Type IV Collagen Induces Podocytic Features in Bone Marrow Stromal Stem Cells *In Vitro*

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Bone marrow–derived stromal stem cells (BMSC) can differentiate along a variety of mesenchymal lines, including mesangial cells. For determining whether BMSC can be induced to differentiate along podocytic lines *in vitro*, canine BMSC were cultured on plastic, type I collagen, and NC1 hexamers of type IV collagen from normal and Alport canine glomerular basement membrane. Results were compared with a mouse podocyte cell line. In the case of the podocyte line, differentiation occurred on all three matrices as indicated by the expression of synaptopodin and CD2-associated protein (CD2AP) and organization of myosin heavy chain IIA into a linear pattern. BMSC proliferated equally well on all matrices, but cells that were grown on type IV collagen NC1 hexamers became larger and stellate. Evidence for podocytic differentiation occurred on all three collagen matrices as indicated by the redistribution of myosin IIA to a linear pattern and expression of synaptopodin, CD2AP, and α-actinin. A punctate distribution of CD2AP was seen only in cells that were grown on normal and Alport glomerular basement membrane NC1 hexamers. Differentiated podocytes expressed the α1, α2, and α5 chains of type IV collagen but at higher levels in cells that were grown on NC1 hexamers. Similar results were obtained in BMSC for the α1 and α2 chains only. The α3, α4, and α6 chains were never detected in the podocyte line or BMSC. These results indicate that BMSC undergo a degree of podocytic differentiation *in vitro* and greater when grown on type IV collagen NC1 hexamers than type I collagen. Alport and normal NC1 hexamers seem equally permissive to BMSC growth and differentiation, suggesting that these processes are not influenced specifically by the α3/α4/α5 network. BMSC may be useful in the development of stem cell–based reconstitution of glomeruli that are damaged by disease and for gene therapy of genetic glomerular diseases such as Alport syndrome.

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Bone marrow–derived stromal stem cells (BMSC) can exhibit considerable phenotypic plasticity, including mesenchymal differentiation (osteocyte, adipocyte, and chondrocyte) and "unorthodox" differentiation (hepatocyte, muscle, and neural) (1–3). BMSC have been shown to take up residence in glomeruli *in vivo* and differentiate into mesangial cells and even podocytes (4–8). Such cells hold promise for reconstitution of diseased glomeruli and could conceivably be used to deliver a transgene to the glomerulus for gene therapy.

It is known that specific microenvironments can induce BMSC to differentiate along particular mesenchymal lines (2). The available data on glomeruli are largely based on experiments that were designed to reconstitute bone marrow with the detection of "stray" cells that repopulated glomeruli. Because these BMSC are physically distant from their bone marrow origin, it is reasonable to postulate that the microenvironment of the kidney promoted the differentiation of such cells into glomerular cells. The conditions that might favor glomerular cell differentiation from these multipotential cells are poorly understood. PDGF-β induces mesangial cell differentiation in cultured stromal stem cells that are grown on type IV collagen (9), but experimental data on differentiation of stromal stem cells into podocytes are lacking.

Many of the proteins that are expressed in mature podocytes are specific or nearly specific to that one cell type and offer an approach to assess podocytic differentiation in cell lines. Podocytes possess a contractile system composed of actin in relation to other proteins, such as myosin IIA and α-actinin-4 (reviewed in 10–13). Synaptopodin is associated with the actin network. The slit diaphragm, the chief size selective filter, is composed of nephrin in association with several other proteins, including CD2-associated protein (CD2AP) and podocin. The main structural protein of the glomerular basement membrane (GBM) is type IV collagen. Type IV collagen is assembled from a family of six distinct chains (reviewed in 14,15), designated α1 to α6. The six type IV collagen chains self-assemble into three basic protomers (each a triple helix) with the composition $\alpha1_2\alpha2$, $\alpha5_2\alpha6$, or $\alpha3\alpha4\alpha5$. These protomers then are organized into

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three distinct networks, $\alpha1/\alpha2$, $\alpha3/\alpha4/\alpha5$, and $\alpha1/\alpha2/\alpha5/\alpha6$, through dimerization at the carboxyl terminal, also referred to as the NC1 domain (15–18). Thus, NC1 domains form hexamers when two adjacent triple helical protomers join. In the GBM, the $\alpha1/\alpha2$ network appears first but is largely replaced by the $\alpha3/\alpha4/\alpha5$ network when capillary loops form. The $\alpha3/\alpha4/\alpha5$ network is believed to originate from podocytes (19), whereas the $\alpha1/\alpha2$ network is produced by all glomerular cells.

Tissue repair is believed to occur in part by multipotential cells that normally reside in organs. Such cells are believed to be replenished in part by BMSC (20). Engraftment of BMSC is enhanced in the setting of organ damage (21). Because mature podocytes are essentially nondividing cells, loss of podocytes through disease is largely irreversible. A diseased glomerulus then might act as a stimulus for repopulation by BMSC. As a step in understanding this process, we set out to determine whether type IV collagen that was extracted from normal and diseased glomeruli provided a microenvironment that influenced differentiation in BMSC.

Materials and Methods

Experimental Design

We compared the effect of different collagen matrices on a well-characterized mouse podocyte line (22) and on BMSC that were derived from normal canine bone marrow. Selected markers were used to assess podocyte differentiation, including actin (23), synaptopodin (24), myosin IIA (25), CD2AP (26), WT1 (22), and podocin (27,28). Extracellular matrix production was assessed in terms of the $\alpha1$ through $\alpha5$ chains of type IV collagen, the isoforms normally found in the GBM, and the $\alpha6$ chain, which in glomeruli is confined to Bowman's capsule. This cell line is usually grown on type I collagen. We also tested NC1 hexamers of type IV collagen derived from normal GBM and compared this with a diseased matrix, specifically NC1 hexamers of type IV collagen derived from Alport GBM. Alport syndrome is a hereditary disorder of type IV collagen in which the GBM usually contains only the $\alpha1$ and $\alpha2$ chains only and lacks the $\alpha3$, $\alpha4$, and $\alpha5$ chains (29–32). As a source of Alport GBM, we used kidneys from a canine model of X-linked Alport syndrome (33–36). In this model, there is total loss of the $\alpha3/\alpha4/\alpha5$ network from the GBM (37).

Collagen Matrices

Type I collagen was obtained from Sigma (St. Louis, MO). Type IV collagen was prepared by collagenase digestion of GBM isolated from the kidneys of normal and affected dogs (aged 2 to 3 mo) by graded sieving, as described previously (38). Collagenase digestion releases NC1 hexamers (15). Western blotting (results not shown) confirmed the expected α chain composition for the normal canine GBM NC1 hexamers ($\alpha1$ through $\alpha5$ chains) and Alport dog GBM NC1 hexamers ($\alpha1$ and $\alpha2$ chains only).

Podocyte Culture

A conditionally immortalized podocyte line (22), a gift from Dr. Peter Mundel (Mt. Sinai School of Medicine, New York, NY), was studied to provide a baseline for assessment of podocytic differentiation. Cells proliferate at 33°C when exposed to 10 U/ml IFN-γ and differentiate at 37°C without IFN-γ. Cells were maintained for 7 d in the undifferentiated state between passages and then were passed onto dishes that were coated with type I collagen (0.1 mg/ml) and normal canine or Alport canine GBM NC1 hexamers (0.02 mg/ml) and allowed to differentiate for 7 d.

Bone Marrow Culture

BMSC were cultured from 20-ml marrow samples that were obtained from normal dogs. Fresh cells were isolated from canine bone marrow aspirates using a density gradient (Lymphoprep, density of 1.077 g/ml; MJS Biolynx, Brockville, ON, Canada) and enriched for stromal stem cells by their adherence to plastic (1–3,20). Stromal stem cells were grown for 7 d on plastic between passages and then were seeded at a density of 6.5×10^4 cells/ml on plastic that was coated with type I collagen, NC1 hexamers from normal canine GBM, or NC1 hexamers from Alport canine GBM. Cells were maintained in α-minimal essential medium with 10% FCS and 1% penicillin/streptomycin at 5% CO_2 and 37°C for 7 d before further study.

Induction of Osteogenic Differentiation

Induction of osteogenic differentiation was carried out according to the published methods (2). Bone marrow cells were cultured for 2 wk in "osteogenic medium" that consisted of α-minimal essential medium that contained 10 mM β-glycerophosphate, 0.2 mM ascorbic acid, and 10^{-8} M dexamethasone. Osteogenic differentiation was assessed by alkaline phosphatase activity. After fixing in 2% paraformaldehyde, cells were washed in detection buffer (0.1 M Tris [pH 9.5], 0.1 M NaCl, 0.05 M $MgCl_2$, and 0.1% Tween 20) and exposed to 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) Liquid Substrate System (Sigma) for 30 min at room temperature.

Immunocytochemistry

For immunostaining, podocytes and BMSC that were grown on the various matrices were fixed in either 2% paraformaldehyde for 5 min (for detection of CD2AP) or cold acetone for 5 min (for detection of other antigens). Cells that were fixed in paraformaldehyde were not permeabilized before fixation. The primary antibodies were directed against CD9 (1:10 dilution; Monosan, Uden, The Netherlands), CD44 (1:50 dilution; Research Diagnostics Inc., Flanders, NJ), CD45 (1:10 dilution; Leukocyte Antigen Biology Laboratory, University of California Davis, Davis, CA), vimentin (1:100 dilution; DAKO, Carpinteria, CA); α-smooth muscle actin (1:20 dilution; Dako), synaptopodin (1:3 dilution; Research Diagnostics), α-actinin (1:100 dilution; Sigma), CD34 (1: 100 dilution; Novocastra, Newcastle upon Tyne, UK), desmin (1:60 dilution; Dako), factor VIII–related antigen (1:100 dilution; Dako), low molecular weight cytokeratin (1:100 dilution; Becton-Dickinson Biosciences, Mountain View, CA), the $\alpha1$ through $\alpha6$ chains of type IV collagen (1:25 dilution) (39), nonmuscle myosin IIA (1:60 dilution; Biomedical Technologies, Stoughton, MA), CD2AP (1:200 dilution), WT1 (1:25 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and podocin (1:1000 dilution). The antibodies to CD2AP and podocin were provided by Dr. Jeff Miner (Renal Division, Washington University, St. Louis, MO) (40) and Dr. Corinne Antignac (Hôpital Necker Enfants Malades, Paris, France) (28), respectively. All antibodies were monoclonal except factor VIII–related antigen, nonmuscle myosin IIA, CD2AP, WT1, and podocin.

For single labeling, all primary antibodies were applied for 60 min (90 min for the type IV collagen chains) followed by the appropriate biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) for 60 min, then fluorescein-streptavidin (1:50 dilution; Vector Laboratories) for 60 min. Normal mouse and dog kidney served as positive controls. For double labeling, myosin IIA was used for the first antibody and detected as above using a biotinylated goat anti-rabbit antibody followed by fluorescein-streptavidin. The second

antibody, either synaptopodin or smooth muscle actin, then was applied for 60 min, followed by rhodamine red X–conjugated AffiniPure goat anti-mouse IgG (1:30 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min.

Northern Blot Analysis

Analysis for type IV collagen chain expression was performed as described previously (41,42) on the mouse podocyte cell line for both undifferentiated and differentiated cells that were grown on type I collagen and type IV collagen matrices. The probes for the α1 through α6 mRNA were cDNA for the respective canine NC1 domains. Mouse kidney mRNA was used as a positive control. A probe for actin was used as a loading control.

Reverse Transcriptase–PCR

For assessment of expression of the six α chains of type IV collagen, reverse transcriptase–PCR (RT-PCR) was performed as described previously (41,42). Total mRNA was extracted from undifferentiated and differentiated canine stromal stem cells, grown on plastic and type I collagen and type IV collagen matrices. RT-PCR was also performed on podocytes for the α3, α4, and α6 chains of type IV collagen using the same method.

Results

Mouse Podocyte Cell Line

Morphologic Change of Cells *In Vitro* on Different Collagen Matrices. After 1 wk in culture, undifferentiated cells that were cultured on matrices of type I collagen and NC1 hexamers of type IV collagen from normal GBM and from Alport GBM all had a similar appearance (Figure 1). Cells were characterized by spindle-shaped nuclei and tight cell contacts. Differentiation was marked by slower cell growth, an increase in cell size, larger and rounder nuclei, and the appearance of cytoplasmic extensions. Differentiated cells all had a similar appearance, regardless of the collagen matrix used.

Expression of Podocyte Proteins. Undifferentiated cells did not express synaptopodin (Figure 1), actin (data not shown), or α-actinin (data not shown) on any collagen matrix, whereas myosin IIA (Figure 1) showed diffuse cytoplasmic staining. After differentiation of the podocyte cell line, all four proteins showed a linear pattern of cytoplasmic staining, regardless of the collagen matrix used. Double immunofluorescent staining of differentiated podocytes was carried out for myosin IIA paired with synaptopodin or actin. In each pair, there was fusion of red and green signals, indicating that all three proteins co-localized (within the limits of resolution of fluorescence microscopy; Figure 2), although the co-localization of synaptopodin with myosin IIA seemed less complete than between actin and myosin IIA (Table 1).

The staining results for CD2AP were influenced by the matrix used. Cells that were cultured on type I collagen expressed CD2AP only upon differentiation (Figure 1), whereas both undifferentiated and differentiated cells that were grown on normal and Alport dog GBM NC1 hexamers stained positively. Moreover, differentiated cells that were on type I collagen and undifferentiated cells that were grown on NC1 hexamers of type IV collagen showed diffuse cytoplasmic staining. In contrast, differentiated cells that were grown on NC1 hexamers of

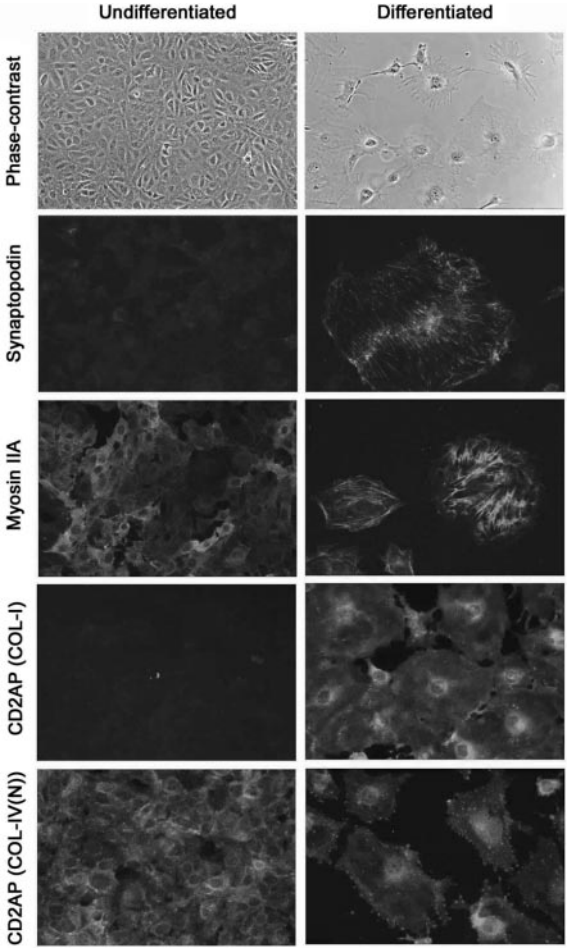

Figure 1. Undifferentiated and differentiated mouse podocyte cell line. The undifferentiated and differentiated cells were cultured for 1 wk on type I collagen (COL-I), NC1 hexamers of type IV collagen from normal glomerular basement membrane [GBM; COL-IV(N)], and Alport GBM [COL-IV(A)] matrices (cells that were grown on type IV collagen from normal GBM are illustrated except for the fourth row, in which cells were grown on type I collagen). (First row) Undifferentiated cells had a similar appearance regardless of the matrix used. Differentiated cells showed an increase in cell size, larger and rounder nuclei, and cytoplasmic extensions but seemed to be the same on all three matrices. (Second row) Immunofluorescent detection of synaptopodin by the podocyte cell line. Undifferentiated cells did not express synaptopodin. Differentiated cells showed linear cytoplasmic staining, as would be seen with localization to the cell cytoskeleton. Similar results were obtained for actin and α-actinin (data not shown). (Third row) Immunofluorescent detection of myosin IIA by the podocyte cell line. Undifferentiated cells on all three matrices showed diffuse cytoplasmic staining. In contrast, in differentiated cells, the distribution of myosin IIA was organized into a linear pattern. (Fourth and fifth rows) Immunofluorescent detection of CD2-associated protein (CD2AP) by the podocyte cell line. Cells that were cultured on type I collagen expressed CD2AP only upon differentiation. Differentiated cells that were grown on type I collagen and undifferentiated cells that were grown on NC1 hexamers of type IV collagen (either normal or Alport GBM) showed diffuse cytoplasmic staining. In contrast, the differentiated cells that were grown on NC1 hexamers of type IV collagen showed bright, bead-like staining with localization of CD2AP toward the cell periphery. Magnification, $\times$200.

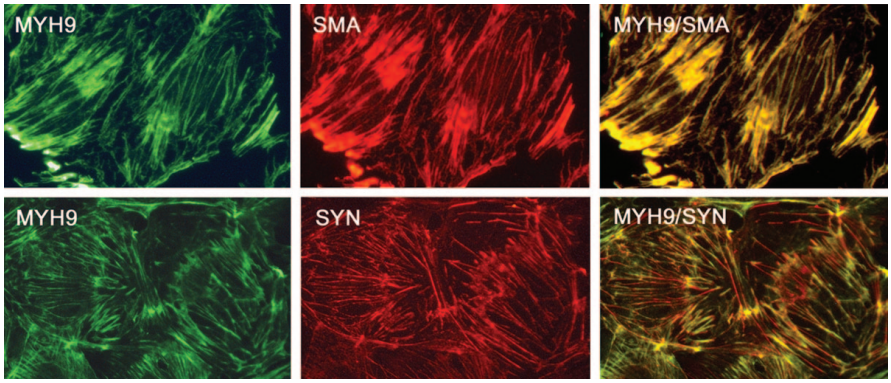

Figure 2. Double immunofluorescent detection of the expression of myosin IIA (MYH9), actin (SMA), and synaptopodin (SYN) by the differentiated podocyte cell line. The merged yellow signal shows co-localization of myosin IIA with actin (top row) and myosin IIA with synaptopodin (bottom row).

Table 1. Comparison of protein expression in murine podocyte cell line and canine BMSC under various culture conditions[a]

Protein	Murine Podocyte Cell Line				Canine BMSC			
	Undiff'd	Diff'd COL-I	Diff'd COL-IV(N)	Diff'd COL-IV(A)	Plastic	COL-I	COL-IV(N)	COL-IV(A)
Synaptopodin	Neg	+L	+L	+L	Neg	+L	+L	+L
Actin	Neg	+L	+L	+L	+C	+L	+L	+L
α-Actinin	Neg	+L	+L	+L	Wk L	+L	+L	+L
Myosin IIA	+C	+L	+L	+L	Wk C	+L	+L	+L
CD2AP	+/−	+C	+P	+P	Wk C	Wk C	+P	+P
WT1	+N	+N	+N	+N	+N	+N	+N	+N
Podocin	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
α1, α2 chain	+C	+C	ND	ND	Wk C	+C	ND	ND
α3, α4 chain	Neg	Neg	ND	Neg	Neg	Neg	ND	Neg
α5 chain	+/−	+	ND	+	Neg	Neg	ND	Neg
α6 chain	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

[a]BMSC, bone marrow stromal stem cells; Undiff'd, undifferentiated podocyte cell line; Diff'd differentiated podocyte cell line; COL-I matrix of type I collagen; COL-IV(N), matrix of NCI hexamers of type IV collagen from normal dog glomerular basement membrane (GBM); COL-IV(A), matrix of NCI hexamers of type IV collagen from Alport dog GBM; +/−, in the undifferentiated podocyte cell line, positive staining in cells that were grown on NCI hexamers but negative staining in cells that were grown on type I collagen; wk, weakly positive; C, diffuse cytoplasmic pattern; L, linear pattern; P, punctate pattern; N, nuclear; ND, not determined.

type IV collagen showed a bright, bead-like staining with localization of CD2AP toward the cell periphery. Both normal and Alport GBM gave the same results. Positive nuclear staining for WT1 was noted in both undifferentiated and differentiated cells on all matrices (results not shown). No cells on any matrix expressed podocin; normal mouse kidney served as a positive control (results not shown).

Collagen Chain Expression. Collagen chain expression was assessed at the protein level in terms of the individual α chains. Both undifferentiated and differentiated cells that were grown on type I collagen expressed the α1 and α2 chains of type IV collagen. Staining could not be assessed on the NC1 hexamer matrices because these already contained the α1 and α2 chains. Expression of both chains was cytoplasmic in a delicate punctate pattern (Figure 3). Undifferentiated cells that were cultured on type I collagen did not express α5 chain. In contrast, diffuse cytoplasmic staining was seen for undifferentiated cells that were grown on NC1 hexamers of type IV collagen from Alport dog GBM, differentiated cells that were grown on type I collagen, and differentiated cells that were grown NC1 hexamers of type IV collagen from Alport dog GBM (Figure 3). The α3, α4, and α6 chains were not detected in undifferentiated or differentiated cells that were grown on type I collagen or Alport dog NC1 hexamers (results not shown). Cells that were grown on normal dog GBM NC1 hexamers could not be evaluated for expression of the α3, α4, and α5 chains, because the matrix contained these chains.

By Northern analysis, signals for the α1, α2, and α5 chains were detected in undifferentiated and differentiated cells that were grown on type I collagen and in NC1 hexamers of type IV

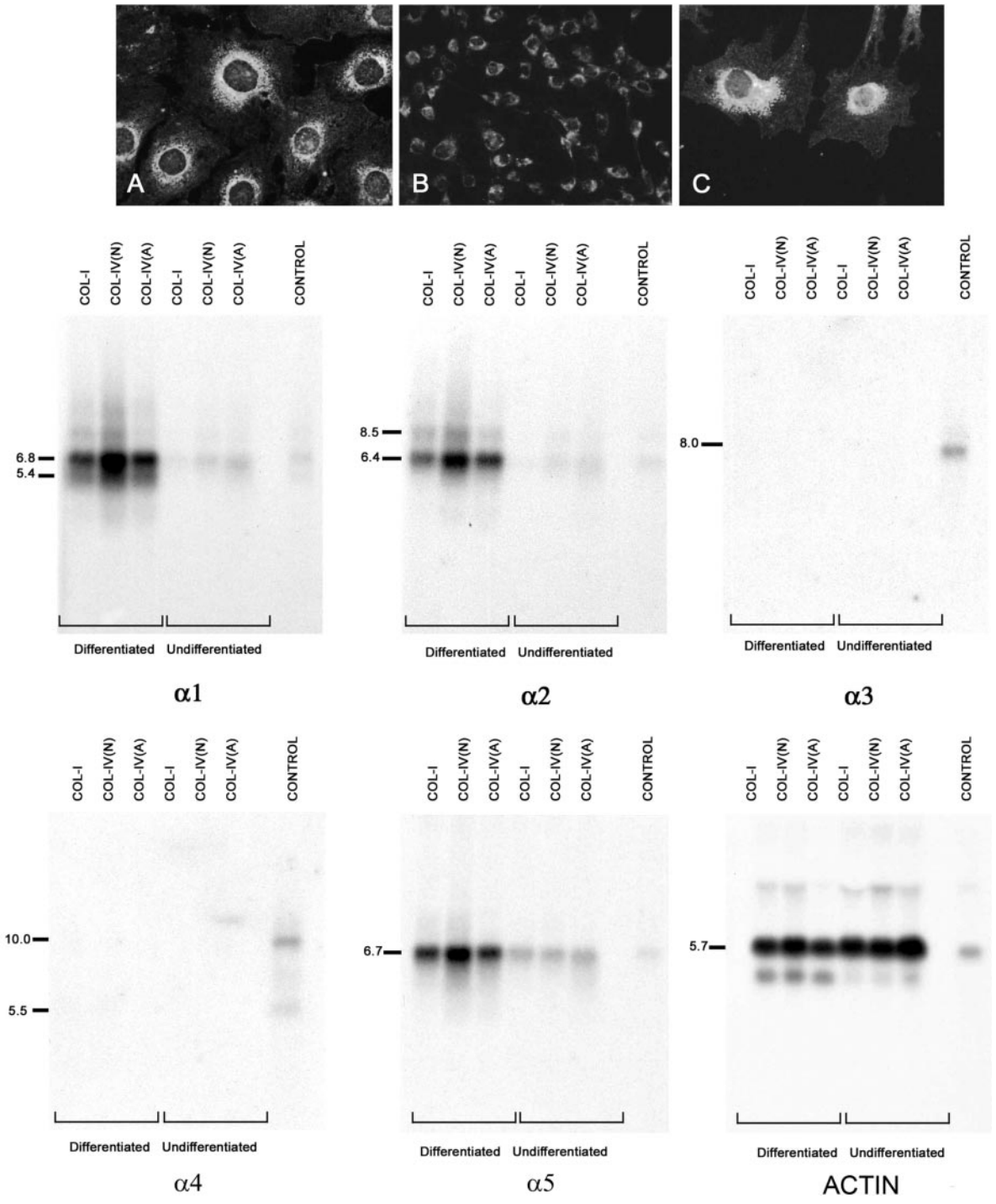

Figure 3. Type IV collagen expression in the podocyte cell line. (Top row) Immunofluorescent detection of the α1 chain of type IV collagen by the differentiated podocyte cell line grown on type I collagen (A) and the α5 chain of type IV collagen by the podocyte cell line, undifferentiated (B) and differentiated (C), when grown on NC1 hexamers of type IV collagen from Alport GBM. Both undifferentiated and differentiated cells showed positive cytoplasmic staining. Cells that were cultured on type I collagen expressed the α5 chain only after differentiation (data not shown). Staining for the α2 chain was identical to the α1 chain (results not shown). The α3, α4, and α6 chains were not detected in undifferentiated or differentiated cells regardless of the matrix used (results not shown). (Middle and bottom rows) Northern blot analysis of α1 through α5 mRNA expression in the undifferentiated and differentiated podocyte cell line. Signals for the α1, α2, and α5 chains were detected in undifferentiated and differentiated cells that were grown on type I collagen (COL-I) and NC1 hexamers of type IV collagen from normal dog GBM [COL-IV(N)] and Alport dog GBM [COL-IV(A)]. Signals were consistently stronger in differentiated cells compared with undifferentiated cells. Expression in differentiated cells that were grown on NC1 hexamers of type IV collagen was higher compared with type I collagen, although there was no obvious difference between NC1 hexamers that were extracted from normal and Alport dog GBM. No signals were detected for the α3 and α4 chains for cells on any of the matrices or for the α6 chain (data not shown). Mouse kidney mRNA was used as a positive control. Probes for actin were used as controls for loading. Magnification, ×200 in A through C.

collagen that were extracted from normal and Alport dog GBM NC1 (Figure 3). Signals were consistently stronger in differentiated cells compared with undifferentiated cells. Differentiated cells that were grown on NCI hexamers of type IV collagen showed higher expression compared with cells that were grown on type I collagen, but there was no appreciable difference between using normal and Alport dog GBM as a type IV collagen source. No signals were detected for the α3, α4, or α6 chain for cells on any of the matrices, either by Northern analysis or by RT-PCR (results not shown).

Canine Stromal Stem Cells

Morphologic Appearance of Cells *In Vitro*. Fewer than 1% of bone marrow cells adhered to plastic. These cells were CD9+, CD44+, CD45− (Figure 4), in keeping with stromal stem cells (43), and were used for further expansion. These cells were positive for vimentin and showed osteogenic differentiation (Figure 4), confirming that stromal stem cells had been subcultured. Cells that were grown on collagen matrices showed only slightly slower rates of growth than cells that were seeded on plastic. Cells showed no apparent difference in growth rate or survival when grown on a type I collagen matrix *versus* NC1 hexamers of type IV collagen from normal or Alport GBM. After 7 d, the majority of cells that were grown on plastic and type I collagen maintained a spindle shape, whereas cells that were grown on NC1 hexamers of type IV collagen from normal and Alport canine GBM became larger and more stellate (Figure 4). There was a degree of overlap between the different matrices, in that approximately 1% of BMSC that were grown on type I collagen also became more stellate, whereas approximately 5% of BMSC that were grown on type IV collagen remained spindle shaped. No difference in appearance was noted using matrix from normal *versus* Alport GBM.

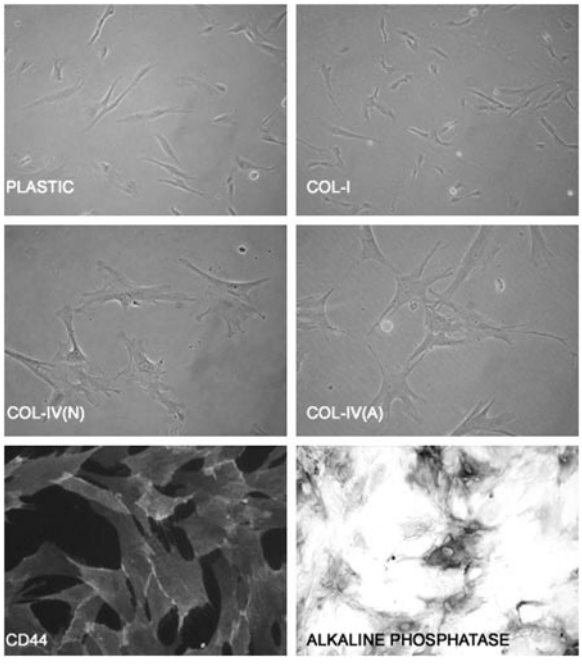

Figure 4. Morphology of bone marrow–derived stromal stem cells (BMSC) in culture. The cells were cultured on plastic, type I collagen (COL-I), and NC1 hexamers of type IV collagen from normal GBM [COL-IV(N)] and Alport GBM [COL-IV(A)] matrices. Cells that were grown on plastic and type I collagen showed a spindle-shaped morphology. In contrast, cells that were grown on NC1 hexamers of type IV collagen from normal and Alport canine GBM were larger and more stellate. No difference in appearance was noted between matrix from normal *versus* Alport GBM. Cultured cells expressed CD44 and under specific culture conditions expressed alkaline phosphatase, demonstrating osteogenic differentiation.

Expression of Podocyte Proteins. By immunohistochemistry, the cultured cells were negative for factor VIII–related antigen, CD34, desmin, and low molecular weight keratin, indicating that the cells were not differentiating along endothelial, mesangial, or epithelial lines (Table 1). Cells that were grown on plastic showed diffuse and disorganized cytoplasmic staining for myosin IIA and no positive staining for synaptopodin (Figure 5). Weak staining for actin (data not shown) and α-actinin (Figure 6) was apparent in a linear pattern. The larger stellate cells that were grown on all three collagen matrices expressed synaptopodin (Figure 5) and myosin IIA (Figure 5), actin (data not shown) and α-actinin (Figure 6). Staining had a linear cytoplasmic distribution, resembling that seen in differentiated podocytes. No difference was apparent among the three collagen matrices with respect to expression of synaptopodin, myosin IIA, actin, and α-actinin within individual cells. However, as mentioned above, very few BMSC that were grown on type I collagen became stellate and expressed these proteins, in contrast to the majority of BMSC that were grown on type IV collagen. Synaptopodin and myosin IIA co-localized in the cytoplasm in double-labeling experiments (Figure 5), as seen with the podocyte cell line. In similar experiments, actin co-localized with myosin IIA (results not shown).

CD2AP was weakly expressed in cells that were grown on plastic, in a diffuse cytoplasmic distribution (Figure 6). For cells that were grown on type I collagen, there was stronger expression but still in a diffuse cytoplasmic pattern. In contrast, in cells that were grown on NCI hexamers of type IV collagen from normal and Alport canine GBM, there was a re-distribution of CD2AP staining to a bright-bead–like pattern. However, this staining tended to remain distributed throughout the cytoplasm, rather than localize to the periphery as seen in the differentiated podocyte cell line. There was positive nuclear staining for WT1 in BMSC regardless of the matrix used (results not shown). No positive staining was seen for podocin in any cells that were grown either on plastic or on any of the collagen matrices; normal dog kidney was used as a positive control (results not shown).

Collagen Chain Expression. By immunohistochemistry, stromal stem cells expressed the α1 and α2 chains in a diffuse cytoplasmic punctate pattern when grown on plastic or type I collagen (Figure 7). The type IV collagen–containing matrices could not be assessed using this technique. None of the stromal stem cells showed positive staining for any of the α3, α4, α5, or α6 chain of type IV collagen, regardless of the matrix used (results not shown). By RT-PCR, signals were obtained for the α1 and α2 chains for all culture conditions, but the signals for

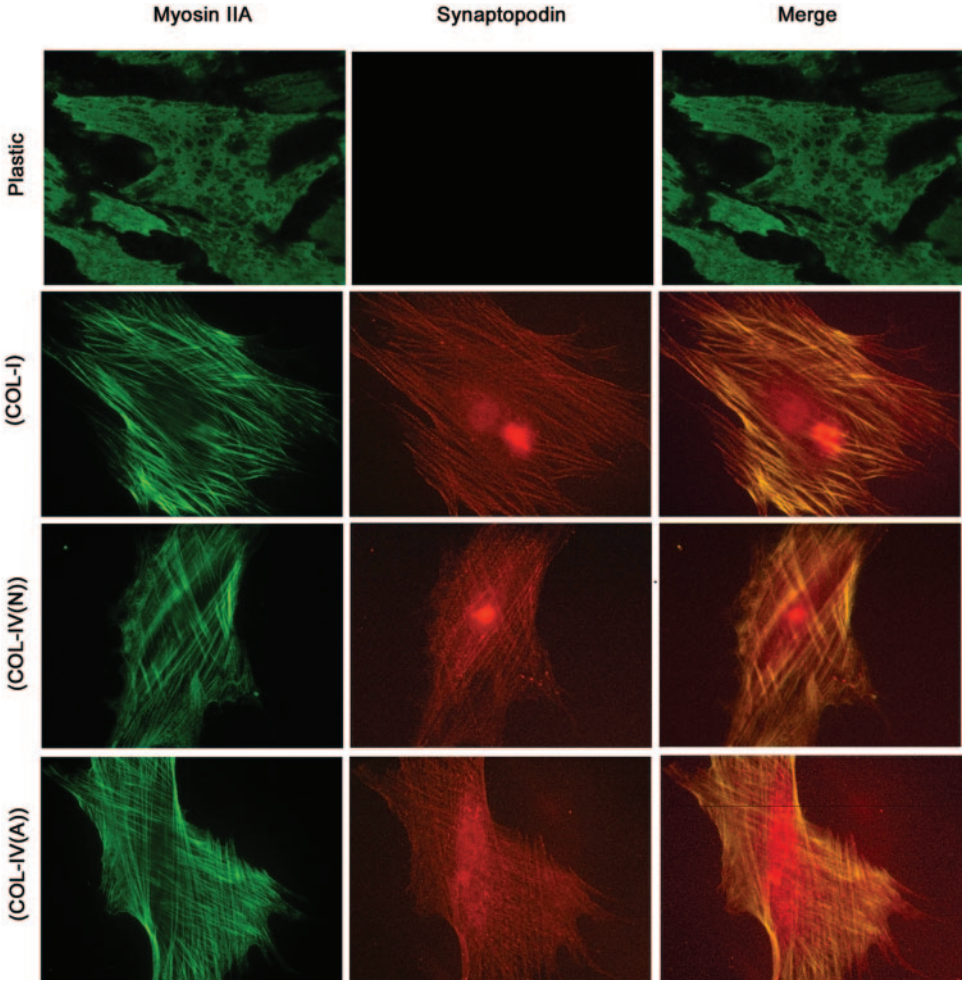

Figure 5. Immunofluorescent localization of myosin IIA and synaptopodin in BMSC after 1 wk in culture on different matrices. Cells that were grown on plastic showed diffuse and disorganized staining for myosin IIA and no positive staining for synaptopodin. Cells that were grown on a type I collagen showed a linear rearrangement of myosin IIA and expression of synaptopodin. Cells that were grown on NC1 hexamers of type IV collagen from normal GBM [COL-IV(N)] and Alport GBM [COL-IV(A)] showed similar linear cytoplasmic staining for both myosin IIA and synaptopodin. The merged yellow signal shows co-localization of myosin II and synaptopodin in cells that were grown on all three collagen matrices. Magnification, ×200.

cells that were grown on plastic were weakest, and expression was highest in cells that were grown on NC1 hexamers of type IV collagen. Similar expression was noted in hexamers that were derived from normal and Alport dog GBM. No signals were obtained for any of the α3 through α6 chains, regardless of which matrix on which cells were grown (Figure 7).

Discussion

BMSC offer a potential for replacing nondividing cells such as podocytes in situations of organ damage (20,21). Very little is known about the potential of such cells in glomeruli, and our goal was to examine differentiation of such cells *in vitro* in response to different matrices, including ones that were derived from normal and diseased glomeruli. We chose Alport syndrome because the composition of the GBM in this disease has been studied extensively, and the availability of a canine model ensured a supply of matrix for the *in vitro* studies. Canine BMSC were easily separated out from marrow samples and grew readily in culture, as has been noted by others (1,20,43,44). The immunophenotype of CD9+CD44+CD45− indicated that BMSC were recovered (43), and this was confirmed by the ability of these cells to undergo osteogenic differentiation under controlled *in vitro* conditions (2).

A mouse podocyte cell line, grown on different matrices, was studied to provide a basis of comparison with BMSC. Although this line typically is grown on type I collagen, the cells grew equally well on a type IV collagen matrix, derived either from normal or Alport GBM. As previously noted, WT1 was expressed in all cells regardless of the degree of differentiation (22). For certain podocyte proteins (synaptopodin, actin, and α-actinin), only the differentiated cell line showed expression, as has been reported previously (22), whereas for myosin IIA, there was a change in distribution as the cell line differentiated. All four proteins showed a linear pattern of cytoplasmic staining, regardless of the collagen matrix used. Myosin IIA co-localized with synaptopodin and actin at the light microscopic

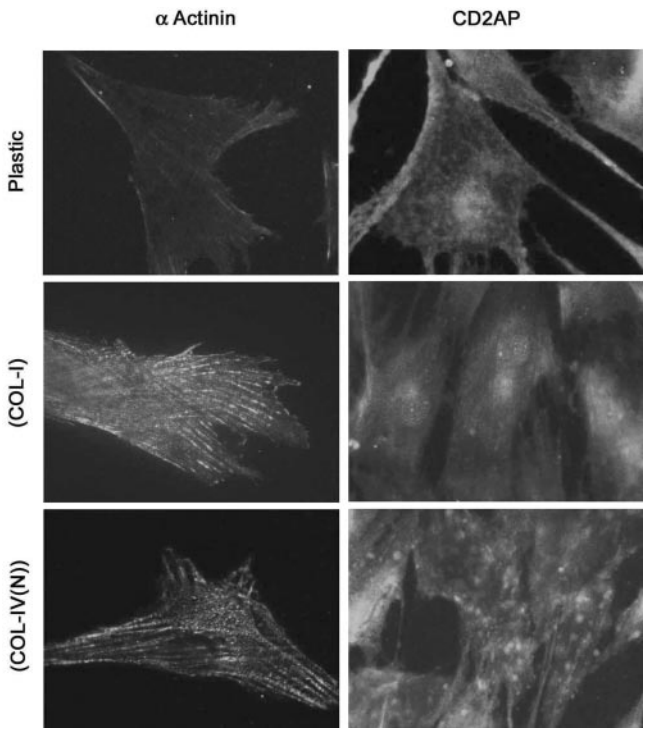

Figure 6. Immunofluorescent localization of α-actinin and CD2AP in BMSC after 1 wk in culture on different matrices. Cells that were grown on plastic showed weak expression of α-actinin and diffuse cytoplasmic staining for CD2AP. Cells that were grown on collagen I upregulated expression of α-actinin but still showed diffuse staining for CD2AP. Cells that were grown on NC1 hexamers of type IV collagen from normal GBM and Alport GBM (data not shown) both showed an upregulation of α-actinin expression compared with plastic-grown cells and a re-distribution of CD2AP staining to a bright-bead–like pattern, but, in contrast to the podocyte cell line, the distribution of CD2AP was not preferentially peripheral. Magnification, ×200.

level. The results suggest that myosin IIA may require one or both of these other proteins to take on a linear distribution, and without this influence, myosin IIA would remain diffusely distributed in the cytoplasm. Synaptopodin, actin, and α-actinin have been shown previously to associate in podocytes (reviewed in 11,12). Whereas myosin IIA is known to be expressed in mature podocytes *in vivo* (25), this is the first demonstration that this protein co-localizes with synaptopodin and actin and likely has a functional relationship with them as part of a contractile apparatus in podocytes.

The only protein that showed a different expression on type I collagen *versus* type IV collagen was CD2AP. The cell line when cultured on type I collagen expressed CD2AP only upon differentiation, whereas when cultured on type IV collagen (from normal or Alport GBM), expression was seen even in the undifferentiated cell line. An additional difference was noted with respect to cellular localization; only differentiated cells that were grown on type IV collagen developed a punctate pattern of expression along the cell periphery. Thus, there is a differential response of the podocyte cell line in relation to the specific matrix to which it is exposed, one that influences the degree of differentiation of these cells. CD2AP is associated with the slit diaphragm complex. Hence, it is reasonable that this protein might show a peripheral localization *in vitro*. The only other slit diaphragm protein tested in this study was podocin, and it was not expressed under any conditions, indicating that differentiation of this murine podocyte cell line is incomplete *in vitro*.

We also analyzed the podocyte cell line for production of type IV collagen. In the GBM, the α1/α2 network appears first but is largely replaced by the α3/α4/α5 network when capillary loops form (37,45,46). The α3/α4/α5 network has been shown to originate from podocytes (19), whereas the α1/α2 network is produced by all glomerular cells. The α1/α2/α5/α6 network in glomeruli is limited to Bowman's capsule, and podocytes would not be expected to produce this network. In our study, the podocyte cell line produced the α1, α2, and α5 chains of type IV collagen. That the α3, α4, and α6 chains were never detected in the cell line is consistent with the cytoplasmic localization of the α5 chain in cultured cells, because this chain needs to form a trimer with the α3 and α4 chains or the α6 chain before it can be exported from the cell into the extracellular matrix (15). However, the α1 and α2 chains, which normally form a trimer that then is exported from the cell, also showed the same cytoplasmic distribution by immunofluorescence; thus, definite conclusions about the ability of the podocyte cell line to export specific type IV collagen trimers into an extracellular matrix cannot be determined by immunohistochemical studies alone and would require further study. At the mRNA level, expression of the α1, α2, and α5 chains was greater in differentiated cells compared with undifferentiated and greater in cells that were grown on type IV collagen compared with type I collagen. There was no appreciable difference in expression between cells that were grown on type IV collagen that were extracted from normal *versus* Alport GBM. Because the normal canine GBM contains the α1 through α5 chains whereas the Alport GBM contains only the α1 and α2 chains (37), the results from the podocyte cell line suggest that the podocyte growth and differentiation are not influenced specifically by the α3/α4/α5 network. This result is in keeping with our earlier finding that the α3/α4/α5 network was not necessary for normal GBM formation (37). Moreover, the chain composition does not seem to affect the viability of the podocytes growing on it. This has implications for Alport syndrome, in which the GBM persistently lacks the α3/α4/α5 network. Our *in vitro* results suggest that podocyte viability is not compromised on the basis of this missing network and that other factors are involved in the eventual podocyte loss and glomerulosclerosis that occurs in this disease.

As markers of podocyte differentiation in BMSC, we studied expression of WT1, synaptopodin, actin and α-actinin, myosin IIA, CD2AP, podocin, and the α5 chain of type IV collagen. Protein expression in BMSC that were grown on plastic tended to resemble that seen in the undifferentiated podocyte cell line, whereas BMSC that were grown on collagen resembled more the differentiated podocyte cell line in terms of expression. For example, although myosin IIA was expressed in BMSC that

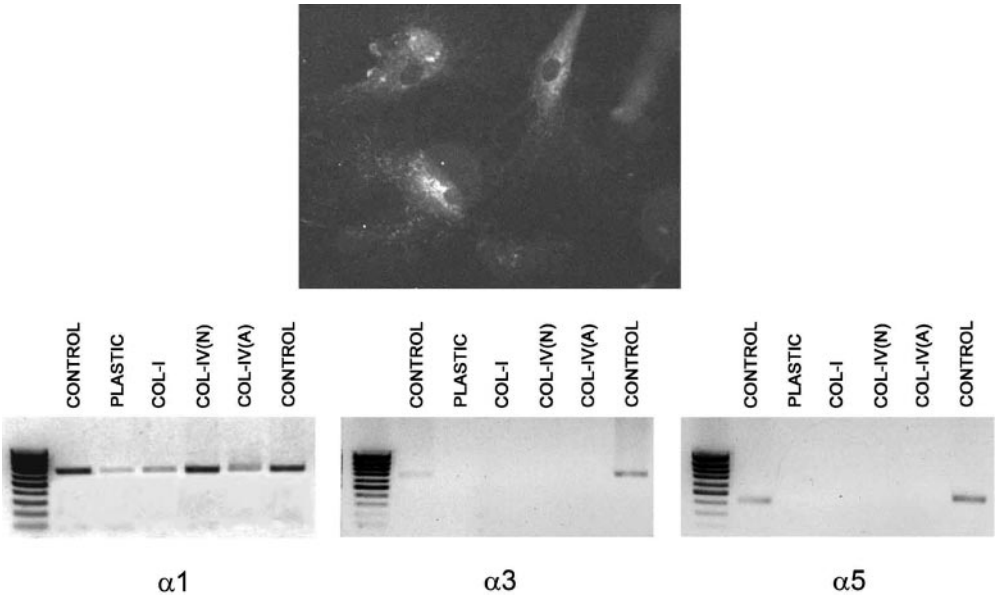

Figure 7. Expression of type IV collagen by BMSC. (Top) Cells showed cytoplasmic staining for the α1 chain of type IV collagen when grown on type I collagen (illustrated). Similar results were obtained for the α2 chain (results not shown). No staining for the α3 through α6 chains was detected on any matrix tested (results not shown). (Bottom) Reverse transcriptase–PCR analysis of type IV collagen expression in BMSC that were grown on plastic, type I collagen I (COL-I), and NC1 hexamers of type IV collagen from normal dog GBM [COL-IV(N)] and Alport dog GBM [COL-IV(A)]. Expression of the α1 and α2 chains of collagen type IV was upregulated in cells that were grown on NC1 hexamers from both normal and Alport dog GBM as compared with plastic and type I collagen. No expression of the α3 through α6 chains was detected on any matrix. Only the α1, α3, and α5 chains are illustrated.

were grown on plastic, the protein did not acquire a linear distribution until cells were grown on type I or type IV collagen. Also, only BMSC cells that were grown on collagen showed expression of synaptopodin. Similar to the podocyte line, there was nuclear expression of WT1 and cytoplasmic co-localization of synaptopodin, actin, and myosin IIA in BMSC. CD2AP in BMSC showed a differential expression on type IV collagen *versus* other matrices. CD2AP was expressed in a diffuse cytoplasmic distribution in BMSC that were grown on plastic and on type I collagen. BMSC that were grown on a type IV collagen matrix developed the bead-like pattern of expression also seen with the podocyte cell line. However, CD2AP tended to remain scattered throughout the cytoplasm rather than localize exclusively to the cell periphery as was seen in the podocyte cell line. This finding might indicate a lesser degree of differentiation in the BMSC or lack of some other protein involved in the translocation of CD2AP to the cell periphery. There was no expression of podocin by BMSC on any matrix. Only the α1 and α2 chains of type IV collagen were expressed by BMSC, regardless of the matrix used. Expression was weakest in cells that were grown on plastic and strongest in cells that were grown on type IV collagen. Cellular distribution was similar to that seen for the podocyte cell line. There was no expression of the α3, α4, or α6 chain of type IV collagen, but in contrast to the podocyte line, there was also no expression of the α5 chain. In fact, of all of the proteins studied, only the cytoplasmic distribution of CD2AP and expression of the α5 chain differed between the podocyte cell line and BMSC.

It can be concluded that BMSC undergo a degree of differentiation along a podocyte lineage as judged by production of proteins that are characteristically expressed by podocytes, including synaptopodin, myosin IIA, α-actinin, actin, and CD2AP. Differentiation is most enhanced when the cells are contact with a type IV collagen matrix. However, differentiation is incomplete as reflected by the lack of expression of podocin and the α3, α4, and α5 chains of type IV collagen and the persistence of a punctate cytoplasmic distribution of CD2AP, rather than migration to the cell periphery. Such findings indicate that other, yet unidentified factors must play important roles in podocyte differentiation and subcellular organization. Because engraftment of BMSC is reported to be enhanced in the setting of organ damage (21), we compared growth and differentiation on matrices that were derived from normal and diseased glomeruli (Alport syndrome in this case). We found that the Alport matrix was equally permissive to cell growth and differentiation.

BMSC offer the therapeutic potential of repairing damaged organs that depend on terminally differentiated cells that no longer can divide, such as podocytes. Thus, BMSC may be useful to reconstitute damaged glomeruli or deliver a transgene to the glomerulus for gene therapy. There are many unanswered questions related to this aim. What is the optimal method of delivery of the cells to glomeruli? Should BMSC be administered in the undifferentiated or partially differentiated state? What is the ultimate fate and potential of these cells in a glomerular environment? The development of successful technologies will allow us to explore the potential of BMSC for

reconstitution of glomeruli and for treatment for Alport syndrome and other glomerular diseases.

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