Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice

Asada, Nariaki; Takase, Masayuki; Nakamura, Jin; Oguchi, Akiko; Asada, Misako; Suzuki, Norio; Yamamura, Ken-ichi; Nagoshi, Narihito; Shibata, Shinsuke; Rao, Tata Nageswara; Fehling, Hans Joerg; Fukatsu, Atsushi; Minegishi, Naoko; Kita, Toru; Kimura, Takeshi; Okano, Hideyuki; Yamamoto, Masayuki; Yanagita, Motoko

Journal of Clinical Investigation (2011)


URL: http://hdl.handle.net/2433/147049
In chronic kidney disease, fibroblast dysfunction causes renal fibrosis and renal anemia. Renal fibrosis is mediated by the accumulation of myofibroblasts, whereas renal anemia is mediated by the reduced production of fibroblast-derived erythropoietin, a hormone that stimulates erythropoiesis. Despite their importance in chronic kidney disease, the origin and regulatory mechanism of fibroblasts remain unclear. Here, we have demonstrated that the majority of erythropoietin-producing fibroblasts in the healthy kidney originate from myelin protein zero-Cre (P0-Cre) lineage-labeled extrarenal cells, which enter the embryonic kidney at E13.5. In the diseased kidney, P0-Cre lineage-labeled fibroblasts, but not fibroblasts derived from injured tubular epithelial cells through epithelial-mesenchymal transition, transdifferentiated into myofibroblasts and predominantly contributed to fibrosis, with concomitant loss of erythropoietin production. We further demonstrated that attenuated erythropoietin production in transdifferentiated myofibroblasts was restored by the administration of neuroprotective agents, such as dexamethasone and neurotrophins. Moreover, the in vivo administration of tamoxifen, a selective estrogen receptor modulator, restored attenuated erythropoietin production as well as fibrosis in a mouse model of kidney fibrosis. These findings reveal the pathophysiological roles of P0-Cre lineage-labeled fibroblasts in the kidney and clarify the link between renal fibrosis and renal anemia.

Introduction

Erythropoietin (EPO) is a hormone that is essential for the production of red blood cells (1). EPO is mainly produced in the kidney after birth, and its production is severely reduced in patients with chronic kidney disease (CKD) (2) with renal anemia. The cloning of the EPO gene and the production of recombinant human EPO revolutionized the management of patients with CKD, providing an opportunity for safe long-term anemia correction, which improves cognitive function, quality of life, exercise capacity, and cardiac function (3). Nowadays, an increasing number of patients with CKD receive erythropoiesis-stimulating agents (ESAs), and annual US prescription sales for ESAs reached 10 billion dollars in 2006. In addition to the hematopoietic function of EPO, increasing evidence suggests that EPO plays a role as a tissue-protective factor in various nonhematopoietic tissues (4), raising the possibility that it will be prescribed for various types of tissue injuries in the near future.

Studies using in situ hybridization and the transgenic mice approach indicate that EPO is mainly produced by the interstitial fibroblasts in the deep cortex and the outer medulla in the kidney (5, 6). Although the essential role of EPO has been well recognized, the identity and developmental origin of EPO-producing cells in the kidneys remain unknown. This has hindered both the generation of EPO-producing cells from stem cells as well as the clarification of the molecular mechanisms underlying the defective production of EPO in patients with CKD—EPO-producing cells may lose their ability to produce EPO after injury, or, alternatively, EPO-producing cells may be lost during CKD.

Fibroblasts are interstitial mesenchymes that structurally support epithelia by producing ECM. Recently, the tissue-resident fibroblasts have attracted considerable attention because of their prominent roles in fibrosis, endocrine, organogenesis, and tumorigenesis (7). In chronic kidney injury, sustained inflammation accompanies the proliferation of interstitial fibroblasts and myofibroblasts (8), leading to renal fibrosis, which is the final common pathway for all CKD and eventually leads to renal failure (9). The origin of scar-producing myofibroblasts is of prime importance in understanding the mechanisms of renal fibrosis, which remain controversial. The contribution of bone marrow–derived fibrocytes (10) and the epithelial-mesenchymal transition (EMT) of injured tubular epithelial cells (11) has been proposed in some studies, but other groups have reported conflicting results (12–16).
Myelin protein zero (P0) is expressed in migrating neural crest cells in the early embryonic stages as well as in Schwann cells, which also originate from neural crest, in later stages (17). In P0-Cre mice, the activation of the P0 promoter induces the Cre-mediated recombination of reporter alleles, permanently tagging the cells arising from P0-Cre-expressing precursors with the expression of indicator genes.

Here, we demonstrate the occurrence of P0-Cre lineage-labeled fibroblasts in the kidney interstitium and their 2 different characteristics — they produce EPO, which possesses hematopoietic and tissue-protective functions in healthy kidney, and they transdifferentiate into scar-producing myofibroblasts and lose their EPO-producing activity after kidney injury, leading to renal fibrosis and renal anemia. We further demonstrate that the attenuated EPO production in scar-producing myofibroblasts can be restored through the administration of neuroprotective reagents, such as low-dose dexamethasone and neurotrophins. A similar effect was also obtained by the administration of a renoprotective reagent HGF. Moreover, the in vivo administration of tamoxifen, a selective estrogen receptor modulator (SERM), restored attenuated EPO production as well as fibrosis in a mouse model of kidney fibrosis.

These findings provide insights that we believe to be novel into the mechanisms of renal fibrosis and renal anemia in CKD and provide clues that assist in the development of therapeutic approaches to treat these disorders.

**Results**

Most fibroblasts in the kidney arise from P0-Cre-expressing precursors. P0-Cre mice were mated to 4 indicator mouse lines: floxed EGFP (18), R26R (19), R26ECFP (20), and R26tdRFP mice (21). Immunostaining of the kidneys of P0-Cre/R26ECFP mice using anti-GFP antibody revealed numerous enhanced cyan fluorescent protein-positive (ECFP+) cells in the cortex and outer medulla coexpressing ECFP (% ECFP/PDGFR-β cells) and the proportion of ECFP+ interstitial cells coexpressing PDGFR-β (% PDGFR-β/ECFP cells). (K) In neonatal kidneys of P0-Cre/floxed-EGFP mice most of the EGFP+ cells were also positive for p75, a neural crest marker. Scale bars: 10 μm (B–I); 100 μm (K).
CD31, a marker for endothelial cells (Figure 1G and Supplemental Figure 7). Very few ECFP⁺ cells expressed tyrosine hydroxylase, a marker for sympathetic nerve fibers, whereas most ECFP⁺ cells did not (Supplemental Figure 2). Instead, ECFP⁺ cells expressed PDGFR-β, a marker for interstitial fibroblasts in the kidney (refs. 9, 23, and Figure 1H). ECFP⁺ cells in the cortex expressed CD73/5′-nucleotidase (CD73/5′NT), a marker for cortical fibroblasts (ref. 9 and Figure 1I). More than 98% of ECFP⁺ cells expressed CD31, a marker for endothelial cells (Figure 1G and Supplemental Figure 3, C–E), which is possibly indicative of their routes of migration. The number of ECFP⁺ cells in the kidneys throughout development (Supplemental Figure 3, A–C), which is possibly indicative of their routes of migration. The number of ECFP⁺ cells in the kidneys increased substantially during development. Thus, it is highly unlikely that the ectopic expression of Cre in the kidney accounts for ECFP⁺ cells in the kidney.

**P0-Cre lineage-labeled cells enter the embryonic kidney during nephrogenesis.** To investigate the route and stage of migration of P0-Cre lineage-labeled cells into the kidney, we analyzed P0-Cre/R26ECFP embryos serially from E11.5, when nephrogenesis is initiated, to the neonatal stage. ECFP⁺ cells in the neonatal kidneys of P0-Cre/flxed-EGFP mice were positive for LacZ (Figure 3, D and E), supporting the idea that EPO-producing cells originate from the P0-Cre-expressing precursors. We further analyzed the kidneys of P0-Cre/R26tdRFP/Epo-GFP mice and found that more than 75% of GFP⁺ cells were also positive for class III β-tubulin, a marker for interstitial fibroblasts in the kidney (refs. 24 and Figure 1K). These results indicate that most, if not all, of the kidney fibroblasts in the cortex and outer medulla are fate-mapped with P0-Cre.

**Figure 2**
P0-Cre lineage-labeled cells in the developing kidney. (A–C) ECFP⁺ cells were first observed in the kidneys of P0-Cre/R26ECFP mice at E13.5. ECFP⁺ cells were predominantly located along the outer capsule and the ureter (arrows) of the kidney. (D–F) The number of ECFP⁺ cells in the kidneys of P0-Cre/R26ECFP mice increased at E16.5, and (F) the cells began to express PDGFR-β. The boxed region in E is shown at higher magnification in F. (G and H) ECFP⁺ cells populating the cortex appeared to surround the territory of Six2⁺ renal progenitor cells. (I) ECFP⁺ cells in the kidney were negative for class III β-tubulin. K, kidney. Scale bars: 100 μm (A, B, D, E, and I); 10 μm (C and F–H).
Figure 3
P0-Cre lineage-labeled fibroblasts produce EPO. (A–C) Stellate-shaped GFP+ cells with projections were detected (A) in the interstitium of the kidneys of Epo-GFP mice and were positive for (B) CD73 and (C) PDGFR-β. (D and E) Analysis of the kidney of P0-Cre/R26R/Epo-GFP mice revealed that GFP+ cells were also positive for LacZ. (F) Analysis of the kidney of P0-Cre/R26tdRFP/Epo-GFP mice revealed that most GFP+ cells were also positive for tdRFP. Scale bars: 10 μm. (G) Sorting EGFP+ cells (P5) out of EGFP− cells (P4) from the kidneys of adult P0-Cre/floxed-EGFP mice. RT-PCR analysis of these populations revealed high expression of Epo and p75 in EGFP+ cells but not in EGFP− cells. Cells were stained either with or without anti-GFP antibody. (H) Sorting p75+ cells (P5) out of p75− cells (P4) from the kidneys of P0-Cre/R26ECFP mice at 2 weeks of age. Cells were stained either with or without anti-p75 antibody. Quantitative PCR analysis demonstrated that the expression of p75, Epo, and ECFP was higher in p75+ cells. Expression of p75 was normalized to that of Gapdh and expressed relative to that in p75− cells.
Five days after obstruction of the ureter, (B) interstitial fibrosis was prominent in the operated kidney (A) compared with that in the control kidney. (C and D) The number of ECFP+ cells was markedly increased in the operated kidneys of P0-Cre/R26ECFP mice compared with that in the control kidney. (E and F) FACS analysis also confirmed the increase in the number of EGFP+ cells in the operated kidneys of P0-Cre/floxed-EGFP mice. Cells were stained with anti-GFP antibody. (G) ECFP/Ki67 double-positive cells were abundant in the operated kidneys of P0-Cre/R26ECFP mice. (H and I) Most ECFP+ cells were also positive for (H) PDGFR-β and (I) α-SMA. (J) Graph illustrating the proportion of PDGFR-β+ or α-SMA+ interstitial cells coinexpressing ECFP (% labeled/ECFP cells) and the proportion of interstitial ECFP+ cells coinexpressing either PDGFR-β or α-SMA (% ECFP/labeled cells). (K and L) The number of ECFP+ cells was also markedly increased in the kidneys of P0-Cre/R26ECFP mice after (K) folic acid nephrotoxicity and (L) severe ischemic reperfusion injury. Most ECFP+ cells were also positive for α-SMA. Scale bars: 10 μm.
of ECFP/Ki67 double-positive cells, indicating that the increase in the number of ECFP+ cells in the operated kidney is at least partially attributable to the proliferation of ECFP+ cells (Figure 4G).

Another possibility is that macrophages and dendritic cells infiltrated the operated kidneys in response to inflammation. This would account for the increase in the number of ECFP+ cells in the operated kidneys. However, ECFP+ cells did not overlap with the inflammatory cells, which infiltrated the operated kidneys in response to inflammation (Supplemental Figure 1, D–F). Instead, these ECFP+ cells were almost exclusively positive for PDGFR-β (Figure 4H), a marker for fibroblasts, and α-SMA (Figure 4I), a marker for myofibroblasts. Quantitative analysis demonstrated that 97% of PDGFR-β+ interstitial cells as well as 94% of α-SMA+ myofibroblasts were positive for ECFP, whereas 97% and 93% of ECFP+ cells were positive for PDGFR-β and α-SMA, respectively (Figure 4J). Similar results were
obtained in other models of renal fibrosis — folic acid nephropathy (Figure 4K) and severe ischemic reperfusion injury (Figure 4L). These results indicate that P0-Cre lineage-labeled fibroblasts are the main source of scar-producing myofibroblasts, contributing to fibrosis after kidney injury.

**EPO-producing fibroblasts also transform into myofibroblasts at the cost of EPO production.** Regardless of the cause of the damage, diseased kidneys invariably suffer from fibrosis with reduced EPO production. The mechanism underlying this defective production of EPO in the diseased kidney remains unknown (27). In situ hybridization detected *Epo* mRNA in the interstitium of the control kidney (Figure 5A, left) but not in the operated kidney at day 14 of UUO (Figure 5A, middle). Suppression of *Epo* mRNA in the operated kidney was evident as early as 12 hours after ligation of the ureter (Figure 5B). Interestingly, the attenuated production of *Epo* mRNA in the fibrotic kidney was restored by the induction of anemia (Figure 5C and Supplemental Figure 10). Similarly, GFP+ cells were almost undetectable in the fibrotic kidneys of *Epo-GFP* mice, whereas many GFP+ cells emerged in the fibrotic kidneys after the induction of anemia (Figure 5D). These GFP+ cells in the fibrotic kidneys were positive for α-SMA (Figure 5D), whereas GFP+ cells in the healthy kidneys were negative for α-SMA (Supplemental Figure 5). These results indicate that transdifferentiated myofibroblasts still possess the capacity to produce EPO after the induction of anemia.

Taken together, the results indicate that the cause of renal anemia is not the loss of EPO-producing cells but is at least partially due to the transdifferentiation of EPO-producing cells into scar-producing myofibroblasts that express a smaller amount of EPO (Figure 6).

These findings led us to seek the possible stimuli to restore the EPO-producing ability in myofibroblasts. Because EPO-producing cells in the kidney possess both renal and neural characteristics, we administered renoprotective and neuroprotective agents to cultured primary myofibroblasts obtained from fibrotic kidneys to determine whether or not the EPO-producing ability could be restored. Among the agents that we tested, we found that the administration of neuroprotective agents, such as low-dose dexamethasone, neurotrophins (neurotrophin-3 [NT-3], NT-4, and brain-derived neurotrophic factor [BDNF]), and the renoprotective agent, HGF, increased EPO production in myofibroblasts (Figure 5E and Supplemental Figure 11). We sought other possible reagents to alleviate the loss of EPO in fibrotic kidney in vivo and found that the administration of tamoxifen, a SERM, restored the attenuated expression of EPO in fibrotic kidney (Figure 5F). SERMs activate the estrogen receptors and function as estrogen agonists in some tissues, such as bone and kidneys, and as estrogen antagonists in other tissues, such as breast. The administration of tamoxifen also restored fibrosis significantly (Figure 5F). These results indicate that the attenuated production of EPO in scar-producing myofibroblasts can be restored (Figure 6).

**Discussion**

In the present study, we demonstrated that EPO-producing cells in healthy kidney and scar-producing myofibroblasts during fibrosis originate from the same P0-Cre lineage-labeled extrarenal cells, which enter the embryonic kidney at E13.5 to become renal fibroblasts and transit from one another depending on the condition of the kidney. A greater understanding of the mechanisms that control the balance between these 2 functions will lead to better treatment for patients with CKD. More importantly, the reversibility of EPO production in the fibrotic kidney (Figure 5, C–F) raised the possibility of a therapeutic approach toward renal anemia (Figure 6).

Neurotrophins are reported to promote neuronal growth and survival in many neuronal populations through the activation of Trk receptor tyrosine kinases (28–30). Furthermore, NT-3 rescues neuronal precursors from apoptosis and promotes neuronal differentiation in embryonic kidney explants (31). Given the origin of EPO-producing cells from P0-Cre–expressing precursors, it is plausible that neurotrophins restore EPO-producing ability in transdifferentiated myofibroblasts. Recently, pharmaceutical strategies to stimulate neurotrophin signaling established proof of principle of these as effective therapeutics for Alzheimer disease (30). Among these strategies, small molecule activators of neurotrophin are appealing and should be tested in the model of renal anemia in the future.

Glucocorticoid exhibits both protective and destructive effects in the nervous system. In excess, glucocorticoid produces neuronal death, whereas low-dose glucocorticoid is neuroprotective. Recently, it has been demonstrated that glucocorticoid provides a neuroprotective effect through the activation of neurotrophin signaling.
Our data support the idea that myofibroblasts emerge from the pro-liferation and transdifferentiation of fibroblasts in the kidney, as described previously. Adult wild-type mice were purchased from Japan SLC. Anemia was induced by bleeding as well as by the administration of phenylhydrazine. UUO and folic acid nephrotoxicity was induced as described previously (47). The GFP signal in Epo-GFP mice (6) were harvested 3 weeks after the operation. Tamoxifen (5 mg/20 g BW) was orally administered everyday from 5 days before UUO to the day of operation. Tamoxifen (5 mg/20 g BW) was orally administered everyday from 5 days before UUO to the day of operation and every 2 days after the operation.

**Methods**

*Animals.* Transgenic mice expressing Cre under the control of the P0 promoter (P0-Cre mice) (17) were mated with floxed-EGFP mice (CAG-CATloxP-stoploxP, EGFP mice; ref. 18) (a gift from J. Miyazaki of Osaka University, Osaka, Japan), R26R mice (19), R26ECFP mice (20) (a gift from F. Costantini of Columbia University, New York, New York, USA), and R26tdRFP mice (21) to obtain double-transgenic mice. Epo-EGFP mice (6) were described previously. Adult wild-type mice were purchased from Japan SLC. Anemia was induced by bleeding (6) as well as by the administration of phenylhydrazine as described previously (47). The GFP signal in Epo-EGFP mice was observed after the induction of anemia. All animal studies were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US NIH).

Kidney disease models. UUO and folic acid nephrotoxicity was induced as described previously (48, 49). Severe ischemic reperfusion injury was induced by 75-minute clamp of the unilateral renal pedicles: the kidneys were harvested 3 weeks after the operation. Tamoxifen (5 mg/20 g BW) was orally administered everyday from 5 days before UUO to the day of operation and every 2 days after the operation.
Flow cytometric analysis. Flow cytometric analysis was performed using BD FACSAria. Mice were anesthetized and then perfused with normal saline.

Kidneys were harvested, and the tissue was minced with a razor blade and digested by 1 mg/ml collagenase A and 0.1 mg/ml DNase type I as previously described (52). The cell suspensions were filtered through a cell strainer to remove debris and were stained with antibodies against GFP (Molecular Probes) and p75 (Advanced Targeting Systems). The cells were fixed in 4% PFA for GFP staining but not for p75 staining.

Flow cytometric analysis. Flow cytometric analysis was performed using BD FACSAria. Mice were anesthetized and then perfused with normal saline. Kidneys were harvested, and the tissue was minced with a razor blade and digested by 1 mg/ml collagenase A and 0.1 mg/ml DNase type I as previously described (52). The cell suspensions were filtered through a cell strainer to remove debris and were stained with antibodies against GFP (Molecular Probes) and p75 (Advanced Targeting Systems). The cells were fixed in 4% PFA for GFP staining but not for p75 staining. Myofibroblast culture. UUO kidneys were harvested 14 days after the operation in the same manner used in the preparation of cell suspension for FACS analysis. Cells from 1 UUO kidney were seeded onto an 8-well dish (BD Bioscience), washed twice, stimulated with various agents 24 hours later, and incubated 48 hours after the stimulation in a hypoxic chamber (1% O2). We confirmed that most, if not all, α-SMA+ cultured myofibroblasts from P0-Cre/R26tdRFP mice were positive for ECFP (Supplemental Figure 6). We also confirmed the presence of EPO protein (0.2-0.75 pg/ml) in the culture supernatant of the cells using ELISA (Mouse/Rat EPO Quantikine ELISA system, R&D Systems).

Statistics. All assays were performed at least 5 times. Data are presented as the mean ± SD. Statistical significance was assessed by Student’s t-test for 2 group comparisons. P < 0.05 is considered significant.

Acknowledgments

We thank Mr. Yanagisawa, M. Nakafuku, R. Kageyama, T. Ohtsuka, Y. Kaziro, T. Nakamura, Y. Nabeshima, S. Nagata, K. Ohta, and A. Economides for their valuable comments and discussion. We also greatly appreciate F. Costantini for the R26ECFP mice, J. Miyazaki for the CAG-CATtdRFP-EFGEF mice, T. Yamamoto for his kind help with in situ hybridization, T. Kuroasaki and H. Kawamoto for the valuable help in the transfer of the R26tdRFP mice, and A.P. McMahon and J.W. Mugford for the FoxD1 antibodies and protocol for immunostaining. We sincerely thank C. Gerle for the critical reading of this manuscript. We are very grateful to A. Hosotani for her excellent technical assistance. This study was supported by the Funding Program for Next-Generation World-leading Researchers; Grants-in-Aid from the Ministry of Education, Culture, Science, Sports and Technology of Japan (Shin-gakuyutsu 21200080); a grant-in-aid for Research on Biological Markers for New Drug Development; Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan (08062855); grants from Astellas Foundation for Research on Metabolic Disorders; Novartis Foundation for the Promotion of Science; Senri LifeScience Foundation; Mochida Memorial Foundation; Takeda Science Foundation; and Japan Foundation for Applied Enzymology.

Received for publication February 25, 2011, and accepted in revised form July 26, 2011.

Address correspondence to: Motoko Yanagita, Career-Path Promotion Unit for Young Life Scientists, E-wing, Kyoto University Graduate School of Medicine, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. Phone: 81.75.753.9310; Fax: 81.75.753.9311; E-mail: motoy@kuhp.kyoto-u.ac.jp.


