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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

***In vivo* nephrogenesis in Lewis rats from endothelial cells of syngeneic liver grafts transplanted into partially excised kidney: The role of transforming growth factor (TGF)-β1 and Thy-1 (CD90)**

OBJECTIVE *In vitro* nephrogenesis using the own tissues is an ideal substitute for allogeneic whole-kidney transplantation. **METHOD** Adult Lewis rats were transplanted syngeneic liver structure grafts, from which hepatocytes had been removed, into an excision site in the left kidney. Either transforming growth factor (TGF)-β1 or epidermal growth factor (EGF) was then injected into the hosts. **RESULTS** Good nephrogenesis was observed in the 7 rats that received a total of 60 μg of TGF-β1 each. Among these, the 4 (53%) rats with the best nephrogenesis showed a high percentage of Thy-1 (CD90)-positive bone marrow (BM) cells (60±5%), measured using a flow cytometer (FCM). In their mesenteric lymph nodes (MLN), the 7 TGF-β1 rats showed reduced percentages of CD3-positive cells (76±1%), calcineurin-regulated nuclear factor of activated T cell c1 (NFATc1)-positive cells (51±19%), and signal transducers and activators of transcription 3 (STAT3)-positive cells (39±14%). On transmission electron microscope (TEM) analyses, endothelial to mesenchymal transition (EndMT) could be observed in liver graft blood vessels, which showed massive proliferation of mesenchymal cells around a destroyed vessel to form a new nephron. On TEM analysis of specimens with pre-fixing staining with NFATc1 antibody-gold, NFATc1 expression was shown in newly generated mesangial cells, podocytes, and tubular cells. Mesenchymal cells had small granules of NFATc1. Aggregated NFATc1 in a large lysosome appeared in well-developed proximal convoluted tubular cells. **CONCLUSIONS** Increased TGF-β1 signaling of EndMT trigger was very important for nephrogenesis, together with high expression of BM Thy-1. EGF injection was associated with less uniform results.

In 1993, transforming growth factor-β1 (TGF-β1) messenger RNA (mRNA) and its peptide were detected in rat metanephroi. It was shown, however, that exogenous TGF-β1 inhibits tubulogenesis.¹ It has also been reported that small phenotype and mothers against decapentaplegic-related proteins (Smads) of the TGF-β superfamily, that are expressed during mouse kidney development from embryonic day 12 (E12) until the end of nephrogenesis at postnatal day 15, play specific roles in determining renal cell fate.² All 8 Smads expressed in the mesenchymal cells of the nephrogenic zone were found to be downregulated once these mesenchymal cells began to undergo epithelialization. Among the 8 Smads, the bone morphogenetic-responsive receptor regulated (R)-Smad1, 5, and 8 were mainly ex-

pressed in the nephrogenic zone. Normal nephrogenesis in the fetus depends on mesenchymal to epithelial interactions in development. The earliest embryonic nephron progenitor cells are regulated by fibroblast growth factor/epidermal growth factor (FGF/EGF) signaling through RAS.³ It is considered that the combined activation of FGF receptors (fgfr) 1 and 2 is absolutely essential for normal function of metanephric mesenchyme (MM).⁴ Fgfr1/2 Mes^{-/-} mice develop a ureteric bud that does not elongate or branch. The EGF receptor initiates the events required for very early ureteric bud branching.

The effects of dexamethasone (DEX) exposure on rat metanephric development have been examined.⁵ When whole metanephroi from E14.5 rat embryos were cul-

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In vivo νεφρογένεση σε αρουραίους Lewis από τα ενδοθηλιακά κύτταρα των γενετικά όμοιων μοσχευμάτων ήπατος που μεταμοσχεύτηκαν σε μερικώς εκτομηθέντα νεφρό: Ο ρόλος του τροποποιητικού αυξητικού παράγοντα (TGF)-β1 και του παράγοντα Thy-1 (CD90)

Περίληψη στο τέλος του άρθρου

Key words

Endothelial to mesenchymal transition
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tured for 2 days in the presence of DEX (10⁻⁵ M), ureteric branching was inhibited. DEX exposure at E14.5/E15.5 *in vivo* induced decreased expression of glial cell line-derived neurotrophic factor (GDNF), a member of the TGF- β family promoting phosphorylation of Ret receptor, and increased expression of bone morphogenetic protein-4 (BMP-4) and TGF- β 1.^{5,6} It was shown that Ret receptor, a protein tyrosine kinase receptor, and its ligand GDNF, but not TGF- β 1, played a promoting role in ureteric branching. Activated c-Ret appeared to mediate epithelial morphogenesis by prolonging cell survival in conjunction with the activation of morphogenic receptors, c-Met and EGF receptor.⁷

In this *in vivo* study, using models of adult Lewis rats, nephrogenesis in a syngeneic liver structure graft was examined. The liver structure graft, from which hepatocytes had been removed, was transplanted into an excision site in the host left kidney, following which TGF- β 1 or EGF was injected into the hosts. The best nephrogenesis was induced in the TGF- β 1 rats. As the nephrogenesis was derived from donor liver endothelial cells, auto-liver grafts were expected to improve the results of nephrogenesis.

MATERIAL AND METHOD

Animals

Lewis (LEW/SsN) rats, which had been purchased from Japan SLC Co, Ltd (Hamamatsu, Japan), were maintained in the animal center of Hamamatsu University School of Medicine. The experimental (Exp) rats were descended from the purchased Lewis rats and the parents of Exp rats were directly related. In this study, only syngeneic Lewis rats were used.

Experimental designs

The Exp systems of this study were classified into 8 subclasses: Exps A, B, C, D, E, F, G, and H. The outline of the Exp systems is shown in table 1. In the 4 males in Exp A, initial mean body weight (BW) was 235 g at the age of 8.4 weeks. From a Lewis male donor, a piece of liver (1.51 g per rat) was cut out, from which liver structure tissue at 0.56 g per rat was made by removing the hepatocytes. At the same time, in each host in Exp A, a part of cortical kidney sized 0.8×0.5 cm was excised from the middle of the left kidney. A piece of liver structure tissue (0.56 g) was then transplanted into the excision site of the left kidney. Five Exp B females had an initial mean BW of 154 g at the age of 8.6 weeks. From a Lewis female donor, a piece of liver structure tissue of 0.45 g and pieces of bone marrow (BM) with bone (total 0.12 g) were prepared for each host in Exp B. After a part of cortical kidney sized 0.4×0.7 cm was excised from the middle of the left kidney, pieces of liver structure tissue and BM were transplanted into the excision site in the left kidney of each host. Four Exp C males had initial mean

BW of 191 g at the age of 7.7 weeks. From a Lewis male donor, a piece of liver structure tissue of 0.32 g and pieces of BM with bone (total 0.23 g) were prepared for each host in Exp C. A part of cortical kidney sized 0.75×0.6 cm was excised from the middle of the left kidney of each Exp C male, into which prepared donor liver and BM tissues were then transplanted. TGF- β 1 peptide (60–66), which was composed of H-Lys-Val-Leu-Ala-Leu-Tyr-Asn-Lys-NH₂, was injected into the hosts in Exp C (Anaspec, Inc, Fremont, CA, USA). During 12 days post-transplantation, TGF- β 1 at 10 μ g per rat was injected subcutaneously into the Exp C rats 7 times (i.e., a total amount of 70 μ g). Five Exp D females showed initial mean BW of 136 g at the age of 7.9 weeks. From a Lewis female donor, a piece of liver structure tissue (0.23 g) was prepared for each host in Exp D. After a part of cortical kidney sized 0.35×0.35 cm was removed from the middle of the left kidney of each Exp D rat, a piece of liver structure tissue (0.23 g) was transplanted into the excision site in the left kidney. The liver structure tissues were prepared in the same way as described for Exp A. For 8 days post-transplantation, TGF- β 1 at 10 μ g per rat was injected subcutaneously into each Exp D rat 6 times (i.e., a total amount of 60 μ g per rat). Five Exp E females had an initial mean BW of 145 g at the age of 7.9 weeks. From a Lewis female donor, a piece of liver structure tissue (0.25 g per rat) was prepared for each host in Exp E. From the middle of the left kidney of each host a part of cortical kidney sized 0.4×0.4 cm was excised, and then a piece of liver structure tissue (0.25 g) was transplanted there. EGF 20-31, which has the amino acid sequence of Cys-Met-His-Ile-Glu-Ser-Leu-Asp-Ser-Tyr-Thr-Cys-OH, was purchased from California Peptide Research, Inc (Napa, CA, USA). During 6 days post-transplantation, EGF at 10 μ g per rat was injected subcutaneously into the Exp rats 4 times (i.e., a total amount of 40 μ g per rat).

In Exp F, G, and H rats, a part of syngeneic cortical kidney was also transplanted in addition to a piece of liver structure tissue, which was used to surround the kidney graft. Four Exp F males showed initial mean BW of 212 g at the age of 8.0 weeks. From a Lewis female donor, a piece of liver structure tissue (0.33 g per rat) and 2 pieces of cortical kidney (0.09×2 g per rat) were prepared for each host in Exp F. In each host, from the middle of the left kidney, a piece of cortical kidney sized 0.40×0.45 cm was removed, following which pieces of liver structure tissue and cortical kidney were transplanted in the excision site in the left kidney. During 7 days post-transplantation, EGF at 10 μ g per rat was injected subcutaneously into each Exp F rat 4 times (i.e., a total amount of 40 μ g per rat). Five Exp G females had an initial mean BW of 137 g at the age of 7.3 weeks. From a Lewis female donor, a piece of liver structure tissue (0.08 g) and a part of cortical kidney sized 1/6 of the whole kidney were prepared for transplantation into each host in Exp G. After a part of cortical kidney sized 0.4×0.4 cm had been removed from the middle of the left kidney of each Exp G rat, pieces of liver structure tissue and kidney were transplanted there. TGF- β 1 at 10 μ g per rat was injected subcutaneously into each Exp G rat 5 times (i.e., a total amount of 50 μ g per rat) during 6 days post-transplantation. Four Exp H males had an initial mean BW of 180 g at the age of 7.4 weeks. From the Lewis male

Table 1. Experimental systems of experimental (Exp) Lewis rats: Exp A to Exp H.

Exp no	Lewis host		Lewis donor graft				EGF (Total µg per rat)	TGF-β1	Following (Day)
	Sex	Total (n)	Sex	Liver*	BM	Kidney			
A	M	4	M	+	-	-	0	0	72
B	F	5	F	+	+	-	0	0	74
C	M	4	M	+	+	-	0	70	71
D	F	7	F	+	-	-	0	60	90
E	F	7	F	+	-	-	40	0	95
F	M	4	F	+	-	+	40	0	70
G	F	5	F	+	-	+	0	50	63
H	M	4	M	+	-	+	12.5	0	55

*Hepatocytes were removed from the whole liver

EGF: Epidermal growth factor, TGF-β1: Transforming growth factor-β1

donor, a piece of liver structure tissue (0.17 g per rat) and a part of cortical kidney sized 1/3 of the whole kidney were prepared for each host in Exp H. From the middle of the left kidney, a part of cortical kidney sized 0.6×0.6 cm was removed from each Exp G rat, following which the pieces of liver structure tissue and kidney were transplanted there. During 2 days post-transplantation, EGF at 10 µg or 2.5 µg per rat (total amount of 12.5 µg per rat) was injected into each Exp H rat. The post-transplantation observation periods in all the Exp rats are shown in table 1.

At the time of sacrifice, measurement was made of the BW of each host and the weight of the right kidneys. Macroscopically, absence of a purulent discharge from the grafted left kidneys was examined to imagine nephrogenesis and kidney graft survival. For electron microscopic analyses, grafts without a purulent discharge were selected.

Histopathological analyses

The left kidneys containing grafts from all the experimental rats were fixed in 10% formalin (Sigma-Aldrich, St Louis, MO, USA). All the tissue sections were stained with hematoxylin-eosin (H-E). A BX51 light microscope equipped with a DP72 digital camera was used for taking light micrographs (Olympus, Tokyo, Japan).

In Exp A-4, Exp D-1 and 4, and Exp G-1 rats, the grafted areas of the left kidneys were fixed in 2% glutaraldehyde for transmission electron microscope (TEM) analysis. In Exp D-6, G-2, and H-2 rats, cell suspensions of the grafted areas were made. These were prestained with calcineurin-regulated nuclear factor of activated T cell c1 (NFATc1) antibody (Ab), the immunogen of which is recombinant protein of human NFATc1 amino acids 197–304, for 10 minutes at 4° C (Biolegend, San Diego, CA, USA). NFATc1 Ab was labeled with 15 nm gold colloidal particles for 5 minutes at 4° C (EY Laboratories, Inc, San Mateo, CA, USA). After washing with phosphate-buffered saline (PBS) once, the cell suspensions stained with NFATc1 Abs-gold were fixed with 2% glutaraldehyde. Sectioned samples, which were stained doubly with uranyl-lead

citrate, were observed with a JEM 12000 TEM (JEOL, Tokyo, Japan).

Flow cytometer (FCM) analyses

More than one million cells were separated from mesenteric lymph node (MLN), BM, and cortical kidney in all the Exp rats, 7 control male (CM) rats, and 5 control female (CF) rats. MLN cell suspensions were divided into 3 aliquots, one of which was stained with CD3 monoclonal antibody (mAb) conjugated with fluorescein-isothiocyanate (FITC) (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). The other MLN suspensions were stained with NFATc1 Ab (Biolegend) or signal transducers and activators of transcription 3 (STAT3) Ab (BD Biosciences, Franklin Lakes, NJ, USA) for 30 minutes at 4° C. Apart from CD3-FITC mAb, samples stained with the latter 2 Abs were also stained with goat anti-mouse IgG-FITC for 15 minutes at 4° C (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). BM cell suspensions were divided into 2 aliquots. Firstly, one of the BM suspension aliquots was stained with anti-human epidermal growth factor receptor (EGFR) Ab conjugated with biotin, which recognizes the extracellular domain of the EGFR associated with tyrosine kinase activation, for 30 minutes at 4° C (Leinco Technologies, Inc, Ballwin, MO, USA). The other BM suspension aliquot was stained with biotin-conjugated mouse anti-rat CD90 (Thy-1)/mouse CD90.¹ (Thy-1.1) mAb, which reacts with the rat BM Thy-1 antigen (CD90) expressed by hematopoietic stem cells, early myeloid and erythroid cells, and immature B lymphocytes, for 30 minutes at 4° C (BD Biosciences, Franklin Lakes, NJ, USA). Furthermore, both of the BM suspensions labeled with the biotin-Abs were reacted with fluorescein streptavidin for 30 minutes at 4° C (Vector Laboratories, Inc, Burlingame, CA, USA). Renal cell suspensions were made from the transplanted left kidney. For most of the rats, renal cell suspensions were made from the non-grafted areas of the left kidney. The renal cell suspensions were divided into 2 aliquots, one of which was stained with EGFR Ab using biotin-fluorescein streptavidin reactions in the way described above. The other renal suspension aliquot was stained with FITC-NFATc1 Ab in the way described above.

Positive cell % of each antigen was measured using an EPICSR XL-MCL system III FCM (Beckman Coulter, Fullerton, CA, USA). On the scattergrams of renal cell suspensions, the monocyte group area in peripheral blood samples was selected as a central area for the renal cell histogram to provide the percentages of positive cell populations. For all the measurements of renal cell suspensions, the fixed bit-map was applied. The percentage of positive cells was calculated as mean \pm standard deviation (M \pm SD) using a scientific calculator, CASIO fx-8000.

RESULTS

At the time of sacrifice when the rats were approximately 4 months old, the liver grafts were examined macroscopically, and then nephrogenesis was confirmed using light microscopic and TEM analyses. Table 2 summarizes the macroscopic findings. In table 2, a purulent discharge from liver grafts was described only on the basis of macroscopic findings, although abscess formation was detected more frequently in light microscopic findings. Four Exp A males (4 months and 1 week old) showed a mean BW of 360 \pm 35 g, and a mean right kidney weight (r-KW) (without grafts) of 1.63 \pm 0.13 g. Two (50%) males, Exp A-3 and 4 (Rat no), did not show purulent discharge from the grafts of their left kidney, but 2 others, Exp A-1 and 2, had macroscopic pus discharge. One male, Exp A-2, which showed complete graft rejection, had BW loss a few days post-transplantation, but recovered gradually. The Exp A-4 male showed nephrogenesis in histopathological analyses. As the 5 control males aged 4 months showed BW of 359 \pm 21 g and r-KW of 1.37 \pm 0.14 g, the r-KW of Exp A was judged to be increased 72 days post-transplantation.

Five Exp B females aged 4 months and 1 week had BW of 218 \pm 7 g and r-KW of 0.99 \pm 0.03 g. All the Exp B females had purulent discharge, and complete graft rejection was observed in the 4 (80%) females, with the exception of the Exp B-5 female with incompletely necrotic graft. Pieces of BM with bone aggravated liver structure tissue rejection. In comparison, 5 control females showed BW of 233 \pm 12 g and r-KW of 0.96 \pm 0.09 g at the age of 4 months. Therefore, in the graft rejection in Exp B, r-KW increase was not clear. Four Exp C males (aged 4 months) showed BW of 320 \pm 12 g and r-KW of 1.44 \pm 0.09 g, in which only 1 (25%) male, Exp C-3, was confirmed to have pus discharge, macroscopically. TGF- β 1, which was injected into the Exp C males 7 times, prevented graft rejection and promoted nephrogenesis, compared with the findings in the Exp B females with no TGF- β 1 administration. However, the histopathological results of nephrogenesis and BM survival were poor in the Exp C males. Seven Exp D females, which were injected with TGF- β 1 6 times, showed BW of 208 \pm 7 g and r-KW of 0.90 \pm 0.05 g at the age of 4 months and 3 weeks. The best nephrogenesis was confirmed by histopathological findings in Exp-D1, 3, 4, and 6, although a small amount of purulent discharge was detected in the other 3 (43%) of the 7 Exp D females. Right kidney enlargement was not observed, and the BW and r-KW were slightly smaller than those of control females. The nephrogenesis of the Exp D-3 female is shown in figure 1a. It was concluded from the results of Exp B and Exp C that pieces of syngeneic BM grafts with bone inhibited nephrogenesis in liver structure tissues. It was also clarified from the results of Exp C and Exp D that TGF- β 1 reacted to promote nephrogenesis in liver structure tissues. Seven Exp E females, which were injected

Table 2. A summary of macroscopic findings at the time of sacrifice of experimental (Exp) Lewis rats.

Exp no (Sex)	Injection		Pus from graft Rate (%)	BW (g)	r-KW (g)
	TGF- β 1	EGF			
Control (M)	–	–	–	359 \pm 21	1.37 \pm 0.14
A (M)	–	–	50	360 \pm 35	1.63 \pm 0.13
C (M)	+	–	25	320 \pm 12	1.44 \pm 0.09
F (M)	–	+	50	341 \pm 14	1.48 \pm 0.03
H (M)	–	+	50	308 \pm 16	1.35 \pm 0.05
Cont (F)	–	–	–	233 \pm 12	0.96 \pm 0.09
B (F)	–	–	100	218 \pm 7	0.99 \pm 0.03
D (F)	+	–	43	208 \pm 7	0.90 \pm 0.05
E (F)	–	+	57	229 \pm 15	0.98 \pm 0.10
G (F)	+	–	20	200 \pm 7	0.79 \pm 0.08

BW: Body weight, r-KW: Right kidney weight, EGF: Epidermal growth factor, TGF- β 1: Transforming growth factor- β 1

with EGF, showed BW of 229 ± 15 g and r-KW of 0.98 ± 0.10 g at the age of 4 months and 4 weeks. Four (57%) of the 7 Exp E females (Exp E-1, 2, 3, and 4) exhibited purulent discharge, in which massive necrosis was detected in 2 (Exp E-2 and 3). In the Exp E-2 female, showing massive necrosis, a subcutaneous tumor mass was already detected 1.5 months post-transplantation. Accumulated substantial glomerular generation was demonstrated histopathologically in 3 females (43%), Exp E-5, 6, and 7. These 3 females showed good survival of accumulated glomerulus at the border with normal host kidney. The Exp E-7 female retained the highest numbers of glomeruli and tubules at the border with host kidney, but this was accompanied by active lymphocyte infiltration, as shown in figure 1b. This case still showed massive apoptotic and necrotic tubulogenesis 95 days post-transplantation. Compared with TGF- β 1 injection, which induced the best reaction to nephrogenesis in this experiment, EGF injection promoted nephrogenesis less uniformly. Nephrogenic tissues survived longer in all the rats at the border with the host normal kidney. In the degenerative stages, tubulogenetic cells fell into apoptosis earlier than glomerulogenetic cells. The nephrogenic tissues began to be rejected earlier than 40 days post-transplantation, because the graft rejection of Exp E-2 was confirmed as a subcutaneous nodule 1.5 months post-transplantation.

Figures 1a and 1b show light micrographs that demonstrate nephrogenesis observed in the Exp D-3 and Exp E-7 females, respectively. Both of these rats were transplanted with only liver structure tissues, but the Exp D-3 female received TGF- β 1 and the Exp E-7 female received EGF. It was shown that TGF- β 1 contributed better to nephrogenesis, without severe lymphocyte infiltration.

To examine the effects of nephrogenesis on syngeneic kidney graft survival, kidney grafts were combined with liver structure tissue grafts by surrounding the kidney grafts with liver structure tissues. Four Exp F males exhibited BW of 341 ± 14 g together with r-KW of 1.48 ± 0.03 g, in which EGF was injected 4 times. Histopathologically, good kidney graft survival was not observed in the rats with milder right kidney enlargement. Pus discharge was detected in the 2 (50%) rats, Exp F-1 and 4. Five Exp G females, which were injected with TGF- β 1 5 times, exhibited BW of 200 ± 7 g together with r-KW of 0.79 ± 0.08 g at the age of 3 months and 3 weeks, in which pus discharge from the graft was observed in 1 (20%) female, Exp G-4. Even in the best graft survival, only apoptotic glomeruli and tubules remained 70 days post-transplantation, which was also confirmed by the TEM analysis described below. TGF- β 1 did not contribute much to the kidney graft survival. However, the r-KW was

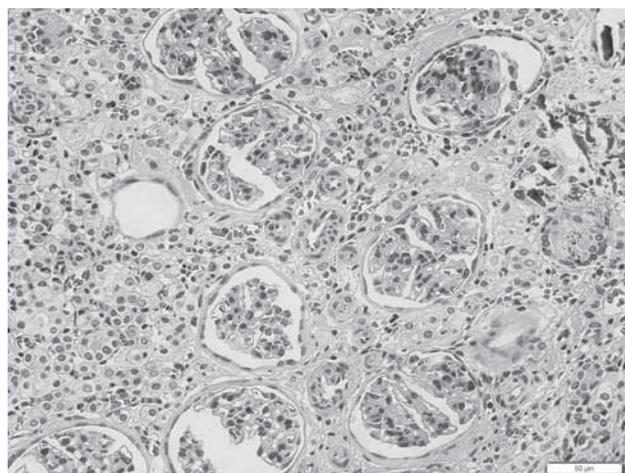


Figure 1a. Light micrograph showing nephrogenesis in the Exp D-3 rat at 90 days post-transplantation, in which a piece of liver structure tissue had been transplanted in the excision site of the left kidney, followed by administration of TGF- β 1. Glomerulogenesis and tubulogenesis are detected in the liver graft. (At the time of sacrifice, on flow cytometry of BM suspension, the Exp D-3 rat showed 51% Thy-1-positive BM cells and 5% EGFR-positive BM cells). (H-E stain).

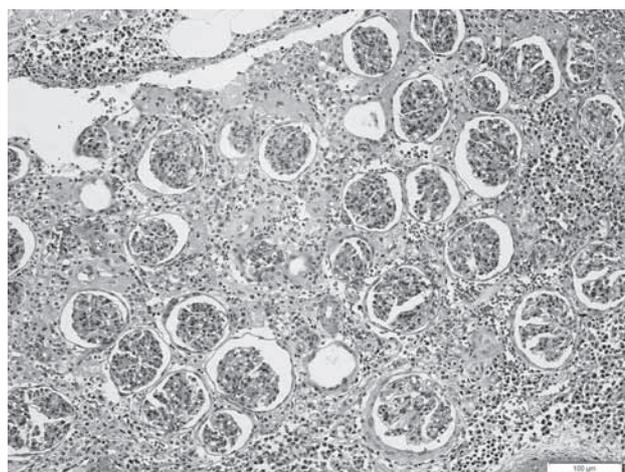


Figure 1b. Light micrograph showing nephrogenesis in Exp E-7 at 95 days post-transplantation, in which a piece of liver structure tissue had been grafted into the left kidney, followed by EGF injection. Accumulated glomerulogenesis and apoptotic tubulogenesis, accompanied by infiltrated lymphocytes, are shown at the border with host kidney. (At the time of sacrifice, on flow cytometry of the BM suspension, the Exp E-7 rat had the highest 49% of Thy-1-positive BM cells, while all the Exp E rats had $39 \pm 10\%$ of Thy-1-positive BM cells). (H-E stain).

rather reduced in the Exp G females; therefore, there was a possibility that, for a while, their kidney grafts functioned together with nephrogenic tissues in the liver structure grafts. Four Exp H males, which were injected with EGF 2 times, showed BW of 308 ± 16 g together with r-KW of 1.35 ± 0.05 g. Two (50%) rats, Exp H-1 and 3, exhibited purulent discharge at the time of sacrifice; Exp H-1 had

completely necrotic graft rejection. EGF effects on kidney graft survival were less favorable than TGF- β 1 effects. It was concluded that kidney graft survival was correlated with nephrogenesis. However, nephrogenic treatment itself did not help so much to prolong kidney graft survival. Although, normal r-KW also was basically related to the weight of excised left kidney not treated normal r-KW could be reflected by the functional results of the modified left kidney. R-KW enlargement was confirmed only in the Exp A males which were simply transplanted with liver grafts. Nephrogenesis in left kidney suppressed the r-KW.

Table 3 shows the FCM results of MLN, in which CD3, NFATc1, and STAT3 Abs labeled with FITC were applied for the FCM analyses of MLN. As control rat data differed between males and females, in table 3, positive cell % data are shown separately for males and females. CD3-, NFATc1-, and STAT3-positive cell % of control males (CM) (73 \pm 3%, 57 \pm 10%, and 41 \pm 5%, respectively) were lower than those of control females (CF) (81 \pm 5%, 96 \pm 5%, and 70 \pm 27%, respectively). Low % of CD3-, NFATc1-, and STAT3-positive cells of host lymphocytes could be expected to be associated with good nephrogenesis on the basis of weak allo-immune reactions. The lymphocytes of the Exp A males had reduced positive cell % of CD3 (68 \pm 6%) and STAT3 (37 \pm 13%), but not of NFATc1 (70 \pm 6%). The lymphocytes of the Exp D females that received TGF- β 1 injection had reduced positive cell % of CD3 (76 \pm 1%), NFATc1 (51 \pm 19%), and STAT3 (39 \pm 14%). The Exp G females with TGF- β 1 injection following kidney graft showed reduced lymphocyte % of CD3-positive (77 \pm 6%), NFATc1-positive (41 \pm 17%), and STAT3-positive (44 \pm 19%), while the Exp C males with

TGF- β 1 injection following BM grafts showed increased lymphocyte % of CD3-positive (83 \pm 12%), NFATc1-positive (87 \pm 9%), and STAT3-positive (77 \pm 13%). TGF- β 1 injection induced immune and intracellular signal suppression of host lymphocytes, although syngeneic BM grafts disturbed the actions, even in the rats injected with TGF- β 1. The Exp E females with EGF injection had reduced lymphocyte % of NFATc1 (68 \pm 24%) and STAT3 (62 \pm 28%), although CD3-positive cell % was increased to 87 \pm 8% in the Exp E females. The Exp F and H males, both of which were grafted with syngeneic kidney, followed by EGF injection, had reduced lymphocyte % of CD3 (72 \pm 4% and 73 \pm 5%, respectively), but had high % of NFATc1 and STAT3. In the Exp F rat, 40 μ g EGF more markedly suppressed STAT3 activity of MLN to 54 \pm 7% than in the Exp H rat injected with 12.5 μ g EGF (79 \pm 5%). The immune suppression of EGF was weaker than that of TGF- β 1, although both of them showed immune suppression.

Table 4 shows the corresponding FCM results of the BM and kidney suspensions. Thy-1 (CD90) and EGFR were measured using biotin-fluorescein streptavidin reactions. NFATc1 Ab was labeled with FITC. To clarify the data changes for the BM and kidney, in table 4, the data are also listed separately for males and females, as shown in table 3. Good nephrogenesis should lead to increased positive cell % of host Thy-1 (CD90) and EGFR. Five CFs had Thy-1-positive BM cells of 44%, 47%, 49%, 54%, and 68%, respectively, and two CMs had 54% and 71%. Thy-1-positive BM cells were generally reduced when liver grafts became necrotic. The lowest % (27 \pm 6%) of Thy-1-positive BM cells was observed in the Exp B females, which showed massive graft

Table 3. Flow cytometry of mesenteric lymph node (MLN) suspension CD3, NFATc1, and STAT3.

Exp no	Sex	Total (n)	Injection		MLN		
			TGF- β 1	HGF	CD3 (%)	NFATc1 (%)	STAT3 (%)
Cont	M	5	-	-	73 \pm 3	57 \pm 10	41 \pm 5
A	M	4	-	-	68 \pm 6	70 \pm 6	37 \pm 13
C	M	4	+	-	83 \pm 12	87 \pm 9	77 \pm 13
F	M	4	-	+	72 \pm 4	93 \pm 4	54 \pm 7
H	M	4	-	+	73 \pm 5	89 \pm 6	79 \pm 5
Cont	F	5	-	-	81 \pm 5	96 \pm 5	70 \pm 27
B	F	5	-	-	88 \pm 6	94 \pm 4	86 \pm 9
D	F	7	+	-	76 \pm 1	51 \pm 19	39 \pm 14
E	F	7	-	+	87 \pm 8	68 \pm 24	62 \pm 28
G	F	5	+	-	77 \pm 6	41 \pm 17	44 \pm 19

EGF: Epidermal growth factor, TGF- β 1: Transforming growth factor- β 1, NFATc1: Calcineurin-regulated nuclear factor of activated T cell c1, STAT3: Signal transducers and activators of transcription 3

Table 4. Flow cytometry of bone marrow (BM) suspension: Thy-1 and EGFR, and renal EGFR and NFATc1.

Exp no	Sex	Total (n)	BM (%)		Kidney (%)	
			Thy-1	EGFR	EGFR (EGFR ^a)	NFATc1 (NFATc1 ^a)
Cont	M	5	62 ^b	6±3	100±0.1 (99±1)	NT ^c
A	M	4	43±4	0.3±0.1	92±7 (80±6)	NT
C	M	4	40±5	0.3±0.1	96±1 (80±4)	95±2 (86±3)
F	M	4	36±10	1±0.3	94±5 (71±18)	NT
H	M	4	57±4	NT	99±0.3 (95±4)	99±0.2 (98±0.1)
Cont	F	5	53±1	13±4	99±0.2 (97±1)	99±1 (98±1)
B	F	5	27±6	1±0.4	97±1 (76±7)	95±3 (80±8)
D	F	7	60±5 ^d	6±3	98±1 (91±6)	NT
			43±3 ^e			
E	F	7	39±10	6±6	97±2 (89±7)	NT
G	F	5	57±7	2±1	100±0.2 (96±1)	100±0.2 (100±0.2)

a: The positive cell % is based on the strongly expressed peaks of control rats (see fig. 2b); b: n=2; c: Not tested; d: N=4; e: N=3
 NFATc1: Calcineurin-regulated nuclear factor of activated T cell c1, EGFR: Epidermal growth factor receptor (EGFR) Ab

necrosis. Thy-1-positive BM cell proliferation was observed in the 4 Exp D females, rats No 1, 3, 4, and 6 (60±5%), while Thy-1-positive cell % was decreased in the 3 Exp D females, rats No 2, 5, and 7 (43±3%), in which purulent discharge from the grafts was observed, compared with the proportion of 53±1% of control females. The Exp C males with TGF-β1 injection following BM grafts, which had poor nephrogenesis, showed 40±5% Thy-1-positive cells. The Exp G females with TGF-β1 injection following kidney graft, which had better nephrogenesis, showed 57±7% Thy-1-positive cells. Both the Exp F males and the Exp E females, which were injected with EGF, showed reduced expression of Thy-1-positive BM cells (36±10% and 39±10%, respectively). EGF injection inhibited Thy-1 expression of host BM cells. EGFR expression of BM cells was suppressed to less than 6% in all the experimental rats, which ranged from 6±3% to 13±4% in control rats. It appears that more than 6% of EGFR-positive BM cells might be required for good nephrogenesis. The EGFR-positive BM cells of the Exp D and Exp E rats were 6±3% and 6±6%, respectively, with better nephrogenesis. From the FCM results of BM cells, it was also judged that TGF-β1 was better than EGF at promoting nephrogenesis. Kidney expression of EGFR and NFATc1 was suppressed in the host kidney with grafts. EGFR expression was paralleled by NFATc1 expression in kidney. In kidney, the Exp D females showed EGFR at 98±1% (91±6%) and the Exp G females showed EGFR at 100±0.2% (96±1%), both of which were injected with TGF-β1. TGF-β1 acted to maintain EGFR expression on the tubular cells of the left kidney. From the FCM results of not only MLN, but also BM and kidney, TGF-β1 injection

was shown to exert a positive effect on nephrogenesis. EGF effects on nephrogenesis were more complicated than those of TGF-β1, based on the FCM results and the histopathological findings.

Figure 2a and figure 2b present the FCM results so that the FCM data can be understood more clearly. NFATc1 and STAT3 of MLN were activated when graft rejection

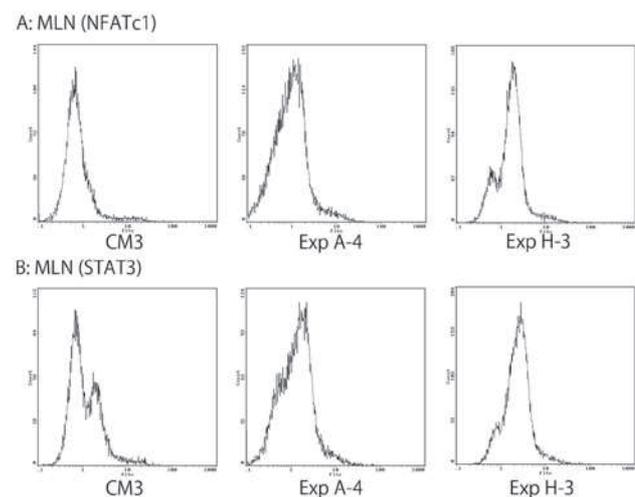


Figure 2a. The flow cytometry (FCM) results of mesenteric lymph node (MLN) suspension. At the top (A), MLN cells were stained with FITC-NFATc1 Ab. CM3, Exp A-4, and Exp H-3 rats showed 57%, 68%, and 81% NFATc1-positive MLN cells, respectively. At the bottom (B), MLN cells were stained with FITC-STAT3 Ab. CM3, Exp A-4, and Exp H-3 rats had 41%, 56%, and 84% STAT3-positive MLN cells, respectively. The border points between negative and positive peaks were decided using the results of Exp H-3 in A and CM3 in B. Parallel immune reactions were observed between NFATc1 and STAT3.

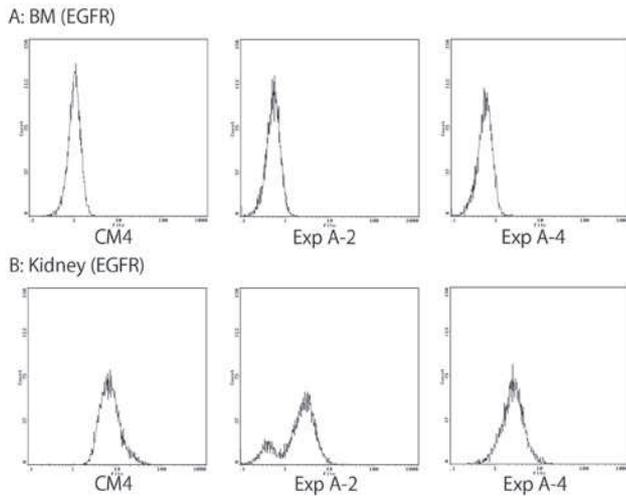


Figure 2b. The flow cytometry (FCM) results of bone marrow (BM) (A) and kidney (B) suspensions, both of which had been stained with fluoresced EGFR Ab. At the top (A), CM4, Exp A-2, and Exp A-4 rats had 10%, 0.3%, and 0.5% EGFR-positive BM cells. At the bottom (B), CM4, Exp A-2, and Exp A-4 rats had 100%, 80%, and 97% EGFR-positive kidney cells, when the border point of negative and positive peaks was decided using the result of Exp A-2. However, they had 100%, 70%, and 82% of EGFR-positive kidney cells, respectively, when the border point of EGFR-positive kidney cells was decided using the strongly positive result of CM4. Liver graft necrotic rejection reduced EGFR expression in BM and kidney.

occurred. It was shown that NFATc1 and STAT3 of MLN were inhibited when nephrogenesis occurred successfully. When there was stress on renal tubular EGFR, BM EGFR was also suppressed. For good nephrogenesis, renal and BM EGFR expression was necessary. The CD3 and Thy-1 results of FCM are not shown in any figures, but for both the positive cells were separated well from the negative cells, with the result that the FCM, positive cell % was calculated more exactly, and Thy-1 expression in host BM cells could be measured correctly.

To demonstrate the sites of NFATc1, nephrogenic cell suspension was stained with NFATc1 Ab-gold complexes at pre-fixing. Figures 3a, 3b, and 3c were obtained from the graft cell suspension of Exp G-2 female, which was injected with TGF- β 1 following a syngeneic kidney graft. However, as the syngeneic kidney graft cells were rejected, the glomeruli shown in these figures were derived from the same nephrogenic tissues. These three figures show glomerular mesangial cells stained positively with NFATc1 Ab-gold. The mesangial cells had high-density granules of NFATc1. Morphologically, two kinds of NFATc1 granules were observed. One pattern was granules with center holes that were negative or weak for NFATc1, while the other pattern was round granules without any NFATc1-negative hole. Another mesangial cell of the glomerulus showed many mitochondria with high-density NFATc1. In

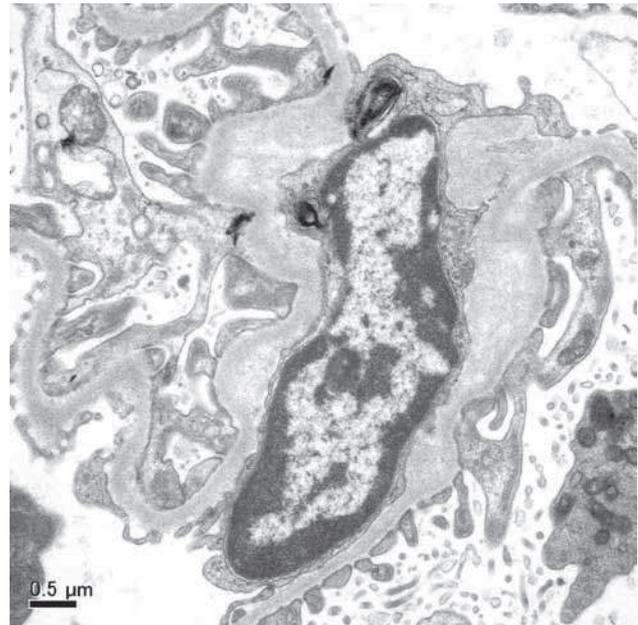


Figure 3a. Electron micrograph derived from the cell suspension of the nephrogenic tissue of the Exp G-2 rat, in which TGF- β 1 had been injected. This separated glomerulus belonging to the nephrogenic tissue was stained with NFATc1 Ab-gold at pre-fixation. The mesangial cell shown in this figure had two high-density NFATc1-positive granules with a central hole that was negative or weak for NFATc1 in the cytoplasm (uranyl acetate-lead citrate double stain).

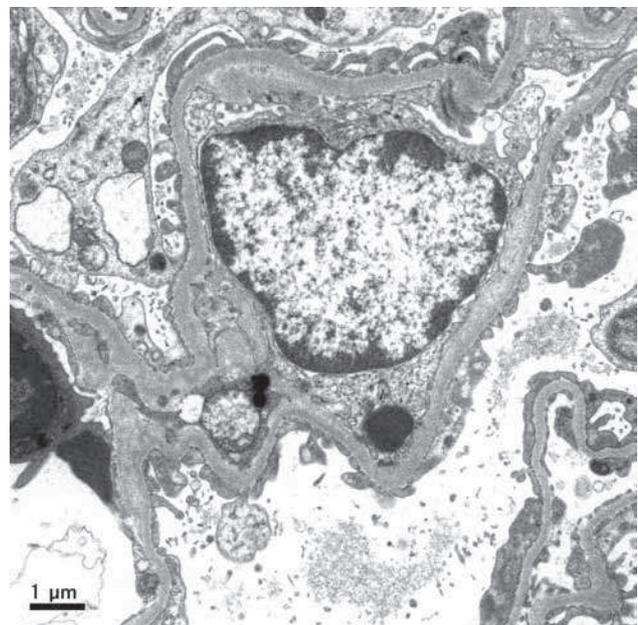


Figure 3b. Electron micrograph of Exp G-2 derived from the same cell suspension as figure 3a, which had been stained with NFATc1 Ab-gold at pre-fixation. This figure shows two kinds of mesangial cells, among which the smaller type was generated later. The other mesangial cell was mature. The small mesangial cell has two high-density round granules stained with NFATc1 Ab-gold. The mature mesangial cell has a large and lower-density granule stained more faintly with NFATc1 Ab-gold (uranyl acetate-lead citrate double stain).

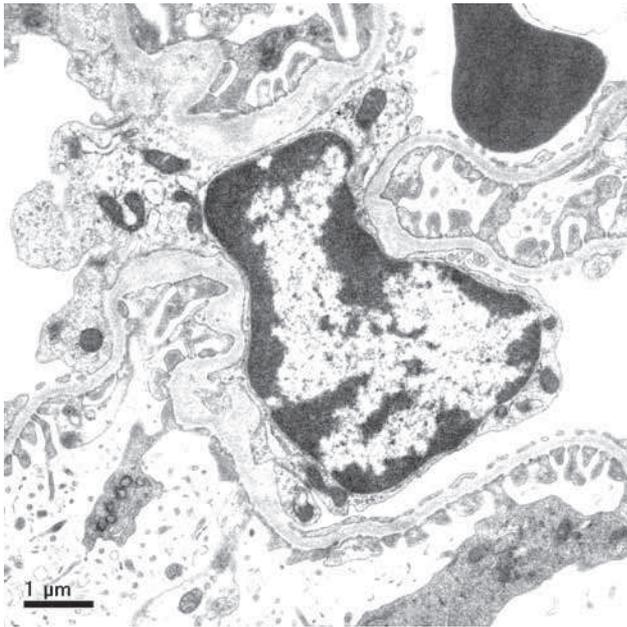


Figure 3c. Electron micrograph of Exp G-2 derived from the same cell suspension as figures 3a and 3b. The mesangial cell has many mitochondria with high-density NFATc1. NFATc1 was also deposited in the mitochondria of podocyte process (uranyl acetate-lead citrate double stain).

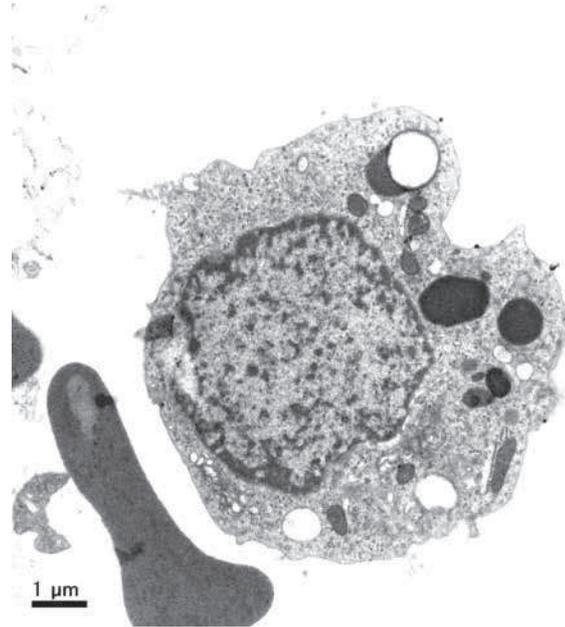


Figure 4b. Electron micrograph derived from the same cell suspension as shown in figure 4a of Exp rat D-6. This more mature tubular cell compared with the figure 4a cell still has small NFATc1-positive granules, but many activated NFATc1 were detected as enlarged granules, in which a lot of small and high-density NFATc1-positive granules were contained (uranyl acetate-lead citrate double stain).

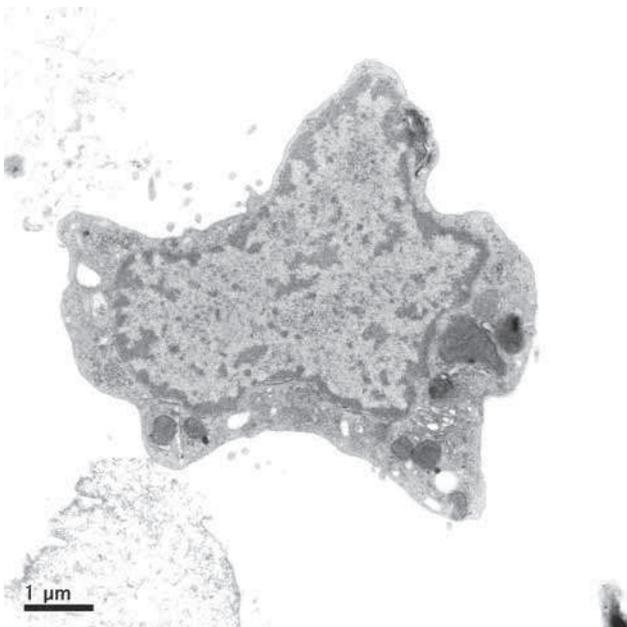


Figure 4a. Electron micrograph obtained from the cell suspension of the nephrogenic tissue of Exp D-6, which had been injected with TGF- β 1 following liver structure tissue transplantation. The preparation was stained with NFATc1 Ab-gold before fixing. A mesenchymal cell is presented in this figure: Several small NFATc1-positive granules can be recognized around mitochondria in the cytoplasm. At the upper right, the cytoplasm also had a collapsing NFATc1-positive granule, similar to those of the mesangial cell shown in figure 3a, but was destroyed. Mesenchymal cells expressed small NFATc1, which was not activated in the cytoplasm, but connected with mitochondria (uranyl acetate-lead citrate double stain).

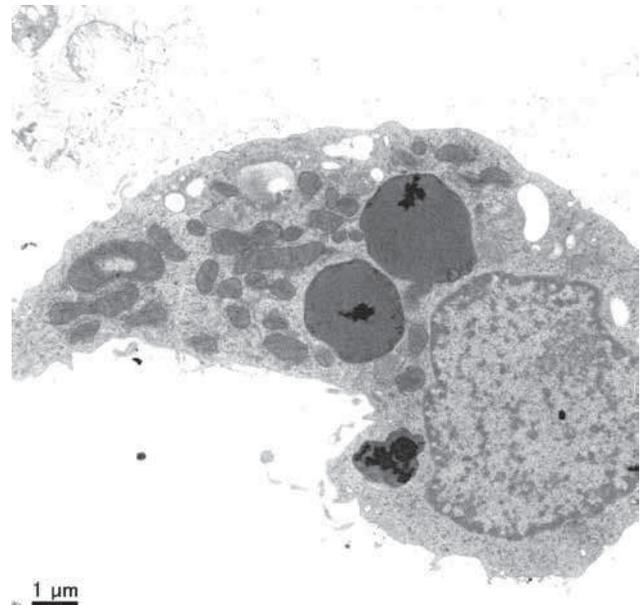


Figure 4c. Electron micrograph for the same cell suspension of the Exp D-6 rat, as shown in figures 4a and 4b. The nephrogenic mature cell of proximal convoluted tubule had three more activated NFATc1-positive granules than in figure 4b. Aggregated NFATc1-positive masses were detected in a large lysosome (uranyl acetate-lead citrate double stain).

the podocyte process, many NFATc1-positive mitochondria were observed. Figures 4a, 4b, and 4c were derived from the nephrogenic cell suspension of the Exp D-6 female, which had been injected with TGF- β 1. Mesenchymal cells, more mature tubular cells than the former, and mature tubular cells showed NFATc1 granules in their cytoplasm. Mesenchymal cells showed small NFATc1-positive granules around or in mitochondria. It was shown that mesenchymal cells had small NFATc1-positive granules with a connection with mitochondria. More mature tubular cells still had small NFATc1-positive granules around or in the mitochondria, but the small NFATc1-positive granules were gradually enlarged in the destroyed mitochondria. Large NFATc1-positive granules were detected in the cytoplasm of more mature tubular cells. In mature tubular cells, NFATc1-positive granules could be described as follows: Mature tubular cells had large lysosomes containing aggregated NFATc1. Newly generated mesangial cells, podocytes, and tubular cells showed NFATc1 with changeable patterns. It was shown that NFATc1 was deposited in mitochondria, and the mitochondria were destroyed by the deposition. High NFATc1 expression on renal cells indicated a kind of destructive metabolism.

To confirm the nephrogenesis more precisely, TEM analyses were applied to specimens from the Exp A-4 male, and the Exp D-1 and -4 females. Figure 5a and figure 5b were

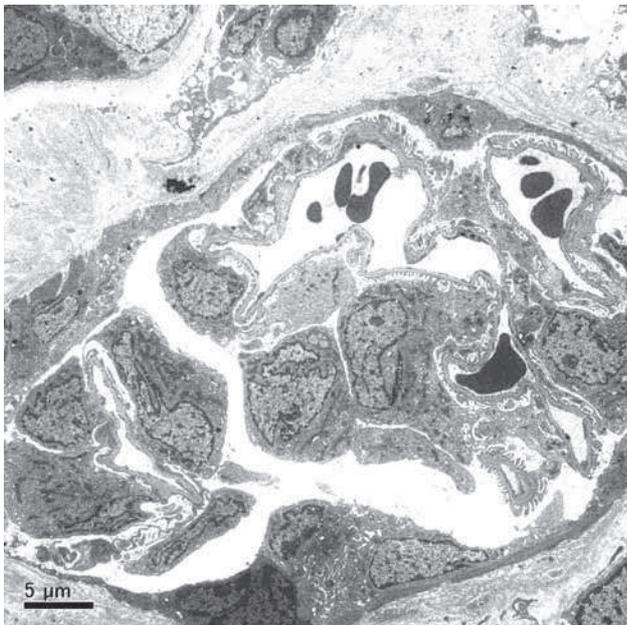


Figure 5a. Electron micrograph obtained from the Exp A-4 rat, which had been transplanted a piece of liver structure tissue, but without TGF- β 1 or EGF injection. An incompletely generated glomerulus is shown (uranyl acetate-lead citrate double stain).

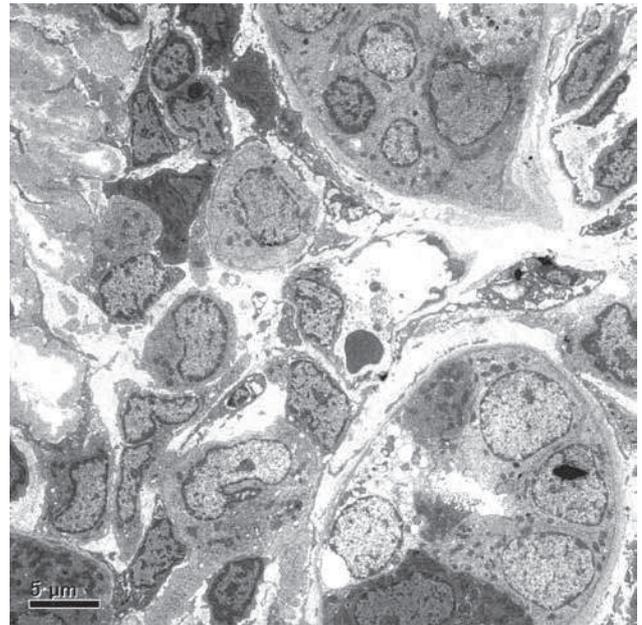


Figure 5b. Electron micrograph obtained from the same nephrogenic tissue of Exp A-4 as shown in figure 5a. Generated renal tubular cells were present diffusely without formation of a complete tubule on the left side of the complete tubules, which contained both newly generated tubular cells and apoptotic tubular cells at the same time (uranyl acetate-lead citrate double stain).

obtained from the nephrogenic area of the Exp A-4 male. The Exp A-4 rat showed Thy-1-positive BM cells of 44% at the time of sacrifice. Incompletely generated glomerular and incompletely generated tubular cells were shown. From renal mesenchymal cells, new glomerular and tubular cells developed in the transplanted liver structure tissues. Detected renal tubules showed a mixture of apoptotic cells and developing new cells. In the Exp A-4 male without TGF- β 1, tubular cells were observed diffusely without forming a complete tubule. Figure 6a and figure 6b were obtained from the Exp D-1 female, which had been injected with TGF- β 1. The Exp D-1 rat showed Thy-1-positive BM cells of 63% and EGFR-positive BM cells of 8% at the time of sacrifice. The nephrogenesis, especially tubulogenesis, of the Exp D-1 female was more active than that of the Exp A-4 male. In the TEM analyses, the female showed renal mesenchymal cells derived from the activated endothelial cells of the vessels in the liver structure graft. TGF- β 1 actively promoted nephrogenesis. Proliferated mesenchymal cells moved out from a donor liver vessel and matured at the outside of the vessel, resulting in new nephron formation. Disrupted endothelial cells had spindle-shaped morphology with projected fibers. New renal tissues were generated from the mesenchymal cells which had transitioned from donor endothelial cells. Figure 7a and figure 7b were ob-

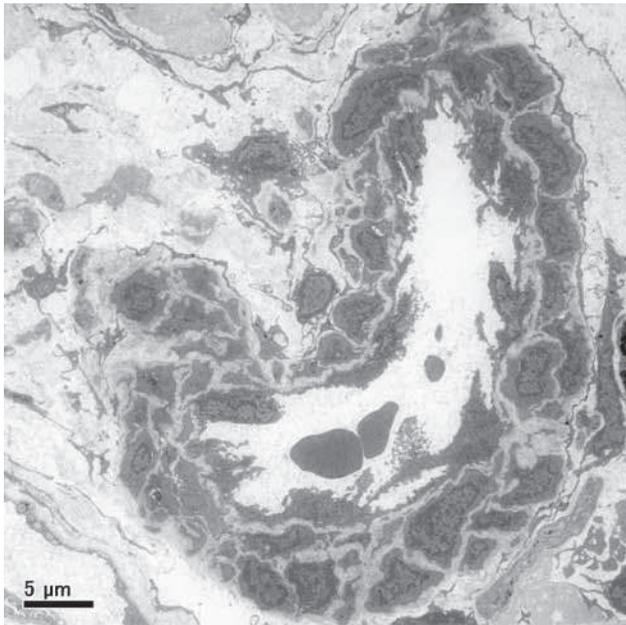


Figure 6a. Electron micrograph derived from the Exp D-1 rat, which had been injected with TGF- β 1 following liver structure tissue transplantation. Many renal mesenchymal cells showing spindle-shaped morphology have proliferated around a vessel in the liver structure graft. In the vessel, the mesenchymal cells have projecting fibrous cytoplasm, which was later disrupted. Multiple layers of mesenchymal cells suggested new glomerular formation (uranyl acetate-lead citrate double stain).

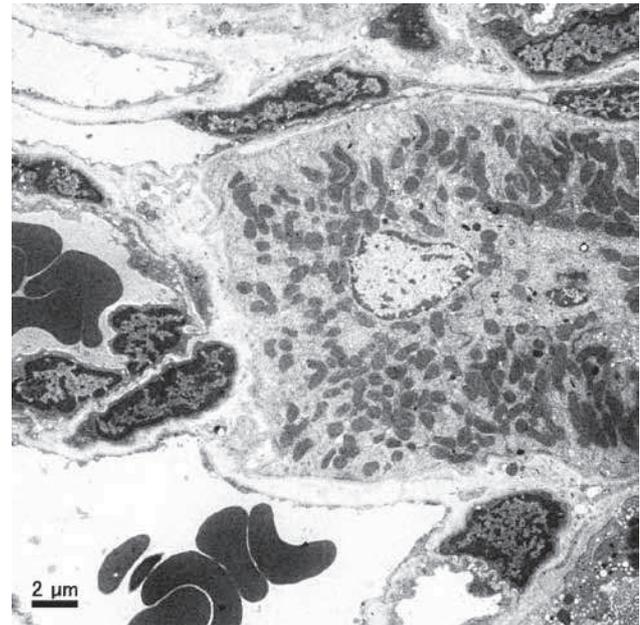


Figure 7a. Electron micrograph derived from the Exp D-4 rat, which had been injected with TGF- β 1 following liver structure tissue transplantation. At least two mesenchymal cells from normal blood vessels, two of which are shown at the left-middle, and one of which is shown at the lower-right, would later penetrate a mature proximal convoluted tubular cell. The two mesenchymal cells had similar morphology to the two activated endothelial cells shown at the left-middle of the picture (uranyl acetate-lead citrate double stain).

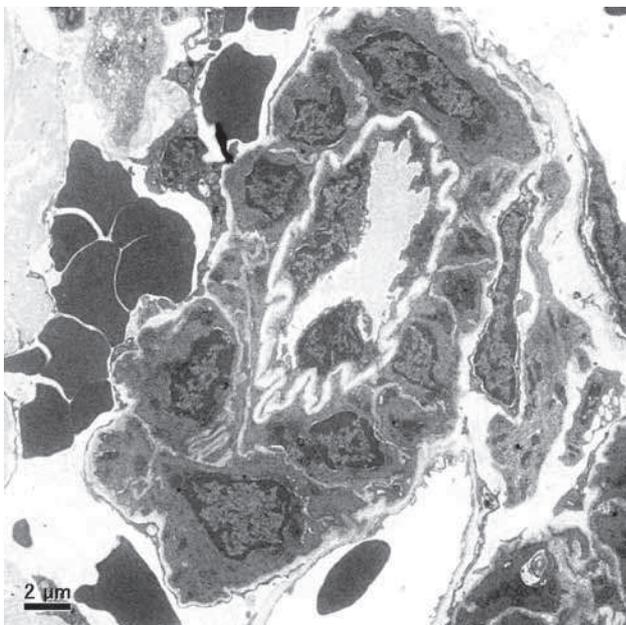


Figure 6b. Electron micrograph obtained from the same nephrogenic area of the Exp D-1 rat as shown in figure 6a, showing more mature mesenchymal cells forming a new renal tubule. Although a few endothelial cells still remained in the blood vessel, the vessel wall would later disappear completely to be replaced by a new tubule (uranyl acetate-lead citrate double stain).

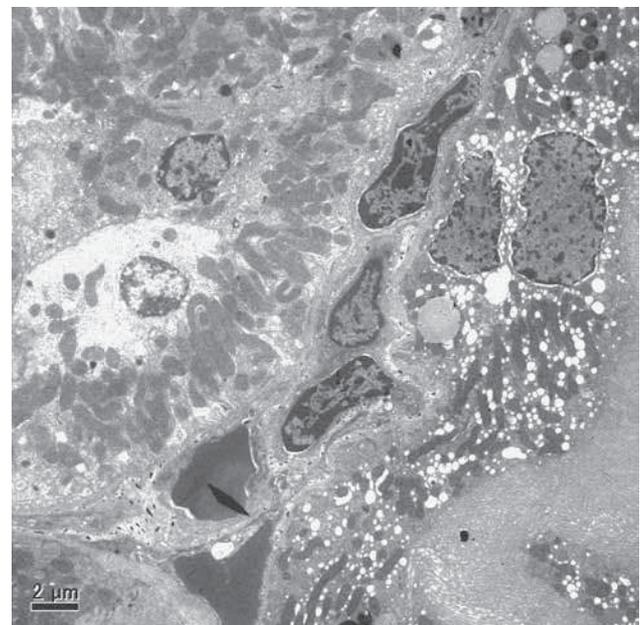


Figure 7b. Electron micrograph from the same Exp D-4 rat as in figure 7a. At the right side of this figure, apoptotic tubular cells are shown, while at the left side, more normal proximal convoluted tubular cells are present. Between the two kinds of tubular cells, 4 immature stem cells, one of which appears only a little at the upper part, are detected without any destroyed vessels. Other two, which are more mature and apoptotic, observed in the apoptotic tubular cells on the right side (uranyl acetate-lead citrate double stain).

tained from the Exp D-4 female, which had been injected with TGF- β 1. The Exp D-4 rat showed 65% Thy-1-positive BM cells and 6% EGFR-positive BM cells at the time of sacrifice. This female's nephrogenic tissue showed that mesenchymal cells moved out from normal graft vessels and penetrated mature proximal convoluted tubular cells. The mesenchymal cells showed a morphological similarity to activated endothelial cells. Several of the mesenchymal cells that moved out from a normal vessel matured at the borders with apoptotic proximal convoluted tubular cells and penetrated the apoptotic tubular cells to repair the tubule, in which host BM-derived mesenchymal cells were suspected on the basis of the high percentage of Thy-1-positive BM cells. Mesenchymal cells reacted not only to develop new nephrons, but also to repair the apoptotic tubule. Endothelial to mesenchymal transition (EndMT) accelerated by TGF- β 1 injection was the most important factor for nephrogenesis in liver grafts.

DISCUSSION

Under exogenous TGF- β 1 administration *in vitro*, human tubular epithelial cells (HUVEC) showed dedifferentiation, undergoing epithelial to mesenchymal transition (EMT).⁸ A study in 2008 reported a possible switch from endothelial cells to mesenchymal profibrotic cells, that is, EndMT, in the perivascular and myocardial interstitial compartments.⁹ The present study showed that a transition of liver vessel endothelial cells to renal mesenchymal cells (EndMT) was triggered in liver structure grafts of adult rats transplanted into kidney, and that TGF- β 1 injection more effectively induced nephrogenesis than EGF injection.

As syngeneic liver structure tissues, from which hepatocytes had been removed, were used as the grafts transplanted into the partially excised host kidney in order to trigger nephrogenesis, the effects of EGF and TGF- β 1 on the liver structure graft itself were considered. It has been described that liver EGF receptor plays critical molecular roles together with c-Met to control liver oval cell proliferation and survival,¹⁰ but c-Met can mediate oval cell proliferation and survival without the help of EGF receptors in adult mice. Conversely to EGF, it has been reported that TGF- β 1 is a major inhibitor of mitogen-stimulated DNA synthesis in cultured hepatocytes.¹¹ Removed liver grafts surely have induced TGF- β 1 mRNA. Furthermore, matricellular protein thrombospondin-1 (TSP-1), which is expressed more by oxidative stress in the endothelial cells of liver grafts, mediates aggressive activation of latent TGF- β 1, resulting in suppression of hepatocyte proliferation. It has also been observed that TSP-1 deficiency accelerated hepatocyte

proliferation via p21 downregulation, accompanied by attenuated levels of phosphorylated Smad2. Liver TSP-1 deficiency, which induces Smad2 attenuation, must react against nephrogenesis, as discussed below. In this study, with TGF- β 1 injection, the remaining hepatocytes were not induced to proliferate at all, while with EGF injection, the remaining hepatocytes were induced to proliferate. Massive liver graft necrosis in the rats that received EGF might hint at the hepatocyte proliferation of the grafts. From the point of view of graft reactions, TGF- β 1 was judged to be better than EGF for nephrogenesis.

In a male not given EGF, but in which massive graft necrosis was induced, c-Met-mediated hepatocyte proliferation was considered to have occurred actively. On the other hand, endothelial cells in males given neither TGF- β 1 nor EGF caused nephrogenesis on the basis of *in vivo* TGF- β 1 pathways. Two (50%) males of the 4, which were not injected with either TGF- β 1 or EGF, did not show graft necrosis, but the other 2 (50%) had liver graft necrosis. Among 7 rats with EGF, in the 3 (43%) of EGF rats showing nephrogenesis, TGF- β 1/Smad2 and 3 signaling pathways must be superior to stimulated EGF/EGF receptor signaling pathways, but the other 4 (57%) had necrotic liver grafts. Even in the rats given TGF- β 1, the 7 rats could be classified as the best nephrogenetic rats (57%) and those (43%) with small necrotic grafts. No pieces of syngeneic kidney wrapped with liver structure grafts could survive longer in the host kidney. It was concluded that at the beginning of liver structure transplantation, the hosts of 40–60% have induced superior TGF- β 1/Smad2 and 3 signaling pathways to EGF-c-Met signaling pathways as an individual difference. Glomerular parietal epithelial cells (GPECs), that is, podocyte precursors lining the inner aspect of the Bowman's capsule, have been shown to revert to embryonic phenotype in response to renal injury.¹² CD24, CD44 and CD29 in GPECs could be indicated to be important cell surface antigens to generation of EMT in adult murine kidney. In this study, in the rats with good glomerulogenesis, strong expression of CD24, CD44 and CD29 surface antigens was suggested in the GPECs. Renal and cardiac mesenchymal stem cells (MSC)-like populations showed strong congruity with BM MSC (Sca1+CD29+CD44+CD90.2+) in adult mice.¹³ In this study, the rats with the best nephrogenesis showed high levels of CD90-positive BM cells. In some control rats, also, high levels of CD90-positive BM cells were measured. Therefore, it was concluded that the rats with high levels of Thy-1 (CD90)-positive BM cells might be expected to show good nephrogenesis. For EndMT, host rats should still have remained high levels of MSC antigens.

Subsequently, the effects of host BM-derived EndMT cells

on nephrogenesis were considered.¹⁴ From the increased percentage of Thy-1 (CD90)-positive BM cells, the high percentage of BM-derived EndMT cells has been deduced. The TGF- β 1 treated rats, which showed good tubulogenesis, but not the EGF treated rats, had a higher percentage of Thy-1-positive BM cells than the control rats even at the time of sacrifice. Although it was difficult to differentiate host-derived BM EndMT cells from donor-derived liver EndMT cells in the liver grafts, a single mesenchymal cell derived from a normal liver vessel was suspected more likely to be a host-derived BM EndMT cell. Host-derived BM EndMT cells must be used for the repair of apoptotic cells. Host BM-derived mesenchymal cells of the B cell line must attenuate allo-immune reactions to the nephrogenic tissues.¹⁵ The FCM results of the MLN suspension demonstrated that T cell immune reactions were suppressed in the TGF- β 1 rats. It has been reported that TGF- β 1 inhibits IL-2-dependent tyrosine phosphorylation of T cells, resulting in the inhibition of T cell proliferation.¹⁶ Conversely, in EGF treated rats, host-derived BM EndMT cells could not be judged clearly. Thy-1-positive BM cells were not increased in the FCM analyses of EGF treated rats. Nephrogenetic cells of EGF treated rats were rejected more severely by host lymphocytes than those of TGF- β 1 treated rats. From the point of view of host-derived BM EndMT and immune reactions, TGF- β 1 injection was also superior to EGF injection for nephrogenesis. As nephrogenic tissues survived longer at the border with the host normal kidney, host renal mesenchymal cells induced by TGF- β 1 appeared to have moved to the liver structure graft and contributed to the survival of nephrogenesis.

TGF- β /Smad signaling mechanisms have been considered in adult rats at the molecular level. Recently, molecular regulation of endothelial cell plasticity by TGF- β was reported in detail.^{17,18} TGF- β signaling mechanisms to lead to EndMT were summarized as follows: TGF- β binds to both the heterotetrameric TGF- β type II receptor complex with TGF- β type I receptor of activin receptor-like kinase 5 (ALK5) and the equivalent complex with ALK1. TGF- β R-Smads (Smad2 and 3) have serine/threonine kinase components. ALK5 phosphorylates the C-terminal phosphates of important substrates TGF- β R-Smads (Smad2 and 3), in which activated Smad2 and 3 form complexes with common-mediator (Co)-Smad4. Smad2, 3, and 4 complexes are translocated to the nucleus to regulate the transcription of specific genes. Smad2, 3, and 4 complexes bind to DNA sequences in Snail1 promoter. C-Myc of a negative regulator of growth inhibitory responses cooperates with Smad complexes to induce expression of transcription factor Snail1, assisted by high-mobility-group

protein A2 (HMGA2) and p300 (co-activator), resulting in EMT. Although TGF- β 1/Smad signaling might be associated with carcinogenesis, donor liver cell EndMT in this study was induced by the expression of Snail1, resulting in nephrogenesis. The complex process of EndMT involves disruption of polarized endothelial morphology into cells with spindle-shaped morphology with formation of actin stress fibers. EndMT ends in reduced cell-cell junction and decrease of fibroblast-specific protein (FSP), a smooth muscle actin (SMA), and neural cadherin (N-cadherin), in which Smad2 and β -catenin cause a critical event. In this study, morphological EndMT changes were demonstrated well in liver graft TEM analyses. ALK1 signaling of endothelial cells stimulates endothelial cell migration and proliferation with the interactions of BMP R-Smads (Smad1, 5, and 8). Mesenchymal cell migration and proliferation were also indicated by the findings of the liver graft TEM analyses in this study.

Non-canonical calcium/NFAT wingless-type (Wnt) signaling is required for complete mesenchymal to epithelial transition (MET) during nephrogenesis by potentially acting Wnt4 expressed in the condensing MM.¹⁹ Among calcium-regulated NFATc proteins, NFATc4 exhibited the highest amounts during early nephrogenesis. G-protein-coupled receptor 54 (Gpr54), which was expressed in condensed mesenchyme at E12.5, and epithelial cells of tubules at E17.5 in mice, regulated BMP7 expression through NFATc1 and specificity protein 1 (Sp1). Dephosphorylated NFATc1 and Sp1 proteins by Gpr54-regulated Ca²⁺ activation can bind to the promoter regions of BMP7 to regulate the transcription. NFATc1 is therefore associated with embryonic kidney branching, nephrogenesis, and glomerular development.²⁰ In this study, glomerulogenetic mesangial cells and podocytes expressed NFATc1 in their cytoplasm without any clear pathological changes. It was reported that activated NFATc1 in glomerular mesangial cells enhanced membrane type 1-matrix metalloproteinase (MT1-MMP) transcription, and caused proteolytic events of acute glomerulonephritis, which might be later resulted in glomerulosclerosis.²¹ Co-operative interactions between NFATc1 and the zinc finger transcription factors located in the MT1-MMP promoter enhanced transcription of MT1-MMP, MMP2 activator. Mutations (P112Q, R895C, and E897K) of transient receptor potential channel C6 (TRPC6), which were associated with constitutive activation of calcineurin-NFAT (NFATc1, 3, and 4)-dependent transcription in podocytes, mediated focal segmental glomerulosclerosis (FSGS).²² The mutant TRPC6 was associated with FSGS through excess activation of calcineurin-NFAT pathway in podocytes. Using generated mice that allowed conditional induction of

NFATc1, it was shown that NFATc1 activation had caused progressive proteinuria and FSGS.²³ As shown by the TEM analyses with staining with NFATc1 Ab-gold at prefixing, NFATc1 apoptosis in renal cell cytoplasm was judged to be an important feature to maintain their prolonged survival. Aggregated NFATc1 was observed in a large lysosome in NFATc1 apoptosis. FCM analyses in this study suggested reduced NFATc1 expression of normal tubule cells, when necrotic graft rejection was observed in the same kidney. In this study, an important role of NFATc1 was shown in

post-development nephrogenesis.

For clinical application in humans, auto-liver structure grafts were suggested to be better than allo-liver structure grafts. Furthermore, *in vitro* nephrogenesis was considered to be better than *in vivo* nephrogenesis in humans, which should be established in the future. Only successful nephrogenetic tissues will be available for human transplantation, and TGF- β 1 side effects in humans can be rendered avoidable.

ΠΕΡΙΛΗΨΗ

In vivo νεφρογένεση σε αρουραίους Lewis από τα ενδοθηλιακά κύτταρα των γενετικά όμοιων μοσχευμάτων ήπατος που μεταμοσχεύτηκαν σε μερικώς εκτομηθέντα νεφρό: Ο ρόλος του τροποποιητικού αυξητικού παράγοντα (TGF)- β 1 και του παράγοντα Thy-1 (CD90)

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ΣΚΟΠΟΣ Η *in vitro* νεφρογένεση με χρήση των κυττάρων του ίδιου του οργανισμού αποτελεί ιδανική υποκατάσταση για αλλογενή πλήρη μεταμόσχευση του νεφρού. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Σε ενήλικες αρουραίους Lewis μεταμοσχεύτηκαν μοσχεύματα γενετικά όμοια με τη δομή του ήπατος, από τα οποία αφαιρέθηκαν τα ηπατοκύτταρα, σε κάποιο σημείο εκτομής του αριστερού νεφρού. Στη συνέχεια πραγματοποιήθηκε έγχυση είτε του τροποποιητικού αυξητικού παράγοντα (TGF)- β 1 είτε του επιδερμικού αυξητικού παράγοντα (EGF) στους ξενιστές. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Σε 7 αρουραίους, οι οποίοι έλαβαν συνολικά 60 μ g του TGF- β 1 ο καθένας, παρατηρήθηκε καλή νεφρογένεση. Μεταξύ αυτών, οι 4 (53%) αρουραίοι με την καλύτερη νεφρογένεση εμφάνισαν υψηλό ποσοστό κυττάρων από το μυελό των οστών ($60 \pm 5\%$) θετικών στο Thy-1 (CD90), που μετρήθηκαν με κυτταρομετρία ροής. Στους μεσευκτέριους λεμφοκύτταρους τους, οι 7 αρουραίοι με τον παράγοντα TGF- β 1 εμφάνισαν μειωμένα ποσοστά κυττάρων θετικών στο CD3 ($76 \pm 1\%$), κύτταρα θετικά στο ενεργοποιημένο T κύτταρο c1 (NFATc1) του πυρηνικού παράγοντα που ρυθμίζεται από την καλσινευρίνη ($51 \pm 19\%$) και μετατροπείς σήματος, καθώς και ενεργοποιητές των θετικών κυττάρων της μεταγραφής 3 (STAT3) ($39 \pm 14\%$). Στις αναλύσεις με το ηλεκτρονικό μικροσκόπιο μεταβίβασης (TEM), θα μπορούσε να παρατηρηθεί μετατροπή ενδοθηλίου προς μεσέγγυμα στα αιμοφόρα αγγεία του ηπατικού μοσχεύματος, τα οποία έδειξαν ογκώδη πολλαπλασιασμό των μεσεγγυματικών κυττάρων γύρω από ένα κατεστραμμένο αγγείο προκειμένου να σχηματιστεί ένας νέος νεφρώνας. Στις αναλύσεις των δειγμάτων με το TEM μέσω χρώσης με το χρυσό αντίσωμα NFATc1 πριν από τον καθορισμό, η έκφραση NFATc1 εντοπίστηκε σε πρόσφατα μεσαγγειακά κύτταρα, σε ποδοκύτταρα και σε σωληνοειδή κύτταρα. Τα μεσεγγυματικά κύτταρα είχαν μικρούς κόκκους NFATc1. Το NFATc1 που συγκολλάται σε ένα μεγάλο λυσόσωμα εμφανίστηκε στα καλώς αναπτυγμένα εγγύς εσπειραμένα σωληνοειδή κύτταρα. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Η αυξημένη σηματοδότηση του παράγοντα TGF- β 1 προκειμένου να τεθεί σε λειτουργία η μετατροπή ενδοθηλίου προς μεσέγγυμα ήταν πολύ σημαντική για τη νεφρογένεση σε συνδυασμό με την υψηλή έκφραση του Thy-1. Η ένεση του επιδερμικού αυξητικού παράγοντα συσχετίστηκε με λιγότερο ομοιόμορφα αποτελέσματα.

Λέξεις ευρητηρίου: Αρουραίοι, Επιδερμικός αυξητικός παράγοντας, Μετατροπή ενδοθηλίου προς μεσέγγυμα, Νεφρογένεση, Τροποποιητικός αυξητικός παράγοντας β 1

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