massive albuminuria with focal glomerulosclerosis was induced in adult SIRPα-mutant mice. These findings demonstrate that SIRPα is involved in regulating the podocyte structure and function at the slit diaphragm region via SHP-2 under physiological and pathological conditions.

CD47, a ligand for SIRPα (Brown and Frazier 2001), is also expressed in the glomerulus; however, its distribution is distinct from that of SIRPα. CD47 is located along the plasma membrane of mesangial cells, but not podocytes. The cytoplasmic domain of SIRPα is dephosphorylated when CD47 binds to the extracellular domain of SIRPα (Johansen and Brown 2007). In the normal glomerulus, mesangial cells cannot attach directly to podocytes due to the intervening thick GBM between the two cells. However, when mesangial cells are injured by an E30 injection, the phosphorylation of SIRPα is decreased markedly because of the interaction between SIRPα and CD47. The complex of SIRPα and CD47 is incorporated by podocytes after mesangiolysis. These findings indicate that the SIRPα-CD47 system plays a role in cell–cell communication in the diseased glomerulus. However, the mechanisms by which CD47 from injured mesangial cells gains access to podocytes currently remain unclear. Membrane shedding is one of the mechanisms involved in the interaction between two molecules because the ectodomain shedding of SIRP-alpha has been reported in cultured cells (Ohnishi et al. 2004). The shedding of microvesicles containing CD47 has also been described in stored red blood cells (Kriebardis et al. 2008). Our findings suggest that CD47 released from injured mesangial cells after mesangiolysis is endocytosed at the basolateral domain of podocytes because signals for CD47 have been detected at the endocytotic pits and in the cytoplasmic vacuoles of podocytes (Fig. 7). The interaction between CD47 and SIRP-alpha induces the dephosphorylation of the tyrosine residues in the cytoplasmic tails of SIRP-alpha as well as the release of SHP-2 from the slit diaphragm region (Motegi

et al. 2003). The separation of SHP-2 from SIRP-alpha is associated with increases in the tyrosine phosphorylation of slit diaphragm molecules, including nephrin, Neph-1, and ZO-1. The activation of Rho kinase is also induced by the dissociation between SIRP-alpha and SHP2. Reorganization of the actin cytoskeleton induced by the activation of

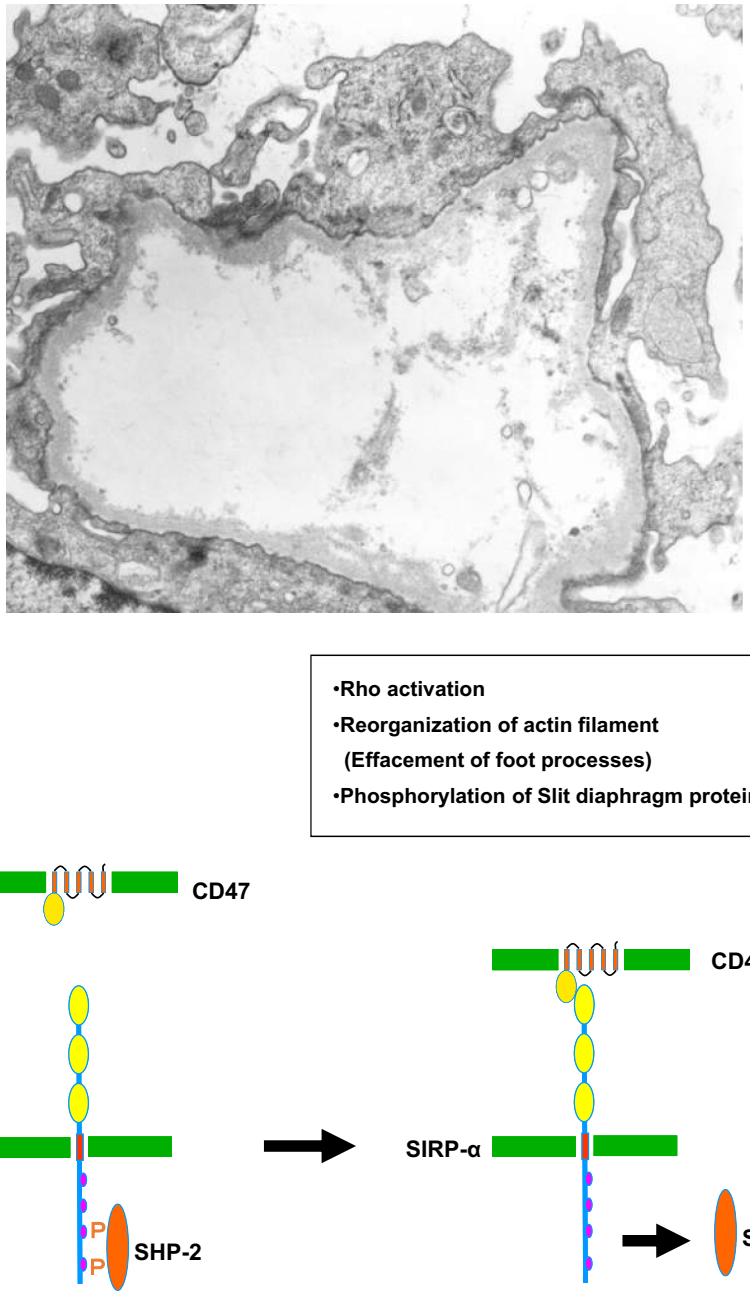

Fig. 7 The CD47-SIRP-slpha system in a diseased glomerulus. In a normal glomerulus, mesangial cells cannot attach directly to podocytes. However, when mesangial cells are injured by an injection of E30, the phosphorylation of SIRP-alpha is decreased markedly because of the interaction between SIRP-alpha and CD47. The complex of SIRP-alpha and CD47 is incorporated into podocytes after mesangiolysis. These findings suggest that the CD47-SIRP-alpha system plays a role in cell–cell communication in the diseased glomerulus. Morphological changes (foot process fusion and the effacement of podocytes) in podocytes are often observed after mesangiolysis induced by an injection of E30, as shown in the *upper panel*. A schematic model for the CD47-SIRP-alpha system in the normal (*left*) and Thy1.1 nephritic glomerulus (*right*) in the *lower panel*. SIRP-alpha is expressed in podocytes, whereas CD47, a ligand of SIRP-alpha, is expressed in mesangial cells. SHP-2 binds to the cytoplasmic region of SIRP-alpha. When mesangial cells are damaged, CD47 is released due to membrane shedding and binds to SIRP-alpha in podocytes. As a result, the dephosphorylation of SIRP-alpha occurs and SHP-2 is released. Subsequent events including the phosphorylation of slit membrane proteins and rearrangement of actin cytoskeletons in podocytes cause proteinuria

rho may be associated with dislocation of the slit diaphragm and the effacement of foot processes, which are constantly observed in the diseased kidney. These findings may help explain how the injection of anti-Thy1.1 antibodies, which cause mesangial cell injury, results in the development of proteinuria.

In conclusion, the CD47-SIRP-alpha system may play an important role in signal transduction between glomerular cells in the diseased kidney.

Conclusion

As described, the mesangial cell, which was previously referred to as the third cell of the renal glomerulus, is a central player in constructing and maintaining the architecture of the complex glomerular capillary network. The interaction between the GBM and mesangial cell process is achieved by specific molecules including EPLIN at opposing mesangial angles, the interspace of which is lacking at the GBM and bridged by the enlarged single cell process of the mesangial cell. The mesangial matrix produced by mesangial cells regulates the growth of their own cells, as shown for laminin alpha1. Mesangial cell injury induces uncontrolled cell growth and phenotypic changes to the cell. Mesangial cell injury is also transduced to podocytes by CD47 and affects glomerular filtration by inducing a change in the phosphorylation of slit diaphragm proteins through the CD47-SIRP-alpha system. Mesangial cells cooperate with glomerular endothelial cells and podocytes and control glomerular functions. In this context, a better understanding of the intercellular signaling system in the renal glomerulus is important for the development of new agents for chronic kidney disease, the number of patients with which has rapidly increased.

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