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H. 知的財産権の出願・登録状況

該当なし

厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書

エンドセリンA受容体遺伝子を発現する心血管細胞の動態に関する研究

研究分担者　西山　功一　東京大学大学院医学系研究科助教

研究要旨
本研究において、ETAR発現細胞の動態解析を行った。ETAR発現心筋細胞が、心形成過程で流入路から上行して心室筋や心房筋に分化する特徴的な細胞系譜を示すこと、ETシグナルによるERK活性化を介して初期の心臓形成に寄与すること、刺激伝導系の形成とも関連する可能性があることが明らかになり、本研究計画の重要な基盤となるとともに、心臓発生のメカニズムを理解する上でも重要な知見となった。さらに、ETARは神経堤細胞、血管平滑筋細胞に発現していることが明らかになった。発現動態に関するこれらの知見は、ETAR遺伝子座へのノックインによる疾患モデルマウスの確立と解析を行う上で不可欠の情報であるとともに、病態形成における細胞系譜の役割を解析する重要なツールになる。

A．研究目的

本研究は、我々がこれまで行ってきたエンドセリンA受容体遺伝子座(ETAR)を標的とするリコンビナーゼ依存性カセット交換法(Recombinase-mediated cassette exchange; RMCE)を用いたマウス遺伝学的研究を基盤として、心および平滑筋細胞におけるETAR発現動態を解析し、ETAR発現細胞の発生学的役割を明らかにするとともに、病態モデルマウス作成に基盤となる情報を提供することを目標とする。

B．研究方法

1．遺伝子改変マウス

ETAR-lacZ, ETAR-EGFPマウスは、既報の通りリコンビナーゼ依存性カセット交換（RMCE）を用いて作成した。即ち、ETAR遺伝子第2エクソンに変異型lox配列（lox71, lox2272）で挟んだneomycin耐性遺伝子を導入したES細胞に対し、上記変異型loxに対応する配列（lox66, lox2272）で挟んだlacZ, EGFP遺伝子断片それぞれを含むプラスミドを電気穿孔法で導入してCreリコンビナーゼ遺伝子を含むアデノウィルスベクター（AxCANCre）を感染させてlox配列に相同組み換えを起こさせ、導入遺伝子をETAR遺伝子座にノックインしたES細胞株を得た。これらよりキメラマウスを作成し、生殖細胞系列に寄与したキメラマウスよりノックインマウスを得た。

マウスは温度23±2° C、湿度50-60%、12時間毎の明暗サイクル下に飼育し、実験に供した。実験は東京大学動物実験規則に則り、東京大学医学系研究科動物実験委員会により承認された実験計画のもとで行われた。

2．β-ガラクトシダーゼ染色によるlacZ発現細胞の可視化

β-ガラクトシダーゼの活性は、全胚固定標本または凍結切片標本において、X-gal (5-bromo-4-chloro-3-indoyl β-D-galactosidase)を基質とした発色反応により検出した。

3．in situハイブリダイゼーション

全胚固定標本または凍結切片標本において、digoxigeninで標識したRNAプローブを用いて通常の方法で行った。

4．免疫染色

凍結切片標本において、一次抗体反応後にペルオキシダーゼ、FITC、ビオチンで標識した二次抗体を反応させ、蛍光または発色反応によって可視化した。

5．蛍光色素標識による細胞の生体追跡

マウス胎生8.25日胚の心流入路領域に対して、蛍光色素PKH67（緑）またはPKH26（赤）をマイクロインジェクションした。その後、DMEM/F2＋50%ラット血清存在下で回転培養を30時間行い、蛍光実体顕微鏡（Leica MZFLIII stereomicroscope＋Hamamatsu digital camera C4742-95）で観察した。

6．EGFP 発現細胞の移植による細胞動態の解析
マウス胎生 8.25 日胚の心流入路 EGFP 発現領域より組織片を切り出し、一部の実験では蛍光色素 SYTO16 で細胞をラベルした後、同じ発生段階のマウス胚の流入路領域に移植した。対照群として、心流出路、心筒領域、尾部の組織を移植片に用いた。移植を受けた胚は、α-MEM＋10%ウマ血清存在下で 低酸素状態（5% CO2、95% N2）で 24 時間培養し、6 と同様に観察した。

C．研究結果

ETAR-lacZ または ETAR-EGFP ノックインマウスを用いて、本研究の標的細胞である ETAR 発現心筋細胞の分布と動態を解析した。ETAR-lacZ/EGFP 発現細胞は、マウス E8.0 日胚の心臓原基腹側において最初に認められ、原始心筒形成期には心臓流入路の腹側に局在していた。その発現は、in situ ハイブリダイゼーションによる ETAR 遺伝子の発現パターンとほぼ一致しており、内在性 ETAR の発現を反映すると考えられた。この初期の発現は、一次心臓予定領域マーカーである Nkx2.5・Mlc2a の発現領域の一部と一致していたが、二次心臓予定領域マーカーである Isl1 とは重ならず、ETAR-lacZ/EGFP 発現細胞は一次心臓予定領域に含まれる細胞集団であると考えられた。心ループ形成期には、ETAR-lacZ/EGFP 発現細胞は左側壁から左心室領域にかけて大彎に沿って分布し、四腔形成期にはその発現は左心室と両心房、さらに右心室の一部に広がった。

マーカー遺伝子発現の経時的解析から、心臓形成初期における ETAR-lacZ/EGFP 発現細胞は、流入路に生じて原始心筒～心ループ形成期に上方へ移動する細胞群と考えられた。また、免疫染色の結果から、ETAR-lacZ/EGFP 発現細胞は心室や心房の作業心筋に分化していくことが示された。細胞移動を証明するため、（ｉ）蛍光色素標識による細胞追跡、（ii）EGFP 発現細胞移植による動態解析を行った。その結果、E8.25 日の心流入路 ETAR-lacZ/EGFP 発現領域に蛍光標識した場合に、30 時間後の観察で ETAR-lacZ/EGFP 発現パターン同様の分布パターンが認められること、同領域の移植によって EGFP 発現細胞の左心室への分布が認められるのに対し、心流出路、心筒領域、尾部の組織を移植片に用いた対照群では同様の分布が認められなかったことから、細胞の上方への移動が特徴的な分布パターンを形成していることが証明された。

一方、ETAR 欠損胚の一部では心室の低形成、心筋における増殖活性および転写因子 Tbx5 の発現低下が見られ、初期の心臓形成の過程で ETAR シグナルは ERK のリン酸化と左心室マーカー Tbx5 の発現を促進していること、Tbx5 の発現は MEK 阻害薬によって抑制されることが示唆された。Tbx5 は Holt-Oram 症候群の原因遺伝子であり、心臓形成において重要な転写因子である。これらの結果から、ETAR シグナルは ERK のリン酸化を介して、心筋細胞増殖や Tbx5 による心室形成に寄与していることが示唆された。

D．考察

心臓における ETAR 発現細胞は、主に心流出路～大血管起始部に寄与する心臓神経堤細胞と、心室心房領域の作業心筋の２つに大別されるが、本研究では後者に関し、心臓形成の初期からその起源となる領域と動態を lacZ/EGFP ノックインマウスを用いて明らかにした。ETAR-lacZ/EGFP 発現細胞は一次心臓予定領域の一部として心流入路の限局した領域に形成され、心ループ形成期には、左側壁から左心室領域にかけて大彎に沿って分布するという特徴を示した。四腔形成期にはその発現は左心室と両心房、さらに右心室の一部に広がったが、この広範囲の発現の多くは、二次心臓領域由来の細胞などが後の段階で ETAR を発現するようになったと考えられる。大彎に沿った特徴的分布パターンと細胞移動はこれまで報告されていないものであるが、流入路における細胞の出現と上行する細胞移動の方向は、発生初期の刺激伝導系の形成と興奮伝播様式と一致しており、刺激伝導路形成への寄与が考えられる。実際、ETAR の最も初期の発現パターンはペースメーカー細胞の特徴である K_f チャネルをコードする HCN4 遺伝子の発現パターンとよく一致しており、現在その関連について解析を進めている。

ETAR 発現細胞の時空間的分布の変化や細胞系譜における位置づけは、心臓発生研究における重要性に加え、本研究の中心である miRNA の発現による機能解析を行う上で、重要な情報を提供すると考えられる。

E．結論

本研究において、本研究の標的細胞である ETAR 発現心筋細胞が、心形成過程で流入路から上行して心室筋や心房筋に分化する特徴的な細胞系譜を示すこと、ET シグナルによる ERK 活性化を介して初期の心臓形成に寄与することを明らかにした。この成果は、心血管系における miRNA 特異的発現による疾患モデルの確立と解析に有

用と考えられる。

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H. 知的財産権の出願・登録状況
該当なし

厚生労働科学研究費補助金（創薬基盤推進研究事業）
分担研究報告書

心血管系の細胞系譜に関する研究

研究分担者　富田　幸子　東京女子医科大学医学部助教

研究要旨
心血管系の病態理解において、先天性心疾患だけでなく、成人以降に発症する疾患においても、その発症原因となる細胞の発生学的系譜や発生過程での異常の観点から理解することの重要性が指摘され始めている。私の研究グループでは、ニワトリ初期胚の神経堤細胞の切除実験を実施したところ冠血管平滑筋の異常を形成することができた。この結果は、最近論争の的になっている冠血管の起源について、神経堤細胞が関与することを裏付けた。一方、ニワトリ胚における領域特異的な蛍光色素標識や血管鋳型作成技術をマウスに応用することによって、心血管系の病態解析の基盤が充実した。これらの実験系は、心血管系の疾患モデルマウスの表現型解析に有用なアプローチ法と視点を提供すると考えられる。

A．研究目的

循環器疾患の発生学的理解は、これまで先天性心疾患に限られていたが、最近は成人以降に発症する疾患においても、その発症原因となる細胞の発生学的系譜や発生過程での異常の観点から理解することの重要性が指摘され始めている。本研究では、鳥類胚における冠動脈の起源に関する研究を通して、マウス発生工学を中心とする本プロジェクトに新しいアプローチ法と視点を提供することを試みた。

B．研究方法

(1) ニワトリ初期胚操作：ニワトリ初期胚の心臓および菱脳部神経堤細胞の切除実験を実施し、冠血管の形態の異常を解析した（下記）。
(2) 血管鋳型の作成：血管系、特に冠動脈の形態解析として、合成樹脂を大動脈近位部から冠動脈入口部に注入し、血管鋳型を作成した。これらの技術を、マウス胚にも適用した。
(3) 心臓細胞の蛍光標識解析：ニワトリ・ウズラ心臓神経堤移植キメラ胚とともに、マウス胚などを用いて、神経堤細胞をはじめとする心臓発生領域に蛍光色素を微小注入し、発生過程における細胞動態を可視化した。

C．研究結果

1．冠血管平滑筋の由来

冠血管平滑筋の由来は、これまで心外膜前駆組織といわれてきたが、最近になってマウスの研究から神経堤細胞が平滑筋の一部を形成することもわかってきた。本年度の研究で、ニワトリ初期胚の心臓および菱脳部神経堤の切除実験を実施したところ冠血管中隔枝とその分枝の異常を形成することができた。

2．心血管系の発生と病態における細胞系譜解析法の確立

鳥類において、神経堤細胞をはじめとする領域特異的な蛍光色素標識や、合成樹脂などの血管内注入による鋳型作成によって、心血管系の発生過程の解析法を改良するとともに、マウスに応用することによって、正常胚と遺伝子変異による異常胚の心血管系の解剖学的解析がより精密に行えるようになった。これにより、発生初期に心流入路に出現するETAR発現細胞が左側壁を上行して左室と両心房に寄与する新たな細胞群である可能性を支持する結果を得た。マウス血管鋳型作成は、研究代表者のグループが作成したマウス解析に応用し成果を得ている。

D．考察

冠血管の起源については、これまで心外膜前駆組織といわれてきたが、神経堤細胞の関与については一定の見解がない。本研究による結果は、冠血管の一部に、心臓および菱脳部神経堤細胞が関与することを裏付けた。この結果は、神経堤細胞を含むETAR発現細胞を標的とする心血管傷害モデルの作成と病態解析を１つの柱とする本プロジェクトにも有用なアプローチ法と視点を提供すると考えられる。次年度には、この平滑筋異常の詳細な検討をするとともに、心臓神

経堤、菱脳部神経堤、心外膜前駆組織の各部位移植ウズラ-ニワトリキメラ胚と神経堤細胞マーカーであるP0-creマウスを利用し、心臓神経堤細胞の詳細なマッピングを作製する予定である。また、鳥類胚で改良された技術のマウスへの応用は、大きさの問題などで困難さは伴うものの、細胞系譜や病態形成に関わる発生基盤の理解に重要なアプローチ法を提供すると考えられる。

E．結論

冠血管の起源に関する新しい知見と心血管系の発生学的解析法は、心血管系における miRNA 特異的発現による疾患モデルの確立と解析に有用と考えられる。

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H. 知的財産権の出願・登録状況

該当なし

別添５

研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

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Identification and developmental analysis of endothelin receptor type-A expressing cells in the mouse kidney

Taro Kitazawa [1], Takahiro Sato [1,2], Koichi Nishiyama, Rieko Asai, Yuichiro Arima, Yasunobu Uchijima, Yukiko Kurihara, Hiroki Kurihara *

Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

The endothelin (Edn) system plays pleiotropic roles in renal function and various disease processes through two distinct G protein-coupled receptors, Edn receptors type-A (Ednra) and type-B (Ednrb). However, difficulties in the accurate identification of receptor-expressing cells in situ have made it difficult to dissect their diverse action in renal (patho)physiology. We have recently established mouse lines in which *lacZ* and *EGFP* are 'knocked-in' to the *Ednra* locus to faithfully mark *Ednra*-expressing cells. Here we analyzed these mice for their expression in the kidney to characterize *Ednra*-expressing cells. *Ednra* expression was first observed in undifferentiated mesenchymal cells around the ureteric bud at E12.5. Thereafter, *Ednra* expression was widely observed in vascular smooth muscle cells, JG cells and mesenchymal cells in the interstitium. After growth, the expression became confined to vascular smooth muscle cells, pericytes and renin-producing JG cells. By contrast, most cells in the nephron and vascular endothelial cells did not express *Ednra*. These results indicate that *Ednra* expression may be linked with non-epithelial fate determination and differentiation of metanephric mesenchyme. *Ednra-lacZ/EGFP* knock-in mice may serve as a useful tool in studies on renal function and pathophysiology of various renal diseases.

Systemic and local circulatory homeostasis is maintained by a balance between vasoconstrictive and vasodilatory factors. The endothelin (Edn) system, composed of three peptide ligands (Edn1, Edn2 and Edn3) and their two G protein-coupled receptors (endothelin receptors type-A (Ednra) and type-B (Ednrb)), is involved in this mechanism (Masaki, 2004; Yanagisawa et al., 1988). In addition to their vasoconstrictive effects, Edns have a diverse set of biological activities such as proliferative effects on various cells, stimulation of hormone release and modulation of central nervous activity. During embryogenesis, the Edn1–Ednra axis regulates craniofacial and cardiovascular morphogenesis, whereas the Edn3–Ednrb axis contributes to melanocyte and enteric neuron development (Kurihara et al., 1999, 1994; Sato et al., 2008b).

The Edn system has been known to play pleiotropic roles in renal (patho)physiology. In the renal vasculature, Edn1 exerts potent vasoconstriction mainly through both Ednra (Hirata et al., 1989; Honing et al., 2000), whereas some vascular beds show an endothelium-dependent vasodilatory response mediated by Ednrb (Matsumura et al., 2000). Edn1 also acts on renal tubules to promote diuresis and natriuresis by several mechanisms via Ednrb (Ahn et al., 2004; Gariepy et al., 2000; Tomita et al., 1993). Furthermore, Edn1 modulates renin secretion from juxtaglomerular (JG) cells (Rakugi et al., 1988). Through these effects, the Edn system has been implicated in the pathophysiology of hypertension and various renal diseases.

To dissect mechanisms underlying these diverse roles of the Edn system, identification of cells expressing the Edn receptors is of fundamental importance. However, accurate description of their expression patterns remains still elusive due to relatively low expression levels and lack of antibodies sufficient for immunostaining. We have recently established mouse lines in which marker genes such as *lacZ* and *EGFP* are 'knocked-in' to the *Ednra* locus (Asai et al., 2010; Sato et al., 2008a). In these mice, the marker gene expression faithfully recapitulates that of the endogenous *Ednra* during embryogenesis. In this study, we analyzed these mice for renal expression to clarify the localization of Ednra and its developmental changes in the kidney.

1. Results

1.1. Isolation of Ednra–EGFP-positive cells and gene expression profiling by RT-PCR

In *Ednra-lacZ* or *-EGFP* knock-in mice, marker gene expression patterns faithfully recapitulate those of endogenous *Ednra*

* Corresponding author. Tel.: +81 3 5841 3498; fax: +81 3 5684 4958.
E-mail address: kuri-tky@umin.ac.jp (H. Kurihara).
[1] These authors contributed equally to this study.
[2] Present address: Yamanaka iPS Cell Special Project, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan.

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expression in the heart and pharyngeal arches (Asai et al., 2010; Sato et al., 2008a). To confirm that *Ednra-lacZ/EGFP* expression also reflects endogenous *Ednra* expression in the kidney, we performed FACS and RT-PCR. Cells were isolated from the E17.5 *Ednra*$^{EGFP/+}$ kidneys and subjected to forward-side selection to preliminarily identify cells (Fig. 1A), FACS analysis using fluorescent lectin revealed that endothelial cells (detected by BS-1), proximal tubules (detected by LTA) and collecting ducts (detected by DBA) were sufficiently collected (Fig. S1), indicating that overall cell populations of the kidneys were properly obtained through our manipulation. After PI-selection to exclude non-viable cells (Fig. 1B), EGFP-positive and -negative cells were sorted for RT-PCR analysis (Fig. 1C). *Ednra*-expressing cells were detected only in the EGFP-positive fraction, while *Ednrb* expression was detectable only in the EGFP-negative fraction (Fig. 1D). This result indicates that the knocked-in EGFP expression appears to faithfully recapitulate endogenous *Ednra* expression and there is little overlapping in the expression of *Ednra* and *Ednrb* in the developing kidney. *Platelet-endothelial cell adhesion molecule-1* (*PECAM1/CD31*; a marker for endothelial cells) and *α-smooth muscle actin* (*αSMA*; a marker for smooth muscle cells) expression was detected only in the EGFP-negative and -positive fractions, respectively (Fig. 1D). This finding coincides with the distinct expression pattern of Edn receptors in vasculature: *Ednra* and *Ednrb* in smooth muscle cells and endothelial cells, respectively (Masaki, 2004).

RT-PCR also revealed that *aquaporin-1* (*Aqp1*; a marker for proximal renal tubules) and *aquaporin-3* (*Aqp3*; a marker for collecting ducts) expression was undetectable in the EGFP-positive fractions (Fig. 1D). Instead, *glial cell line-derived neurotrophic factor* (*GDNF*; a marker for undifferentiated mesenchyme) and *renin-1* (*Ren1*; a marker for juxtaglomerular cells) expression was found only in the EGFP-positive fractions (Fig. 1D). These results indicate that *Ednra*-expressing cell population is likely to include vascular smooth muscle cells, JG cells and undifferentiated mesenchymal cells.

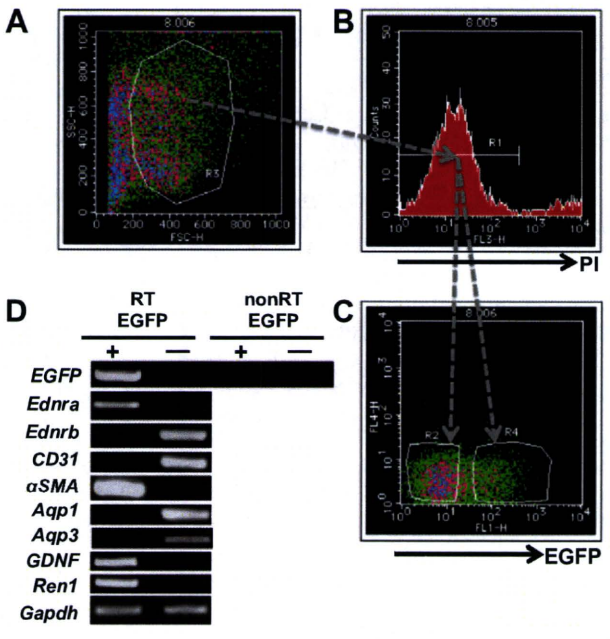

Fig. 1. Characterization of *Ednra-lacZ/EGFP*-expressing cells in the kidney. (A–C) Cells from the E17.5 *Ednra*$^{EGFP/+}$ embryonic kidneys were sorted into EGFP-positive and EGFP-negative fractions. Cells are subjected to forward-side selection for preliminary identification of the cells (A), next PI selection was performed to exclude nonviable cells (B), and finally EGFP selection was carried out to identify EGFP-expressing and EGFP-non-expressing cells (C). Gated R2 and R4 regions correspond to fractions of EGFP-negative and -positive cells, respectively. (D) RT-PCR analysis of EGFP-positive and -negative cells from the E17.5 kidneys. *Ednra* was detected only in the *Ednra–EGFP*-positive fraction. Expression of *Ednrb*, *CD31*, *αSMA*, *Aqp1*, *GDNF*, and *Ren1* was also analyzed. *Gapdh* was used as an internal control.

1.2. LacZ- and EGFP-labeling reveals the renal expression pattern of Ednra and its developmental changes

To analyze the expression pattern of *Ednra* in the kidney, we performed β-galactosidase staining on *Ednra-lacZ* knock-in embryonic and adult kidneys. *LacZ*-expressing cells were detected as early as E12.5 in mesenchyme around ureteric buds, although their expression levels were low compared to those in the lung and testis interstitium, where *Ednra* was most abundantly expressed at this stage (Fig. 2A and B). At E15.5 (Fig. 2C–E) and E18.5 (Fig. 2F–H), when basic expression patterns of *lacZ* are almost the same, *lacZ* expression was broadly distributed mainly in the medullary interstitial mesenchyme (Fig. 2C, D and F). By contrast, *lacZ* expression was relatively low in the cortical nephrogenic region (Fig. 2C, E and F). In higher magnification images, *lacZ*-expressing cells were detected in the vessel wall (Fig. 2G) and in the JG region encompassing intraglomerular mesangium and afferent and efferent arterioles (Fig. 2D–F). In adult sections, *lacZ* expression was apparently much sparser than in embryonic ones (compare Fig. 2I to C and F). At higher magnifications, *lacZ*-positive cells were observed around vessels (Fig. 2J) and in the JG region (Fig. 2K) as in embryonic sections. But, compared to developing kidneys, *lacZ*-positive cells were much decreased in the interstitium and inside the glomerulus. Throughout kidney development, renal tubular epithelium was not stained for β-galactosidase. These observations are consistent with the results of RT-PCR analysis described above.

To confirm that the *lacZ* expression patterns faithfully recapitulate the endogenous expression of *Ednra* in the kidney, we performed in situ hybridization on E18.5 kidney sections. *Ednra* expression was mainly distributed in the medullary interstitial region (Fig. S2A and B). High magnification images detected *Ednra* expression in the medullary interstitium (Fig. S2C and D), vessels (Fig. S2E) and the JG region (Fig. S2F). By contrast, renal tubules are apparently *LacZ*-negative (Fig. S2C–F). These patterns are largely identical to *Ednra-lacZ* expression patterns shown in Fig. 2.

To further dissect the characters of *Ednra*-expressing cells in the kidneys, we performed double immunostaining on *Ednra–EGFP* knock-in embryos and adult mice. We used CD31 and αSMA as markers for endothelial and smooth muscle cells, respectively. At E18.5, EGFP-expressing cells were detected around CD31-positive vascular endothelial cells and in the JG region (Fig. 3A–A‴). CD31-negative glomerular mesangial cells also showed EGFP expression (Fig. 3A–A‴). αSMA-positive cells are broadly observed along vessels and they are EGFP-positive (Fig. 3B–B‴). In the JG region, αSMA expression fades away as previously described (Sauter et al., 2008), and only EGFP signals remain (Fig. 3B–B‴). In addition, αSMA-negative and EGFP-positive cells were found in the interstitium (Fig. 3B–B‴).

In adult sections, EGFP signal was sparse, as seen in β-galactosidase staining. EGFP-expressing cells were detected in the vascular medial wall underlying CD31-positive endothelial cells (Fig. 3C–C‴) and in the JG region (Fig. 3D–D‴). Interestingly, EGFP expression in glomerular mesangial cells and the interstitium apparently was much less than in the developing kidney. To determine the character of *Ednra*-expressing cells in the interstitium, we observed some sections and checked approximately 1500 EGFP-positive cells, and found all the EGFP signals adjacent to CD31 signals (Fig. 3E–E‴), indicating that these *Ednra*-expressing cells are likely to be pericytes surrounding the descending vasa recta.

Taken these results together, *Ednra* expression in vascular smooth muscle cells and JG cells remain after birth, while

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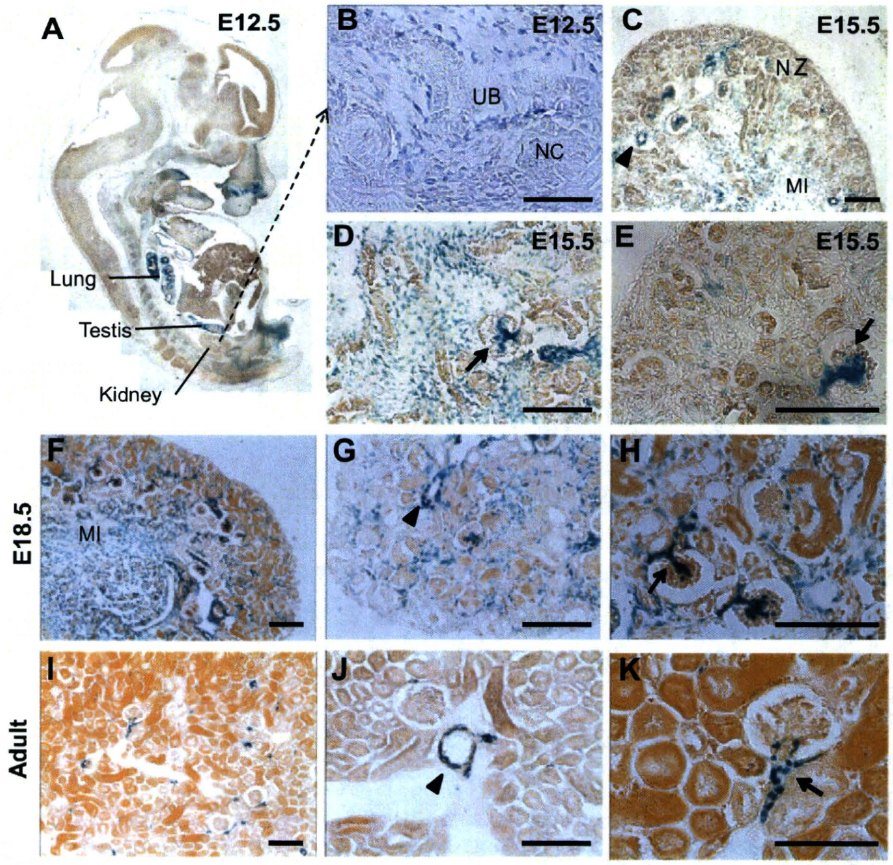

Fig. 2. β-Galactosidase staining in the *Ednra-lacZ* embryonic and adult kidneys. (A and B) A section of an E12.5 embryo stained for β-galactosidase activity. A is a low magnification image of the whole embryo and B is a high magnification image of the renal region of A. Renal *LacZ* signals can be detected but they are much weaker than those of the lung and testis. Signals are detected in mesenchyme around ureteric buds (UB), but not in nephrogenic condensates (NC). (C–E) Sections of the E15.5 kidneys stained for β-galactosidase activity. C is a low magnification image. (D and E) Higher magnification images of the medullary interstitial region and cortical nephrogenic region, respectively. (F–H) Low (F) and high (G and H) magnification images of E18.5 kidney sections stained for β-galactosidase activity. Basic expression pattern of *LacZ* in the E18.5 sections is the same with that of E15.5 ones. Abundant *LacZ* expression is detected throughout the medullary interstitium (C, D and F), but, compared with this, few signals can be detected in the nephrogenic mesenchyme of the cortical region (E). *LacZ* signal are detected around vessels (C, G, arrowheads) and the JG region including small vessels and intraglomerular mesangial cells (E, H, arrows). Renal tubules are apparently *LacZ*-negative. (I–K) Low (I) and high (J and K) magnification images of kidney sections of adult (2 months) mice stained for β-galactosidase activity. *LacZ*-expressing cells are detected around vessels (J, arrowhead) and in the JG region with adjacent small vessels (K, arrow), but are not detected in renal tubules as in embryonic sections. Compared to the developing kidney, *lacZ*-expressing cells were much decreased in the interstitium and inside the glomerulus (I). All the sections are counterstained with orange G. MI, medullary interstitium, NZ, nephrogenic zone. Scale bars: 100 μm.

expression in developing interstitial mesenchyme may be confined to pericytes.

1.3. Inclusion of renin-producing cells in Ednra–EGFP-positive cell population

Next we performed double immunostaining for EGFP and renin on *Ednra*$^{EGFP/+}$ kidney sections to confirm that renin-producing cells are Ednra-positive. At E18.5, renin-expressing cells were found not only in the JG region (Fig. 4A–A‴) but also in vessels outside the JG region (Fig. 4B–B‴) as previously described (Sauter et al., 2008). These renin-producing cells were always found to express EGFP (Fig. 4A–A‴ and B–B‴). In the adult kidneys, renin expression became restricted to cells within the JG region, which were also included within EGFP-positive cell population encompassing the afferent and efferent arterioles (Fig. 4C–C‴).

2. Discussion

In the present study, *Ednra*-positive cells in the kidney were clearly distinguished by marker gene expression. Renal *Ednra* expression was first observed in mesenchymal cells around the ureteric bud around E12.5. Thereafter, *Ednra* expression was broadly distributed in vascular smooth muscle cells, JG cells and mesenchymal cells in the interstitium until neonatal stages. After growth, the expression became confined to vascular smooth muscle cells, pericytes and renin-producing JG cells. By contrast, most cells in the nephron and vascular endothelial cells did not express *Ednra*. This pattern is quite distinct from that of *Ednrb*, which is abundantly expressed in tubular epithelial cells and vascular endothelial cells (Chow et al., 1995; Nangaku et al., 2002; Terada et al., 1992).

Kidney development initiates with the interaction between the Wolffian duct and metanephric mesenchyme (Vainio and Lin, 2002). The Wolffian duct generates the ureteric bud, which then invades the metanephric blastema to induce nephrogenic epithelial condensates destined to develop into nephrons. On the other hand, stromal cell progenitors that are not destined to nephrons are thought to become interstitial mesenchyme, vascular smooth muscle cells and JG cells (Humphreys et al., 2010; Kobayashi et al., 2008; Maric et al., 1997; Sequeira Lopez et al., 2004, 2001). Thus, *Ednra* expression may be linked with non-epithelial fate determination and differentiation of mesenchyme. This is in sharp

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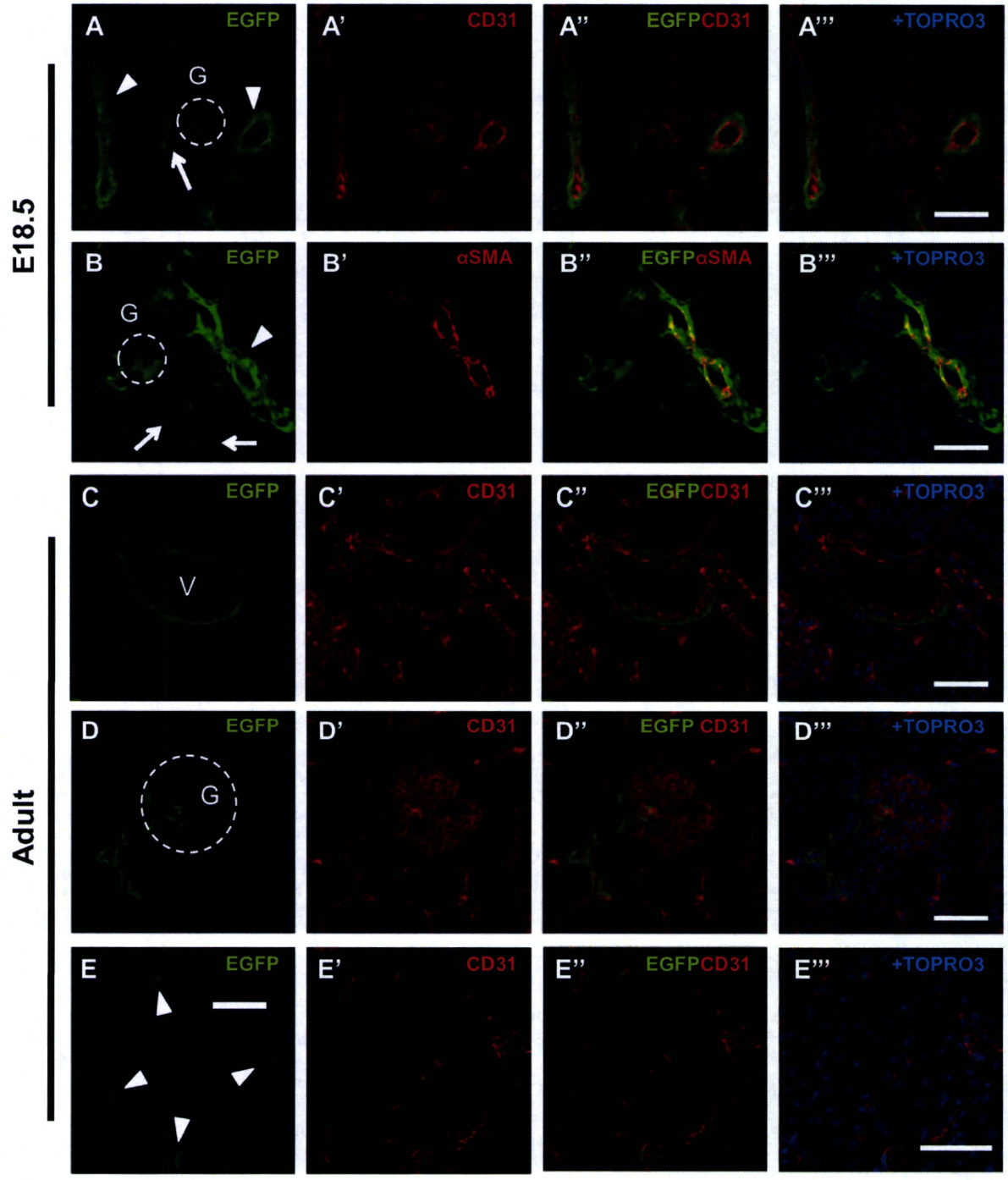

Fig. 3. Expression patterns of EGFP, CD31 and αSMA in *Ednra-EGFP* kidneys. Sections of E18.5 (A–A‴, B–B‴) and adult (C–C‴, D–D‴, E–E‴) kidneys immunostained for EGFP (A–E, A″–E″; green), CD31 (A′, A″, C′, C″, D′, D″, E′, E‴; red), αSMA (B′, B″; red), and merged with TO-PRO-3 staining for nuclei (A‴–E‴; blue). In E18.5 kidneys, EGFP-positive cells are detected around CD31-positive vascular endothelial cells (A–A‴, arrowheads), and in the JG region (A–A‴, arrow). EGFP was co-expressed with αSMA in vessels (B–B‴, arrowhead), but not in the JG region (B–B‴, around glomerulus). EGFP-positive but αSMA-negative cells in the interstitium are indicated with arrows (B–B‴). In the adult kidneys, EGFP-positive cells are detected around CD31-positive endothelial cells (C–C‴) and in the JG region (D–D‴). Compared to the embryonic kidneys, EGFP signals are much decreased inside the glomerulus (D–D‴). EGFP-positive cells in the interstitium are associated with CD31-positive endothelial cells (E and E‴). G: glomerulus. Scale bars: 50 μm.

contrast to the expression of Ednrb, which is abundantly expressed in tubular epithelial cells that are derived from nephrogenic mesenchyme.

In addition to vascular smooth muscle cells, renin-producing JG cells shows intense staining for Ednra expression. This result is consistent with previous finding that Edn directly inhibit cAMP-dependent renin production through Ednra in cultured JG cells (Ryan et al., 2002). JG cells were postulated to derive from smooth muscle cells in the past (Owen et al., 1995). However, Sequeira Lopez et al. have shown that renin-producing precursor cells differentiate into a diversity of cells including smooth muscle cells (Sequeira Lopez et al., 2004, 2001). Furthermore, Matsushita

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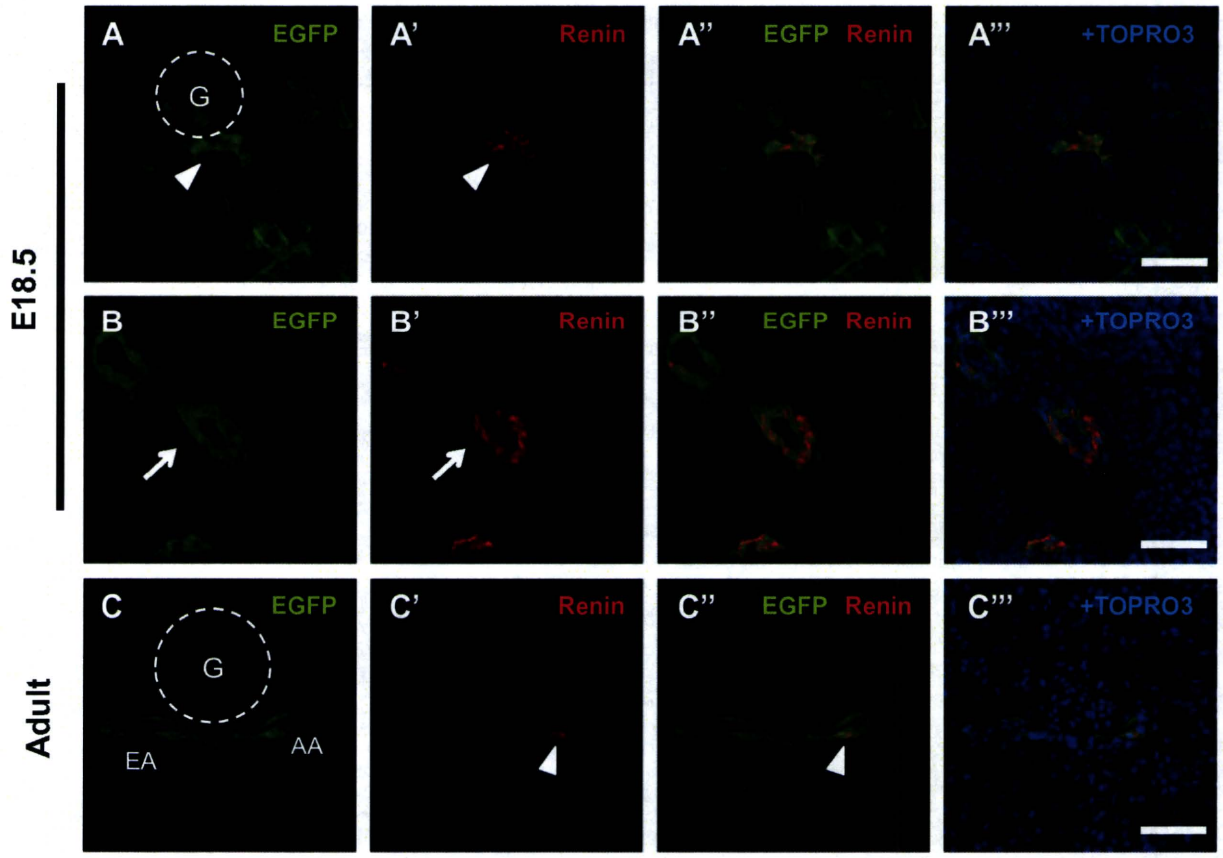

Fig. 4. Co-localization of EGFP and renin in *Ednra–EGFP* kidneys. Sections of kidneys of E18.5 (A–A‴, B–B‴), and adult (C–C‴) mice, immunostained for EGFP (A–C, A″–C‴; green), renin (A′–C′, A″–C″; red), and merged with TO-PRO-3 staining for nuclei (A‴–C‴; blue). In both the E18.5 (A–A‴) and adult (C–C‴) JG region, rennin-producing cells are EGFP-positive (arrowheads). In E18.5 kidneys, rennin-producing cells are also detected in vessels outside the JG region, which are also EGFP-positive (arrows) (B–B‴). G: glomerulus. Scale bars: 50 μm.

et al. have demonstrated the presence of mesenchymal stem cells that may give rise to smooth muscle cells through renin-producing precursors (Matsushita et al., 2010). Although the origin of JG cells and their relationship to smooth muscle cells in the lineage hierarchy is still controversial, *Ednra* expression may serve as a hallmark for non-epithelialized metanephric descendants.

In the present study, *Ednra* expression appears to decrease in mesangium and interstitium after growth. On the other hand, Ednra has been implicated in various diseases involving these cell populations, such as glomerulonephritis and renal intestinal fibrosis (Brochu et al., 1999; Sorokin and Kohan, 2003). In these pathological conditions, *Ednra* expression may be re-activated in proliferative mesangial and/or mesenchymal cells, where Ednra may mediate a mitotic signal to contribute to disease progression.

It has been known that the Edn system is deeply involved in various renal (patho)physiology. However, dissection of its diverse action is difficult possibly because their different effects on the nephron and vasculature can hardly be discriminated (Dhaun et al., 2006). *Ednra*-knock-in mice may serve as a useful tool in such studies by enabling us to identify and isolate *Ednra*-expressing cells in various conditions.

3. Materials and methods

3.1. Mice

Ednra$^{lacZ/+}$ (*lacZ*-knock-in) and *Ednra*$^{EGFP/+}$ (*EGFP*-knock-in) mice, described previously (Asai et al., 2010; Sato et al., 2008a), were maintained on an ICR-background. Mice were housed in an environmentally controlled room at 23 ± 2 °C, with a relative humidity of 50–60% and under a 12L–12D light cycle. Genotypes were determined by PCR on tail-tip or amnion DNA using specific primers. Embryonic ages were determined by timed mating with the day of the plug being embryonic day (E) 0.5. All the animal experiments were reviewed and approved by the University of Tokyo Animal Care and Use Committee.

3.2. Cell sorting

Kidneys were collected from E17.5 *Ednra*$^{EGFP/+}$ and wild-type embryos and dissected into pieces. Then the kidneys were incubated in D-MEM (Wako) containing 1 mg/ml of collagenase (Sigma) at 37 °C for 60 min. After disaggregated in 0.05% trypsin–EDTA solution (Sigma) to obtain single-cell suspensions, cells were subjected to hypotonic treatment for hemolyzation and resuspended in an appropriate volume of FACS buffer (5% fetal bovine serum/PBS). For cell sorting, the cells were passed through a cell strainer (BD Bioscience) and sorted into EGFP-positive and EGFP-negative cells using a FACS VantageSE flow cytometer (BD Bioscience). The data were analyzed with CellQuest software (BD Bioscience). For FACS analysis, the cells collected form wild-type embryos were lectin stained. The cells were incubated with FITC-conjugated lectin from *Bandeiraea simplicifolia* (BS-1) (Sigma), FITC-conjugated *Dolichos biflorus* aggulutinin (DBA) (J-Oilmills), or biotin-conjugated *Lotus tetragonolobus* aggulutinin (LTA) (Vector) on ice for 30 min. The cells incubated with biotin-conjugated

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LTA were washed with an excess amount of FACS buffer, and incubated with PE-conjugated streptavidin (BD Bioscience) on ice for 30 min. The cells were washed and resuspended in FACS buffer again at an appropriate concentration, and passed through a cell strainer before FACS analysis. Analyses were performed on a FACS VantageSE flow cytometer, and data were analyzed with CellQuest software. In the assay, electronic gating was set to exclude nonviable cells with propidium iodide (PI) (Sigma) staining after cellular fractionation on the basis of forward versus side scatter.

3.3. RT-PCR

After cell sorting, EGFP-positive and EGFP-negative cells were subjected to conventional RT-PCR. Extraction of total RNA, reverse-transcription, and conventional PCR was performed as described previously with minor modifications (Asai et al., 2010). PCR on the resulting cDNA was performed using the primers 5′-GACGTAAACGGCCACAAGTTCA-3′ and 5′-GAACTCCAGCAGGACCA TGTGATC-3′ for *EGFP* (product size, 608 bp: annealing temperature, 65 °C), 5′-ACGCTGGCCTTTCG-3′ and 5′-CTGAGCAGTTCACA CCGGTTCTTATC-3′ for *Ednra* (product size, 603 bp: annealing temperature, 62 °C), 5′-CACAGTGCTGAGTCTTTGTGCTCT-3′ and 5′-ACCTATGGGTTCGGGGACAG-3′ for *Ednrb* (product size, 157 bp: annealing temperature, 60 °C), 5′-AGGACAGACCCTTCCACCAA-3′ and 5′-AATGACAACCACCGCAATGA-3′ for *CD31* (product size, 206 bp; annealing temperature, 62 °C), 5′-TGCCGAGCGTGAGAT TG-3′ and 5′-AATGAAAGATGGCTGGAAGAGAG-3′ for *αSMA* (product size, 193 bp; annealing temperature, 62 °C), 5′-GGCTATGTG-CAGTGTCATGTC-3′ and 5′-CTGTGATATGCCAGTGGTCAG-3′ for *Aqp1* (product size, 462 bp; annealing, 62 °C), 5′-ATCAAG CTGCCCATCTACAC-3′ and 5′-GGGCCAGCTTCACATTCTC-3′ for *Aqp3* (product size, 559 bp; annealing temperature, 60 °C), 5′-CCAGA-GAATTCCAGAGGGAAAGGT-3′ and 5′-CAGATACATCCACACCG TTTAGCGG-3′ for *GDNF* (product size, 338 bp; annealing temperature, 60 °C), 5′-ATCCCGCTCAAGAAAATGCC-3′ and 5′-TGTGTCA CAGTGATTCCACC-3′ for *Ren1* (product size, 416 bp: annealing temperature, 62 °C), 5′-GGTGTGAACCACGAGAAATAT-3′ and 5′-AGAT-CCACGACGGACACATT-3′ for *Gapdh* (product seize, 334 bp: annealing temperature, 60 °C).

3.4. β-Galactosidase staining

lacZ expression was detected by staining with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) for β-galactosidase activity. Section staining was performed as described previously with minor modifications (Nagy et al., 2003). Sections were counterstained with 1% orange G (Sigma).

3.5. In situ hybridization

Sections (12 μm) were prepared from frozen mouse kidney samples. Treatment for in situ hybridization was as described with minor modifications (Ishii et al., 1997). The *Ednra* probe has been described previously (Sato et al., 2008a).

3.6. Immunohistochemistry

Immunohistochemistry of sections was performed as described previously with minor modifications (Makita et al., 2008). Embryo cryosections (12 μm) were immunostained using the following antibodies: rat monoclonal anti-GFP (Nacalai Tesque, Kyoto, Japan; 1:200), rabbit anti-GFP (Medical and Biological Laboratories, Nagoya, Japan; 1:250), rat anti-CD31 (BD Pharmingen, 1:200), mouse anti-αSMA (Sigma, 1:500), goat anti-Renin (Santa Cruz, 1:100). Signals were visualized with Rhodamine Red- or FITC-conjugated secondary antibodies specific for the appropriate species. When visualizing signals with anti-mouse secondary antibody, we used M.O.M. Blocking Reagent (Vector) to reduce background staining. Nuclei were visualized with TO-PRO-3 (Molecular Probes).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gep.2011.04.001.

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