

Title: Human iPSC-derived nephron progenitor cells treat AKI and CKD in mouse models

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OVERLINE:

One Sentence Summary: Expanded human iPSC-derived nephron progenitor cells have therapeutic potential for AKI and CKD by secreting reno-protective factors.

Editor's summary:

Abstract: The number of patients requiring dialysis therapy continues to increase worldwide due to the lack of effective treatments for chronic kidney disease (CKD). Furthermore, no curative treatments for acute kidney injury (AKI) have been established. The therapeutic effects of human induced pluripotent stem cell-derived nephron progenitor cells (hiPSC-NPCs) on AKI have been reported in mice but not clinically confirmed. There are also no reports examining the therapeutic potential of hiPSC-NPCs on CKD. Although large numbers of uniform hiPSC-NPCs are required for cell therapies for AKI and CKD, effective expansion cultures remain to be developed. Here, we established a CFY (CHIR99021, FGF9, and Y-27632) medium for cell culture that enables more than 100-fold proliferation of hiPSC-NPCs from multiple hiPSC lines in two passages. We demonstrated that hiPSC-NPCs expanded by CFY or their conditioned medium alone attenuate kidney injury and improve survival in cisplatin-induced AKI mice. We also observed that hiPSC-NPCs prevented kidney functional decline, interstitial fibrosis, and senescence in aristolochic acid-induced CKD mice. In addition, we discovered c-MET as a specific cell surface marker for hiPSC-NPCs and confirmed that purified c-MET⁺ hiPSC-NPCs have therapeutic effects on AKI and CKD. Furthermore, we found that hiPSC-NPCs exerted their therapeutic effects in AKI and CKD mice by secreting vascular endothelial growth factor A (VEGF-A). Together, expanded hiPSC-NPCs are useful cell therapies for AKI and CKD, and may open new avenues for treating kidney diseases.

Main Text:

INTRODUCTION

Chronic kidney disease (CKD) is a pathological condition characterized by glomerulosclerosis and interstitial fibrosis with chronic renal dysfunction, which can be caused by a variety of kidney diseases. CKD reduces the quality of life and prognosis of patients by also increasing the incidence and mortality of heart diseases and stroke (1, 2). In addition, the increased number of patients with CKD is one of the causes of worsening medical economics due to the increased number of patients on dialysis and patients with various complications associated with kidney diseases (3). Acute kidney injury (AKI) has recently attracted attention as a cause of CKD (4). AKI can develop suddenly, and affected kidneys are typically expected to recover fully after a certain period. However, a recent meta-analysis demonstrated that AKI worsens to CKD and end-stage renal disease (ESRD) in 25.8% and 8.6% of patients, respectively (4). Furthermore, AKI has been reported to increase multiple organ failure with high mortality and cardiovascular events (5, 6). Therefore, therapeutic interventions during the acute and subacute AKI phases, which determine the development of CKD and ESRD, are essential (4).

Cell therapy has recently received much attention as a treatment for AKI and CKD, but clinical trials using mesenchymal stem cells (MSCs) have not shown satisfactory effectiveness (7). Furthermore, safety concerns exist since several studies reported that MSCs differentiated into adipose tissues in the glomeruli, promoted breast cancer metastasis, and had the potential to increase renal interstitial fibrosis after cell transplantation (8-10). Human induced pluripotent stem cells (hiPSCs) can be used to produce nephron progenitor cells (NPCs) that are capable of differentiating into kidney lineage cells (11-16). Furthermore, cell therapies using hiPSC-derived NPCs (hiPSC-NPCs) attenuate the kidney damage caused by AKI and prevent renal interstitial

fibrosis after AKI in mice (14, 15, 17). In addition, from a safety standpoint, they are an expected new source of cell therapy for kidney diseases because hiPSC-NPCs differentiate only into kidney lineage cells. However, hiPSCs require costly reagents for their maintenance and differentiation into hiPSC-NPCs, which may vary in quality (15, 18). Therefore, expansion cultures that enable a large supply of NPCs with homogeneous quality are required. Brown *et al.* reported that human embryonic stem cell (hESC)-derived NPCs lose their ability to differentiate into glomeruli after two passages (19). Tanigawa *et al.* could not passage ITGA8⁺PDGFRA⁺SIX2⁺ NPCs generated from SIX2-reporter hiPSC lines more than once, nor could they maintain the cells for longer than two weeks (20). Although Li *et al.* succeeded in expanding SIX2⁺ hiPSC-NPCs generated from a SIX2-reporter hiPSC line in NPSR medium for two months, Tanigawa *et al.* reported difficulty in reproducing the results (20, 21). Furthermore, the expansion culture conditions developed by Li *et al.* and Tanigawa *et al.* can be used only for NPCs generated from SIX2-reporter hiPSC lines, which limits their use (20, 21). Hence, there are no expansion culture protocols applicable to multiple hiPSC lines (table S1). Moreover, hiPSC-NPCs are highly heterogeneous (22), and cell surface markers that purify expandable NPC populations have not been identified, thus making it ever more difficult to achieve long-term expansion cultures of hiPSC-NPCs. Therefore, cell therapeutic strategies for AKI and CKD requiring large numbers of hiPSC-NPCs have been difficult to develop. To address these key issues for clinical application, we aimed to develop methods to expand hiPSC-NPCs that maintain therapeutic efficacy, identify cell surface hiPSC-NPC markers for eliminating off-target cells, determine the therapeutic effects of expanded hiPSC-NPCs and CM produced by hiPSC-NPCs on kidney diseases, and elucidate their therapeutic mechanisms.

RESULTS

hiPSC-NPCs display therapeutic effects on AKI in mice

We first examined NPCs derived from human fetal kidneys (hfNPCs), with the differentiation potential to form kidney organoids, and expanded in NPSR medium using AKI model mice (21). Since it was reported that mouse embryonic kidney-derived NPCs (mNPCs) differentiated towards kidney organoids for 2 days in an air-fluid interface culture have therapeutic effects (21), we differentiated hfNPCs for 2 days in the same culture condition (fate-specified hfNPCs; FS-hfNPCs) and transplanted them into the renal subcapsules of cisplatin-induced AKI (cis-AKI) mice (fig. S1A). The FS-hfNPC transplantation suppressed the elevation of blood urea nitrogen (BUN) and serum creatinine (S-Cre) and histological changes associated with AKI, such as tubular necrosis, tubular dilation, urinary cast, and loss of tubular borders (fig. S1, B to D), suggesting that highly pure hNPCs have therapeutic potential for AKI.

We then transplanted hiPSC-NPCs generated using our previously reported differentiation method into the renal subcapsules of cis-AKI mice after differentiating them towards kidney organoids for 2 days (hiPSC-FS-NPCs) but found no obvious therapeutic effects (16) (Fig. 1, A to F). Considering possible differences in the differentiation status of hiPSC-NPCs compared with expanded hfNPCs, we transplanted hiPSC-NPCs without induction towards kidney organoids into the renal subcapsules of cis-AKI mice. Without induction, the therapeutic effects (Fig. 1, E to H) and the survival of the mice were improved compared to control mice with saline injection (Fig. 1I). These results suggest that hiPSC-NPCs, as well as differentiated hfNPCs, have therapeutic potential on AKI in mice.

Expanded hiPSC-NPCs show therapeutic effects on AKI

Three million hiPSC-NPCs were used in the transplantations (Fig. 1, E to I). Considering the differences in body and kidney sizes between mice and humans, an expansion culture that produces a much larger number of homogeneous hiPSC-NPCs is required for clinical application. We first cultured hiPSC-NPCs in NPSR medium, which allows for the long-term expansion of mNPCs and hfNPCs (21), but found the expression of NPC markers and differentiation capacity into kidney organoids to substantially reduce after one passage (Fig. 2, A to C). These observations may result from NPSR medium proliferating off-target cells that emerged during the differentiation process of hiPSC-NPCs.

We found that the $OSR1^+SIX2^+$ NPC population was increased when the culture was switched from monolayer to suspension culture to form cellular aggregates using the WNT agonist CHIR99021, fibroblast growth factor (FGF) 9, and the Rho-kinase inhibitor Y-27632 in the kidney organoid formation of hiPSC-NPCs (fig. S2A), suggesting that the combination of CHIR99021, FGF9, and Y-27632 may help hiPSC-NPCs mature and stabilize their properties. We thus examined a suspension culture with CHIR99021, FGF9, and Y-27632 and found that the expression of several NPC markers was increased or maintained even after one passage (Fig. 2, A and B, and fig. S2, B and C). Moreover, the percentage of $OSR1^+SIX2^+$ cells at each passage was substantially increased, and the ability of the expanded hiPSC-NPCs to form kidney organoids was retained (Fig. 2, C to E, and fig. S3, A and B). We named this medium condition that enables the expansion culture of hiPSC-NPCs “CFY medium.” To evaluate the ability of expanded hiPSC-NPCs to differentiate into kidney tissues *in vivo*, we next transplanted them into the renal subcapsules of immunodeficient mice, finding that they developed $NPHS1^+$, $PODOCALYXIN$ ($PODXL$) $^+$ or $WT1^+$ glomeruli, LTL^+CDH1^- proximal tubules, $BRN1^+DBA^-$ Henle's loop and $BRN1^+DBA^+$ or $CDH1^+LTL^-$ distal tubules (fig. S3, C to F) and that the glomeruli were

vascularized by CD31⁺ host vessels (16, 21) (fig. S3, C and F). These results indicated that expanded hiPSC-NPCs retain their differentiation potential to kidney lineage cells (16). Although hiPSC-NPCs showed a complete ability to form kidney organoids up to the second passage in CFY medium *in vitro* (Fig. 2, C and E), their ability gradually decreased thereafter (Fig. 2C). This property may be related to lower expression of NPC markers *PAX2*, *HOXA11*, and *SALL1* after the second passage (fig. S2B). Next, we examined the number of cells seeded per well to optimize the conditions for efficient cell proliferation and found that cell aggregates generated by seeding 0.2×10^5 cells per well showed a higher cell proliferation rate after 4 days of cell seeding compared to 0.6×10^5 and 1.5×10^5 cells per well (fig. S4, A and B). We also found that the number of hiPSC-NPCs seeded at 0.2×10^5 cells per well increased to about 90% the number at 0.6×10^5 and 1.5×10^5 cells after 6 days of cell seeding (fig. S4, A and C). It is known that oxygen supply becomes insufficient when the distance from the surface of the cell aggregates exceeds 200 μm (23). Therefore, the lower cell proliferation rate of the larger hiPSC-NPC aggregates may result from a smaller oxygen supply, indicating the importance of seeding a small number of hiPSC-NPCs in the expansion culture. Consistently, hiPSC-NPCs seeded at 0.2×10^5 cells per well proliferated more than 100-fold (Fig. 2F).

Next, we examined the roles of each factor contained in CFY medium. Y-27632 was critical for the formation of hiPSC-NPC aggregates, and FGF9 was involved in NPC marker expression and cell proliferation. CHIR99021 was crucial for NPC marker expression but had little effect on cell proliferation (fig. S4, D and E). Each factor was essential for the expansion of hiPSC-NPCs. Furthermore, we verified whether the expansion culture method using CFY medium could be reproduced at an independent laboratory and found that hiPSC-NPCs could be expanded with maintained ability to form kidney organoids (fig. S4, F and G). We then examined the therapeutic

potential of hiPSC-NPCs expanded until the second passage in CFY medium on cis-AKI mice (Fig. 2G). The expanded hiPSC-NPCs attenuated kidney injury according to both blood tests and histological findings (Fig. 2, H to J) and improved the survival rate of the mice (Fig. 2K).

c-MET is a cell surface marker for hiPSC-NPCs with therapeutic effects on AKI

The high heterogeneity of hiPSC-NPCs has been reported (22), as have differences in the differentiation efficiency and gene expression patterns (15, 18). Therefore, a purification method for hiPSC-NPCs to remove undifferentiated hiPSCs and off-target cells is required for clinical application. We performed RNA-sequencing (RNA-seq) to identify cell surface markers necessary for the purification of OSR1⁺SIX2⁺ cells and listed the genes encoding transmembrane proteins specifically expressed in OSR1⁺SIX2⁺ cells compared to OSR1⁻SIX2⁻ cells. Although the gene expression of previously reported NPC surface markers (*ITGA8*, *NGFR*, *ROBO2*, and *NCAM1*) (20, 21, 24, 25) was not highly specific to the OSR1⁺SIX2⁺ cell population, we found that *c-MET* was preferentially expressed in OSR1⁺SIX2⁺ cells compared to OSR1⁺SIX2⁻ and OSR1⁻SIX2⁻ cells among genes encoding transmembrane proteins (fig. S5A). Validations based on RNA-seq data showed that c-MET was highly specific to the OSR1⁺SIX2⁺ cell population, indicating its usefulness for purifying hiPSC-NPCs with the ability to differentiate into kidney organoids (fig. S5, B to E). Similar to unpurified hiPSC-NPCs, c-MET-purified hiPSC-NPCs maintained their ability to differentiate into kidney organoids over two passages, and the rates of OSR⁺SIX2⁺ and c-MET⁺ cells increased with each passage (fig. S6, A and B). Furthermore, c-MET-purified hiPSC-NPCs were able to expand more than 100-fold with two passages (fig. S6C). We transplanted c-MET-purified hiPSC-NPCs after two passages into the renal subcapsules of cis-AKI mice and

found that the transplants prevented the deterioration of kidney function and histological findings (fig. S6, D to G) and improved the survival rate (fig. S6H).

Next, we generated NPCs from hiPSCs derived from individuals with homozygous human leukocyte antigen (HLA)-haplotypes (HLA-homo-iPSCs), which are expected to reduce the immunological rejection of allogeneic transplantations for recipients with three identical HLA locus haplotypes (HLA-A, HLA-B, and HLA-DR) on at least one side (26). We purified c-MET⁺ and c-MET⁻ cells from poorly differentiated HLA-homo-iPSC-derived NPCs (HLA-hNPCs; fig. S7, A and B). We found that HLA-hNPCs purified with c-MET⁺ and expanded for two passages differentiate into kidney organoids and show therapeutic efficacy in cis-AKI mice compared to c-MET⁻ purified, twice-passaged HLA-hNPCs (fig. S7, A to G). These results indicated that purification with c-MET was effective for NPCs derived from various hiPSC lines and for NPCs that did not show complete differentiation.

hiPSC-NPCs exhibit therapeutic effects on aristolochic acid (AA)-induced CKD mice

First, we transplanted mNPCs into the renal subcapsules of aristolochic acid-induced CKD (AA-CKD) model mice, which show slowly progressive kidney injury and fibrosis, a phenotype that recapitulates Chinese-Herb nephropathy (CHN) in humans (27, 28) (fig. S8A). Although we previously reported that mNPCs treated with CHIR99021 and FGF2 for 2 days have potent therapeutic effects on AKI (21), mNPCs without this treatment more effectively prevented CKD progression (fig. S8B). We also found that therapeutic effects are reduced when fewer mNPCs are transplanted (fig. S8B). Importantly, mNPC transplantation suppressed kidney fibrosis (fig. S8, C and D), suggesting that maintaining the NPC state without induction towards kidney organoids is crucial for CKD treatment.

Following these results, compared to control mice with saline injection, we found that the transplantation of untreated hiPSC-NPCs into AA-CKD mice suppresses kidney functional decline without changes in body weight (Fig. 3A, B and fig. S8E). Furthermore, AA-CKD mice with transplanted hiPSC-NPCs showed milder kidney fibrosis (Fig. 3, C to E) and a tendency for reduced urinary albumin (Fig. 3F) compared to control mice with saline injection. It was reported that glomeruli are characterized by the collapse of capillaries and wrinkling of the glomerular basement membrane in CHN (28). We confirmed that these pathological findings and the glomerular fibrosis in AA-CKD mice were also suppressed by the transplantation of hiPSC-NPCs or mNPCs (fig. S8, F and G).

Previous studies reported that increased DNA damage and oxidative stress caused by the accumulation of uremic toxins and cell injury promote the senescence of kidney cells in CKD and that senescent kidney cells acquire properties known as senescence-associated secretory phenotype (SASP), which promotes the secretion of pro-fibrotic and pro-inflammatory mediators, leading to progressive fibrosis of the kidney (29-32). Other studies reported that AA-induced mitochondrial damage induces apoptosis in the proximal tubules and that mutations in mitochondrial DNA are involved in aging (33, 34). Therefore, considering the possibility that AA administration accelerates kidney senescence, we examined the senescence phenotypes in renal tissues four months after AA administration, finding that senescence-associated β -galactosidase (SA- β -Gal) activities were reduced by the transplantation of hiPSC-NPCs into AA-CKD mice (Fig. 3, G and H). In addition, hiPSC-NPCs decreased the expression of a senescence-associated metalloprotease, matrix metalloproteinase 13 (*Mmp13*), as well as age-related stress response genes in the *p53* tumor suppressor pathway, including *p16^{INK4a}*, *p21^{CIP1}*, activating transcription factor 3 (*Atf3*) and growth arrest and DNA-damage-inducible 45 beta (*Gadd45B*), in treated CKD mouse kidneys.

Furthermore, the expression of canonical SASP factors, such as interleukin-6 (*Il-6*), plasminogen activator inhibitor 1 (*Pai-1*), and transforming growth factor beta 1 (*Tgfb1*), and BTG anti-proliferation factor 2 (*Btg2*), which is a tumor suppressor gene with the capability of triggering senescence, was reduced four months after the transplantation (35) (Fig. 3I), suggesting that the therapeutic effects of hiPSC-NPCs are partly by preventing kidney senescence in CKD.

Additionally, to address the potential long-term effects of c-MET expression, with particular attention on its association with renal cell carcinoma, we conducted a three-month post-transplant evaluation. This evaluation included blood counts, biochemical examinations of mice transplanted with hiPSC-NPCs compared to control mice with saline injection, and a detailed histological assessment focused on the potential emergence of renal cell carcinoma. Statistical differences in BUN and glucose amounts were observed between transplanted and control mice. However, the BUN amounts of both groups were not statistically different from those of normal NOD-SCID mice examined by the breeder (table S2). Although the blood glucose of both groups was higher than that of normal mice examined by the breeder, the transplanted group was closer to normal mice than the control. Other measurements revealed no differences between the two groups (table S2), suggesting that hiPSC-NPC transplantation does not affect blood cell counts and blood chemistry. Moreover, histological assessments showed no evidence of tumor formation nor any obvious histological differences between the two groups (table S3). On the other hand, approximately 11.6% of cells within transplants were NPC-derived differentiated renal lineage cells, such as glomerulus- and renal tubule-like structures, by examination of section HE staining images and immunostaining analysis showed the presence of PODXL^+ glomeruli, $\text{LTL}^+\text{CDH1}^-$ proximal tubules, $\text{BRN1}^+\text{DBA}^-$ Henle's loop, and $\text{LTL}^-\text{CDH1}^+$ and $\text{BRN1}^+\text{DBA}^+$ distal tubules (fig. S9, A to C). Immunostaining analysis also revealed the presence of host mouse-derived blood

vessels within the transplants of hiPSC-NPCs, which indicates that transplants are not only nourished by invading mouse blood vessels but also connected to the host vascular network (fig. S9D). Section immunostaining of grafts at three months post-transplantation demonstrated that most cells continued to express key NPC markers OSR1, SIX2, PAX2, and SALL1, as well as c-MET, but not proliferation marker Ki67 (fig. S9E).

We further evaluated whether the therapeutic effects of c-MET-purified hiPSC-NPCs observed in AKI were also present in CKD model mice. We sorted poorly differentiated HLA-hNPCs into c-MET⁺ and c-MET⁻ populations by flow cytometry (fig. S7, A to C) and transplanted these sorted cells into CKD model mice to evaluate their therapeutic effects (fig. S10A). The results showed that transplantation of c-MET⁺ HLA-hNPCs had more robust effects than c-MET⁻ HLA-hNPCs in suppressing kidney functional decline and fibrosis (fig. S10, B to E).

Secreted factors by hiPSC-NPC aggregates exert reno-protective effects

We examined whether the reno-protective effects of hiPSC-NPCs are obtained by integration into the host kidney. Compared to subcutaneously transplanted hiPSC-NPCs that disappeared after 3 weeks, although hiPSC-NPCs transplanted into the renal subcapsules were engrafted for over 6 months and expressed NPC markers, they did not migrate or integrate into the host kidney parenchyma (figs. S9, A to E, and S11, A and B). Furthermore, since therapeutic effects were obtained in both kidneys of mice even though hiPSC-NPCs were transplanted unilaterally (fig. S12, A and B), we hypothesized that factors secreted by hiPSC-NPCs might exert reno-protective effects in both AKI and CKD model mice. We thus tested treatment with 500 μ L of conditioned medium (CM) collected from 96-well plates with the expansion culture of hiPSC-NPCs three times a day on cis-AKI mice but found no therapeutic effects (fig. S13A).

Next, we considered mimicking the *in vivo* conditions of the treated mice, in which the transplanted mNPC or hiPSC-NPC aggregates fused to form large cell aggregates, covered the surface of host mouse kidneys and exerted therapeutic effects (figs. S9, A, B, and E), and generated giant aggregates by fusing around 700 hiPSC-NPC mini aggregates that were 400 μ m in diameter (Fig. 4, A and B). Although these giant hiPSC-NPC aggregates expressed NPC and proliferation markers and retained the ability to differentiate into kidney organoids after long-term cultures (Fig. 4, B to F), we assumed they would induce apoptosis and cytotoxicity due to poor oxygen and nutrient supply. However, we found low cleaved caspase-3 (CC3) expression in giant hiPSC-NPC aggregates on day 10 after expansion culture. Furthermore, giant hiPSC-NPC aggregates on day 10 after expansion culture showed no difference in LDH release compared to aggregates formed in a 96-well format (Fig. 4, G and H). Additionally, the CM of giant hiPSC-NPC aggregates showed therapeutic effects in attenuating kidney injury and improved survival rates in cis-AKI mice (Fig. 4, I to L). Furthermore, we confirmed that the therapeutic potential of CM was maintained even after freeze-thawing at -80°C (fig. S13, B to D). These results suggest that renoprotective factors play crucial roles in the therapeutic effects of hiPSC-NPCs.

VEGF-A-mediated angiogenesis underlies the therapeutic effects of hiPSC-NPCs in AKI

To elucidate the therapeutic mechanisms of hiPSC-NPCs for AKI, we conducted RNA-seq of hiPSC-NPCs showing therapeutic effects and hiPSC-FS-NPCs with attenuated therapeutic effects. A comparison of their gene expression profiles found 622 differentially expressed genes (DEGs), of which 453 and 169 were up- or downregulated in hiPC-NPCs, respectively (Fig. 5A and data file S2). A gene ontology (GO) analysis of DEGs identified several terms related to

circulatory system development enriched in hiPSC-NPCs compared to hiPSC-FS-NPCs (Fig. 5B and data file S3).

Because the therapeutic effects of hiPSC-NPCs on AKI were attributed to secreted factors (Fig. 4, I to L and fig. S13, C and D), DEGs encoding secreted proteins, including *VEGFA*, a reported therapeutic factor in cell therapy for AKI (36), were selected for further analysis (data file S4). Immunostaining analysis showed that VEGF-A was localized in the cytoplasm of hiPSC-NPCs, and an enzyme-linked immunosorbent assay (ELISA) confirmed that hiPSC-NPCs secreted VEGF-A into the culture supernatant (Fig. 5, C and D).

The sustained release of recombinant human VEGF165 protein from biodegradable hydrogels transplanted to the renal subcapsules partially recovered kidney function in cis-AKI mice (Fig. 5, E and F). Additionally, mice treated with a sustained release of VEGF165 protein exhibited a higher microvessel density (MVD), reflecting the density of CD31⁺ peritubular capillaries (PTCs), and fewer CC3⁺ apoptotic cells compared to controls (Fig. 5, G and H). We subsequently evaluated the systemic effects of VEGF inhibition by orally administering axitinib, a potent inhibitor of VEGF receptors (37), to cis-AKI mice treated with hiPSC-NPCs and found that the therapeutic effects were reduced, with a decrease in MVD and an increase in CC3⁺ apoptotic cells (fig. S14, A to F).

To further assess the direct effects of VEGF-A signaling in the kidneys of mice transplanted with hiPSC-NPCs, we quantified the phosphorylation amount of tyrosine 1175 at the mouse VEGF receptor 2 (VEGFR2), a critical site for mediating VEGF-induced endothelial cell proliferation (38). Comparative analysis showed that mouse VEGF signaling was elevated in the hiPSC-NPC transplant group compared to the PBS-treated control group (fig. S15, A and B). Furthermore, the detection of human VEGF-A in the serum of mice transplanted with hiPSC-NPCs

(fig. S15C) suggested that transplanted cells secreted human VEGF-A, which likely entered systemic circulation.

Next, we evaluated both kidneys of cis-AKI and AA-CKD mice that had hiPSC-NPCs transplanted into their left renal subcapsules. The results showed that the reduction of MVD was suppressed, and the number of CC3⁺ apoptotic cells was decreased in both kidneys of cis-AKI mice (fig. S15D). Additionally, the suppression of MVD reduction was also observed in both kidneys of AA-CKD mice (fig. S15 E). These findings suggest that VEGF-A secreted by transplanted hiPSC-NPCs circulated systemically and exerted reno-protective effects on the non-transplanted kidneys, thereby mitigating renal damage.

***VEGFA* knockout hiPSC-NPCs exhibit reduced therapeutic effects on both AKI and CKD mouse models**

Subsequently, we generated *VEGFA* knockout (KO) hiPSCs using the CRISPR-Cas9 regulated by transcription and nuclear shuttling (CRONUS) system (39) (Fig. 6A). Sanger sequencing of genomic DNA isolated from the generated hiPSC line confirmed a single nucleotide insertion in the targeting site (Fig. 6B). By ELISA, we quantified the concentrations of VEGF-A in the culture supernatants of both wild-type (WT) and *VEGFA* KO hiPSC-NPCs and confirmed the absence of VEGF-A in *VEGFA* KO hiPSC-NPCs (Fig. 6C). . The transplantation of *VEGFA* KO hiPSC-NPCs into the renal subcapsules of cis-AKI mice nullified the therapeutic effects seen with transplantation of WT hiPSC-NPCs, such as higher MVD, reduced CC3⁺ cells, and improvement of kidney damage (Fig. 6, D to I).

VEGF-A plays a pivotal role in renal microcirculation, and its loss is associated with the development of renal fibrosis (40-43). To investigate whether VEGF-A is also involved in the

therapeutic effects of hiPSC-NPCs on CKD, *VEGFA* KO hiPSC-NPCs were transplanted into the renal subcapsules of AA-CKD mice. The *VEGFA* KO hiPSC-NPC transplant group showed attenuated improvement in kidney function at three months post-transplantation compared to the saline group (Fig. 6, J and K). A histological examination revealed that the *VEGFA* KO hiPSC-NPC transplant also failed to suppress renal fibrosis or improve MVD (Fig. 6, L and M). These results suggest that VEGF-A contributes to the therapeutic effects of hiPSC-NPCs on both AKI and CKD.

CM of giant hiPSC-NPC aggregates exerts reno-protective effects through VEGF-A

Finally, we examined whether VEGF-A is one of the reno-protective factors in the CM of giant hiPSC-NPC aggregates (giant-CM). First, we showed that giant-CM contained VEGF-A and that VEGF-A could be substantially inhibited by bevacizumab (Avastin), a potent neutralizing antibody for VEGF (fig. S16A). We subsequently confirmed that the inhibition of VEGF-A in giant-CM by 10,000 ng/mL of bevacizumab attenuated the therapeutic effects in cis-AKI mice (fig. S16B). These results suggest that hiPSC-NPCs inhibit the progression of renal injury primarily by secreting VEGF-A.

We then examined whether the therapeutic effects of giant-CM are exclusively exerted by VEGF-A. We administered the giant-CM and the same concentration of VEGF-A protein as that contained in giant-CM to cis-AKI mice and found giant-CM to have more potent therapeutic effects (fig. S16C), suggesting that giant-CM contains secreted factors other than VEGF-A with reno-protective effects. Therefore, we compared 96-well plate-derived CM (96-well-CM), which showed poor therapeutic effects, with giant-CM by mass spectrometry. This analysis identified 288 secreted proteins in giant-CM and 212 in 96-well-CM, and of these secreted proteins, 112

were unique to giant-CM (fig. S16D and table S4). Among these proteins, VEGF-A was present in both types of CMs but, as confirmed by ELISA, was secreted in slightly higher amounts by giant aggregates (fig. S16E). Furthermore, out of 112 unique proteins included in giant-CM, 14 proteins are directly involved in vascular development (fig. S16F), suggesting that whereas VEGF-A contributes to the therapeutic outcomes, the broader spectrum of angiogenic proteins in these CMs likely synergized with VEGF-A to enhance vascularization processes. On the other hand, secreted proteins unique to giant-CM contained a diverse protein profile, including not only those involved in angiogenesis but also those involved in cell motility and growth (fig. S16G and data file S5). These results indicate that the therapeutic effects of giant-CM are exerted not only by promoting tissue regeneration through angiogenesis but possibly through multifaceted actions, including modulation of the immune environment.

DISCUSSION

In this study, we demonstrated that hfNPCs expanded in NPSR medium have therapeutic effects on AKI. However, numerous issues, such as ethical concerns, the need for immunosuppression, and unexpected infections, must be resolved before clinical application using human fetal kidneys could occur. In contrast, hiPSC-NPCs have the potential to address all these issues. Moreover, we showed that, unlike NPSR medium, our newly identified CFY medium enables the maintenance of NPCs derived from multiple hiPSC lines that can effectively treat kidney diseases (table S1). The therapeutic effects of hiPSC-NPCs on AKI have been reported (*14, 15, 17*), but this study demonstrates that they are also effective for CKD. In addition, we revealed that hiPSC-NPCs exert anti-senescence effects as a possible mechanism of inhibiting CKD progression. On the other hand, it was reported that the prognosis and renal outcome of AKI

worsen in correlation with elevated early diagnostic markers, including tubulointerstitial damage markers (4, 44), thus requiring early therapeutic intervention to reduce the progression of the tubulointerstitial damage at the stage of subclinical AKI before S-Cre elevation. In this study, we developed a highly reproducible method to obtain CM with therapeutic effects on AKI from giant hiPSC-NPC aggregates that mimic *in vivo* conditions. To attain a broader range of therapeutic effects, however, it may be necessary to establish a treatment method in which CM obtained from hiPSC-NPCs is directly administered because CM is likely to exert its effects through complex interactions associated with multiple reno-protective factors. Hence, a standby surgical procedure involving the renal subcapsular transplantation of hiPSC-NPCs, which can secrete reno-protective factors for extended periods, may be appropriate for CKD. In contrast, in the early stages of AKI onset, the administration of hiPSC-NPC-derived CM or constituent reno-protective factors rather than the surgical transplantation of hiPSC-NPCs into the renal subcapsules may prevent the deterioration of kidney function and improve the outcome of patients with AKI, which can otherwise progress to CKD that necessitates continuous renal replacement therapy such as dialysis. Moreover, the components of hiPSC-NPC-derived CM may contain seeds for new drug development.

In this study, we identified multiple factors at the protein level that may contribute to the therapeutic effects of hiPSC-NPC-derived CMs by mass spectrometry analysis. This proteomic approach has provided a deeper understanding of the complex milieu of therapeutic proteins in CM, supporting the potential of a multi-factorial basis for the observed therapeutic outcomes. On the other hand, we demonstrated that VEGF-A plays a crucial role in the therapeutic effects of hiPSC-NPCs on both AKI and CKD. In addition, RNA-seq revealed several DEGs between

hiPSC-NPCs and hiPSC-FS-NPCs, including known reno-protective factors, such as *SDF1* and *STC1* (45, 46) (data file S4).

The roles of VEGF-A in the therapeutic mechanisms of hiPSC-NPCs discovered here are consistent with previously reported findings demonstrating VEGF-A's reno-protective effects in cellular therapy for AKI (36) and its involvement in renal microcirculation and fibrosis (47). The use of axitinib and bevacizumab to inhibit VEGF-A signaling further supports the importance of VEGF-A in mediating the observed improvements in renal injury. In severe AKI, functional changes of injured PTCs and pericytes lead to vascular rarefaction, hypoxic renal tubules, and the production of hypoxia-inducible factor (HIF)-1 α (48). In addition, PTC damage and rarefaction are hallmarks of CKD and are often accompanied by declining kidney function in various renal disorders (49, 50). In this study, CD31⁺ PTCs were reduced in the kidneys of cis-AKI and AA-CKD mice, but VEGF-A secretion by transplanted hiPSC-NPCs resulted in increased CD31⁺ PTCs and therapeutic effects. These results suggest that VEGF-A secreted by hiPSC-NPCs increases vascular endothelia and protects the kidneys from hypoxia caused by renal injury.

It has been recently reported that VEGF-A has effects beyond angiogenesis, such as antioxidant effects against skin aging (51) and autophagy activation (52). To date, although there have been few reports of effects of VEGF-A other than angiogenesis in the kidney, it is necessary to focus on these effects beyond angiogenesis and fully elucidate the mechanisms of reno-protection. Therefore, building on our current findings emphasizing the roles of VEGF-A in the treatment of kidney diseases, future research should comprehensively analyze how identified proteins interact synergistically with VEGF-A to enhance the therapeutic effects of hiPSC-NPCs in kidney disease treatment. Understanding the complex network of interactions involving VEGF-

A and its associated proteins will provide valuable insights into optimizing regenerative strategies and uncovering new therapeutic targets for both AKI and CKD.

We examined the appropriate transplantation site for hiPSC-NPCs that secrete renoprotective factors by comparing renal subcapsular transplantation with subcutaneous transplantation previously established for autografts after parathyroidectomy in clinical practice. Our data showed that hiPSC-NPCs transplanted to renal subcapsules engrafted and survived longer than subcutaneously transplanted hiPSC-NPCs. Furthermore, renal subcapsular transplantation of hiPSC-NPCs may be safer than systemic administration or renal parenchymal injections of stem cells in that hiPSC-NPCs do not integrate into the host kidneys and can be removed in the case of adverse events. Therefore, we postulate that renal subcapsules represent an appropriate transplantation site for the long-term therapeutic effects and safety of hiPSC-NPCs.

Both mNPCs and hfNPCs can be expanded for several months in NPSR medium and show therapeutic effects after being stimulated to differentiate into kidney organoids (21). In contrast, hiPSC-NPCs cannot be expanded in NPSR medium (20), and their therapeutic effects on AKI are more potent without differentiating into kidney organoids, suggesting that certain characteristics, including gene expression profiles, are different between hfNPCs and hiPSC-NPCs. It has been pointed out that hiPSC-NPCs show distinct gene expression patterns depending on the differentiation methods, hiPSC lines, induction efficiency, and even between experimental batches (15, 16, 18, 22). Therefore, an optimal expansion culture condition for hiPSC-NPCs generated by each differentiation method should be developed.

To address variations in induction efficiency and gene expression patterns among hiPSC-NPCs, we focused on identifying and characterizing cell surface markers that could aid their purification and subsequent application in the clinic. We identified c-MET as a marker crucial for

the purification of hiPSC-NPCs to enhance their therapeutic potential. In mice, the addition of hepatocyte growth factor (HGF), a ligand for c-MET, to metanephric mesenchyme promotes epithelialization (53), and c-MET is expressed with HGF in induced mesenchymal cells during early differentiation and disappears as differentiation proceeds, indicating that c-MET is expressed in embryonic NPCs (54). However, it was difficult to perform long-term expansion cultures of hiPSC-NPCs even following purification by c-MET, similar to mNPCs and hfNPCs (21). In addition, hiPSC-NPCs contained both c-MET⁺ and c-MET⁻ subpopulations, suggesting that they are heterogeneous at various differentiation stages. Such diversity in differentiation status may be a determining factor hindering expansion culture. It is thus necessary to develop methods that identify and purify bona fide NPC populations from hiPSC-NPCs with an equivalent differentiation status to hfNPCs to allow for long-term expansion culture.

It was recently reported that kidney function declines through aging (55), and various complex factors, including the proliferation of tertiary lymphoid nodes, exacerbate kidney damage (56). Furthermore, CKD-induced senescent kidney cells exacerbate kidney damage by acquiring phenotypes such as SASP (27, 28, 31), suggestive of a vicious circle between CKD and kidney aging progresses to inflict further kidney damage. As an anti-aging approach using stem cells, a previous report showed that extracellular vehicles produced by cord blood-derived MSCs ameliorate kidney damage and fibrosis in aging mice (57). Another study reported that MSCs suppress kidney senescence caused by renal ischemia due to renal artery stenosis (58). However, no report has examined the therapeutic potential of stem cells on CKD-induced senescence due to non-ischemic mechanisms or the use of hiPSC-NPCs as a therapeutic approach to reversing kidney senescence. In this study, we showed that in addition to alleviating kidney functional decline and fibrosis, the renal subcapsular transplantation of hiPSC-NPCs prevents kidney senescence in CKD,

possibly through the TGF- β 1/Smad2/3 signaling pathway (59). These results indicate that cell therapies using hiPSC-NPCs have the potential to break the cycle that accelerates the progression of kidney damage by preventing not only the deterioration of kidney function in CKD that worsens through aging but also the kidney aging caused by CKD. Thus, kidney aging may be a future therapeutic target for CKD.

The development of an expansion culture method of hiPSC-NPCs may open up entirely new therapeutic fields for AKI and CKD and make a contribution to the drug screening area. Microphysiological systems (MPS) were recently noted as promising drug screening platforms that more closely resemble biological environments (60-62). Our expansion culture method of hiPSC-NPCs could supply MPS with large numbers of homogeneous cells and kidney organoids derived from various hiPSC lines, including disease-specific hiPSCs, thus helping to establish more accurate drug screening systems. Therefore, the findings and techniques illustrated in the current study could become a cornerstone for the emergence of therapeutic approaches to various kidney diseases.

Our study has several limitations. One is the use of NOD-SCID mice for hiPSC-NPC transplantation, which does not adequately validate the patient immune response observed in clinical settings. Although long-term use of immunosuppressive agents is essential for allogeneic hiPSC-NPC transplantation, their use may lead to side effects, such as diabetes, accelerated renal fibrosis, and increased infectious morbidity. Furthermore, immunosuppressive agents limit the use of live attenuated vaccines developed for existing infectious diseases, such as tuberculosis, yellow fever, and polio, as well as those emerging in the future. In addition, the effects of immunosuppressive agents on hiPSC-NPCs have not been examined adequately, and their potential to reduce the therapeutic efficacy of hiPSC-NPCs and induce the differentiation of

hiPSC-NPCs into off-target cells has not been fully validated. We believe that one promising strategy to mitigate immunological reactions is the use of HLA-homo iPSC lines to reduce the likelihood of immune rejection by the host. Additionally, hypoinmunogenic or low antigenic hiPSC lines are other innovative approaches to minimize immune responses, thus enhancing the compatibility and acceptance of transplanted cells. The transplantation of NPCs derived from these hiPSC lines into human-immunized mice will demonstrate the utility of these approaches (63). Therefore, further validation of the impact of immunosuppressive agents on hiPSC-NPCs and research to reduce the use of immunosuppressive agents are necessary.

Although we extended the evaluation period to three months and continued to observe no evidence of tumorigenesis or differentiation into non-target cells, thus affirming the initial safety of the hiPSC-NPC transplantation, nonetheless potential risks associated with longer-term outcomes, including the possibilities of tumor formation or differentiation into unintended cell types, have not been adequately evaluated. In addition, factors that predict the differentiation of hiPSC-NPCs into off-target cells have not been identified, making it difficult to monitor risks such as tumor formation. Therefore, aside from extended observations, future studies are needed to develop quality control markers to monitor the risk of off-target cell formation and to improve the purification of hiPSC-NPCs. Although we have developed methods to remove undifferentiated cells to substantially reduce the risk of teratoma formation and abnormal tissue development (64), further technical improvements are needed to ensure the safety and specificity of cell-based therapies.

In this study, although we have demonstrated the therapeutic efficacy of hiPSC-NPCs in cis-AKI and AA-CKD mouse models, their broader clinical applicability would benefit from testing in additional AKI and CKD models. To date, no stratification markers for hPSC-NPCs have

been identified. Therefore, to progress to clinical trials, we must determine the pathological conditions for which hiPSC-NPCs have therapeutic effects to define kidney diseases that are ideal indications for treatments based on their use.

In conclusion, we developed an expansion culture for hiPSC-NPCs and presented new therapeutic approaches for both AKI and CKD. Furthermore, we have developed a new therapeutic strategy for AKI using hiPSC-NPC-derived CM. Although cell-based therapies for kidney disease, mainly MSCs, have yet to show positive results in clinical trials, adding hiPSC-NPCs as a new option for cell-based therapies for the kidney will undoubtedly further advance this field. Furthermore, as hiPSC-NPCs exert their therapeutic effects by stimulating multiple reno-protective factors, the complete identification of such factors and their interactions would facilitate the development of even more effective therapies against kidney disease by enhancing their functions.

MATERIALS AND METHODS

Study design

The main objectives of this study were to develop expansion culture and purification methods for hiPSC-NPCs, determine the therapeutic effects of expanded hiPSC-NPCs and CM produced by hiPSC-NPCs on kidney diseases, and elucidate their therapeutic mechanisms. Sample sizes were empirically estimated after referring to previous reports (15, 21, 37, 65) and considering variations in their results and the statistical power needed, with minimizing the number of experimental animals used in mind.

First, we determined the sample size based on a previous report using mNPCs (21) in hfNPCs since hfNPCs were cultured in the same expansion medium as mNPCs. We randomly assigned 5 male NOD.CB17-*PRKDC*^{SCID}/J (NOD-SCID) mice to each group to determine the

therapeutic effects of FS-hfNPCs on cis-AKI mice. We then examined kidney function and renal pathological changes in each mouse group 4 days after cisplatin administration, as previously reported (14, 21). Next, the sample size was determined by referring to previous reports using hiPSC-NPCs (15) in non-expanded hiPSC-NPCs since these cells were not derived from fetal kidneys and culture conditions differ from those for mNPCs and hfNPCs (21). Eleven male NOD-SCID mice were randomly assigned to each group to determine the therapeutic effects of non-expanded hiPSC-NPCs on cis-AKI mice. We then examined kidney function and renal pathological changes in each mouse group 4 days after cisplatin administration. Since one mouse in the saline group died on day 3 after the administration, the statistical analysis was performed on 10 and 11 mice in the saline group and the groups transplanted with hiPSC-NPCs or hiPSC-FS-NPCs, respectively. Based on the results of this analysis, we reconsidered the sample size for subsequent studies. To determine the therapeutic effects of WT and *VEGFA* KO hiPSC-NPCs on cis-AKI mice, 8 and 6 male NOD-SCID mice were assigned, respectively, with kidney function and renal pathological changes examined 4 days after cisplatin administration. Since there are no reports on the therapeutic effects of reno-protective factors on cis-AKI mice using the CM of hiPSC-NPCs, 11 male C57BL/6J mice were assigned to each group and examined 4 days after cisplatin administration in the same manner as described above. To validate the contribution of VEGF-A secreted from hiPSC-NPCs to the therapeutic effects on cis-AKI mice, 5 male NOD-SCID mice were randomly assigned per group based on a previous report using axitinib to inhibit VEGFR-2 phosphorylation in xenograft tumors (37). Each group was examined 4 days after cisplatin administration, as described above. To investigate the effects of sustained VEGF-A release from renal subcapsules on kidney function in cis-AKI mice, by referring to a previous report of the sustained release of bioactive substances from gelatin hydrogels (65), gelatinized

recombinant human VEGF165 proteins were administered under the left renal subcapsules of 7 male NOD-SCID mice with cisplatin-induced AKI. Based on these results, 3 male C57BL/6J mice with cisplatin-induced AKI were randomly assigned to each group to investigate the effects of CM in which VEGF-A was inactivated with bevacizumab on kidney function. Kidney function was evaluated in the same manner as described above.

In our evaluation of the therapeutic effects of hiPSC-NPC transplantation on CKD, the gradual progression of kidney damage in AA-CKD mice led to some mice being excluded from the analysis during the 5-month observation period. Thus, the sample size was empirically estimated by considering the variability of the results and the statistical power required, and 15 male NOD-SCID mice were assigned to each group. These mice were examined for kidney function 12 days and 1, 2, 3, and 4 months after AA administration, with changes in gene expression and kidney pathology, including examination of cellular senescence, and urinary protein levels determined at 4 months. To investigate the therapeutic effects of *VEGFA* KO hiPSC-NPCs on AA-CKD mice, 6 male NOD-SCID mice were randomly assigned to each group. Kidney function at 12 days and 1, 2, and 3 months after AA administration and renal pathological changes 3 months after administration were investigated in these mice.

Mice were purchased from a commercial supplier and randomly assigned, but the studies were not blinded. No data points were excluded from the analyses except for the study on AA-CKD mice in which one mouse was excluded from the saline group.

Experiments using hiPSCs were approved by the Ethics Committee of the Department of Medicine and Graduate School of Medicine, Kyoto University. Informed consent was obtained from the donor from whom hiPSCs were generated according to the guidelines of the Declaration of Helsinki. All experiments using human fetal kidneys were approved by the Institutional Review

Board (IRB) Committee of the Salk Institute and conducted at the Salk Institute by researchers belonging to the institute. All animal experiments conducted at Kyoto University were approved by the Kyoto University Animal Care Committee and performed in accordance with the institutional guidelines. All animal experiments conducted at the Salk Institute were performed under the ethical guidelines of the Salk Institute, and animal protocols were reviewed and approved by the Salk Institute Institutional Animal Care and Use Committee (IACUC).

Cisplatin-induced acute kidney injury (AKI) mouse model

NOD-SCID mice (male, 8 to 12 weeks old) were transplanted with human fetal kidney-derived NPCs expanded and differentiated with 5 μ M CHIR99021 and 200 ng/mL FGF2 on an 8.0 μ m pore polycarbonate membrane (Transwell #3422, Corning) for 2 days (FS-hfNPCs) (21), non-expanded hiPSC-NPCs, or expanded hiPSC-NPCs, or injected with saline (Control) into the left renal subcapsules 24 hours after the subcutaneous administration of 21 mg/kg cisplatin (Wako). The renal subcapsular transplantation was performed as described previously (21). Blood and kidney tissue samples were collected 4 days after cisplatin administration. Blood samples were assayed for urea and creatinine amounts using DRI CHEM 7000V (Fujifilm) as kidney function parameters. Collected kidney samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Tissue sections (8-10 μ m thick) of paraffin-embedded samples were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Tubular necrosis, urinary casts, tubular dilation, and the loss of tubular borders were assessed in non-overlapping fields (high-power field; HPF) as described previously (14, 21). Multiple histological sites were assessed to compile a comprehensive view of the tissue response. For treatment with CM (described below), C57B6/J mice were intraperitoneally treated with 16 mg/kg cisplatin, followed 24 hours later by the

intraperitoneal administration of 500 μ L CM or CM treated with bevacizumab (Selleck Biotech) every 8 hours for 3 consecutive days. Non-conditioned CFY medium was used as a control. Blood and kidney tissue samples were collected 4 days after cisplatin administration and the treatments described above. NOD-SCID and C57B6/J mice were purchased from Oriental Bio Service, Inc. and Shimizu Laboratory Supplies Co., Ltd., respectively. All mice were housed at the laboratory animal facility of the Center for iPS Cell Research and Application (CiRA), Kyoto University, under specific pathogen-free (SPF) conditions. The room had controlled temperature (21-25°C), humidity (45–55%), and light (12-hour light-dark cycle), and the mice were provided ad libitum access to food and water.

Aristolochic acid (AA)-induced chronic kidney disease (CKD) mouse model

After 50 mg AA (Sigma-Aldrich) was completely dissolved in 20 mL DMSO, 480 mL PBS filtered through a 0.22 μ m pore size filter was added to the solution to produce 100 μ g/mL AA solution. NOD-SCID mice (male, 8 to 12 weeks old) received 2 mg/kg AA solution intraperitoneally 6 times every other day (days 0, 2, 4, 6, 8, and 10), followed by the left renal subcapsular administration of expanded mouse embryonic kidney-derived NPCs (mNPCs), mNPCs differentiated with 5 μ M CHIR99021 and 200 ng/mL FGF2 on an 8.0 μ m pore polycarbonate membrane for 2 days (FS-mNPCs) (21), hiPSC-NPCs, or saline (Control) on day 12. Renal subcapsular transplantation was performed as described previously (21). In groups transplanted with expanded mNPCs or FS-mNPCs, blood samples were collected 1, 3, and 5 months after AA administration, with kidney tissue samples after 5 months. In the hiPSC-NPC transplant group, blood samples were collected 1 and 4 months after AA administration, with kidney tissue samples after 4 months. Blood and kidney tissue samples were treated as described

above. A part of the collected kidney samples was fixed in 4% PFA and embedded in paraffin. Tissue sections (8-10 μm thick) of paraffin-embedded kidney samples were stained with Masson-Trichrome (MT), Picro-Sirius Red (SR), or with anti- α -SMA. Another part of the collected kidney samples was freshly embedded in OCT compound and rapidly frozen on ethanol chilled with dry ice. Unfixed 8-10 μm thick frozen sections were stained with SA- β gal using a commercially available assay kit (Cellular Senescence Assay Kit; Cell Biolabs). Multiple histological sites were assessed to compile a comprehensive view of the tissue response. Amounts of kidney fibrosis were scored by quantifying the percentage of MT- and Picro-Sirius Red-stained and α -SMA immunostained areas and normalized by the area of the kidney regions captured in the HPF images.

Expansion culture for hiPSC-NPCs

After dissociating the hiPSC-NPCs differentiated in 2D culture, cellular aggregates were formed in low-attachment 96-well plates (Nunc/Sphera #174929, Thermo Fisher Scientific) in suspension culture with Y-27632 added to the factors used in Stage 6 (200 ng/mL human FGF9 and 1 μM CHIR99021) of a previously reported differentiation method for hiPSC-NPCs (16). The medium for expansion culture consisting of 1 μM CHIR99021, 200 ng/mL human FGF9, and 10 μM Y-27632 was defined as CFY medium. Aggregates derived from hiPSC-NPCs were cultured in 96-well plates with CFY medium changed every 2 days, dissociated by Accumax (Innovative Cell Technologies) every 6 to 8 days, and passaged as aggregates. Information on growth factors, low-molecular-weight compounds, and reagents used in this study is listed in table S5.

Conditioned medium (CM) preparation

After dissociating hiPSC-NPCs differentiated in 2D culture using Accumax treatment for 10 min at 37°C and gentle pipetting, single hiPSC-NPCs were seeded in 24-well format SPHERICALPLATE 5D (Kugelmeiers Ltd.) at 7×10^6 cells/well. Approximately 700 mini-aggregates per well formed after 2 days of culture with CFY medium were transferred to each well of low-attachment 24-well plates (PrimeSurface #MS-09240, Sumitomo Bakelite Co., Ltd.), where mini-aggregates fused to generate a giant aggregate. The approximately 30-degree tilt of low-attachment 24-well plates helped shape the giant aggregate by fusing mini-aggregates. The medium was changed every 2 days with 1 mL CFY medium for 8 days. The CM obtained from each medium change on days 2, 4, 6, and 8 was stored at 4°C after filtration through a 0.22 μm membrane filter (Millex-GP SLGPR33RS, Millipore). The CM collected on those days and stored at 4°C were mixed and promptly administered to cis-AKI mice as described above. A portion (around 800 μm^2) cut from a giant hiPSC-NPC aggregate was used for immunostaining or further differentiated into kidney organoids in the same way as previously described for hiPSC-NPC-derived aggregates formed in low-attachment 96-well plates (16, 21). Information on growth factors, low-molecular-weight compounds, and reagents used in this study is listed in table S5.

Statistical analysis

All quantitative data are represented as means $\pm$ SD. Student's *t*-test or Mann-Whitney U-test was used to compare the means of two groups depending on the conditions. One-way ANOVA followed by the Tukey-Kramer test was used for multiple comparisons among groups. Significant differences were labeled in the figures as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

List of Supplementary Materials

Materials and Methods

Figures S1 to S16

Tables S1 to S9

References (66-70)

Data file S1 to S5

MDAR reproducibility checklist

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Author contributions: T.A. and K.O. conceived and designed the study and obtained funding. T.A., K.T., M.R., C.I., and M.M. cultured hiPSCs and differentiated them into hiPSC-NPCs. T.A., K.T., and M.R. planned all animal experiments. T.A., K.T., M.R., C.I., and M.M. generated AKI and CKD mice and transplanted hiPSC-NPCs into these model mice to evaluate the therapeutic effects on kidney function and histological findings. T.A. and M.M. treated AKI mice with hiPSC-NPC-derived conditioned medium or recombinant VEGF-A. K.T. transplanted hiPSC-NPCs into AKI mice treated with axitinib. M.R. measured albuminuria in CKD mice. T.A., C.I., and M.M. performed flow cytometry of hiPSC-NPCs for data analysis and cell purification. T.A., K.T., C.I., and M.M. performed the pathological experiments, including immunostaining and quantitative data analyses. T.A., K.T., and M.M. performed western blotting and ELISA. T.A. and C.I. evaluated the localization and viability of transplanted hiPSC-NPCs based on luciferase expression. K.T., C.I., and M.M. performed RNA isolation, qRT-PCR, and RNA-seq. K.T. analyzed RNA-seq data from hiPSC-NPCs and cells differentiated into kidney organoids for 2 days. J.W. and A.W. performed RNA-seq of cells fractionated with OSR1 and SIX2 from hiPSC-NPCs and analyzed

the data. T.A. and M.M. evaluated the effects on systemic organs after hiPSC-NPC transplantation. M.I. performed mass spectrometry on the conditioned medium of hiPSC-NPCs. T.A., K.T., and M.I. analyzed the mass spectrometry data. Y.T. generated and provided hydrogelatinized hVEGF165. K.T. administered hydrogelatinized hVEGF165 to AKI mice. K.T. generated *VEGFA* KO hiPSC lines and transplanted *VEGFA* KO hiPSC-NPCs into AKI and CKD mice. C.M. and R.Y. designed and experimented to replicate the hiPSC-NPC expansion culture method in an independent facility. T.A., Z.L., and J.C.I.B. designed studies using hfNPCs. T.A. and Z.L. performed animal experiments using expanded hfNPCs and analyzed the data. T.A., K.T., and K.O. supervised all studies and interpreted data. T.A., K.T., and K.O. wrote the manuscript with input from all authors. All authors critically reviewed and edited the final version of the manuscript.

Competing interests: T.A. is a founder and scientific advisor of Rege Nephro Co., Ltd. K.O. is a founder and member of the scientific advisory boards of iPS Portal, Inc., and a founder and chief scientific advisor of Rege Nephro Co., Ltd. R.Y. is a founder and chief technical officer of Physios Biotech, Inc. M.R. is an employee of Rege Nephro Co., Ltd. T.A., A.W., and K.O. hold a patent (“Novel Renal Progenitor Cell Marker and Method for Concentrating Renal Progenitor Cells Using Same,” patent application number PCT/JP2019/028648, filed July 23, 2019). T.A., M.R., and K.O. have a patent application pending (“Medium for Culturing Expanding Nephron Progenitor Cells, Method for Culturing and Expanding Nephron Progenitor Cells, and Method for Producing Renal Organoids,” patent application number PCT/JP2022/000564, filed Jan. 11, 2022). T.A. and K.O. have a patent application pending (“Therapeutic agent for renal disease and method for producing same,” Japanese patent application number 2024-187809, filed Oct. 24, 2024). K.T., C.I., M.M., C.M., J.W., Z.L., M.I., A.W., Y.T., and J.B. declare no competing interests.

Data and materials availability: All data associated with this study are present in the paper or supplementary materials. The NCBI GEO accession number for the RNA sequencing data reported in this paper is GSE276956. OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) and *VEGFA* knockout hiPSC line are available from K.O. under a materials transfer agreement (MTA). hiPSC lines derived from individuals with homozygous human leukocyte antigen (HLA)-haplotypes (HLA-homo-iPSCs) are available from the CiRA Foundation under a collaborative research agreement (<https://www.cira-foundation.or.jp/e/provision-of-ips-cells/manufacturing-flow/>).

Figures

Fig. 1. Human iPSC-derived NPCs (hiPSC-NPCs) without induction to kidney organoids show therapeutic effects on cisplatin-induced AKI in mice. (A) Flow cytometry analysis of two NPC markers, OSR1 and SIX2, in hiPSC-NPCs after Stage 6 of a previously reported differentiation method ($n=5$) (16). (B) Bright-field (BF) and fluorescent images showing the morphology and expression of OSR1 and SIX2 in an aggregate formed from hiPSC-NPCs. Scale bar, 200 μm . (C) A BF image showing an hiPSC-NPC-derived kidney organoid. Scale bar, 500 μm . (D) Immunostaining analysis of an hiPSC-NPC-derived kidney organoid for PODOCALYXIN (PODXL), *Lotus Tetragonolobus* Lectin (LTL), and CADHERIN 1 (CDH1) as markers for podocytes, proximal tubules, and distal tubules, respectively. Scale bar, 100 μm . (E) Schematic representation of the experimental timeline for the transplantation of hiPSC-NPCs and hiPSC-derived fate-specified NPCs (hiPSC-FS-NPCs) in a cisplatin-induced AKI model in NOD-SCID mice. s.c., subcutaneous injection. (F) Blood urea nitrogen (BUN) and serum creatinine (S-Cre) amounts in wild-type (WT) mice and cisplatin-induced AKI mice receiving the indicated

treatments ($n=10-11$ per group). (**G** and **H**) Hematoxylin and eosin (HE) and Periodic acid-Schiff (PAS) staining of kidney sections from WT mice and cisplatin-induced AKI mice receiving the indicated treatments (**G**) and the quantitative evaluation of histological findings ($n=15$, 15 HPF images from 10-11 mice; **H**). Scale bars, 200 μm . (**I**) Kaplan-Meier survival curves of cisplatin-induced AKI mice receiving the indicated treatments. The p -value was assessed by the log-rank test comparing hiPSC-NPC transplantation and saline control groups ($n=10-11$ per group). HPF, high power field. Data are represented as means $\pm$ SD in (**F** and **H**). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and not significant (NS) by one-way ANOVA with Tukey-Kramer test for (**F**) and unpaired t -test for (**H**). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. 2. Expanded hiPSC-NPCs improve renal injury and survival rate of cisplatin-induced AKI mice. (**A**) Fluorescent images showing changes in the expression of OSR1 and SIX2 in hiPSC-NPCs passaged every 6 days under human NPSR (hNPSR) or CFY medium. Scale bar, 200 μm . (**B**) Changes in the gene expression of NPC markers in hiPSC-NPCs after each passage in hNPSR or CFY medium ($n=4$ per group). (**C**) BF images of kidney organoids derived from hiPSC-NPCs after each passage in hNPSR or CFY medium. Scale bars, 500 μm . (**D**) Flow cytometry analysis of OSR1- and SIX2-expressing cells (left panel) and cell number of total, OSR1⁺SIX2⁺, and OSR1⁺SIX2⁻ cells (right panel) in hiPSC-NPCs after each passage in CFY medium ($n=3$ per group). (**E**) Immunostaining analysis of kidney organoids derived from hiPSC-NPCs after each passage in CFY medium for PODXL, LTL, and CDH1. Scale bars, 200 μm . (**F**) Cumulative cell number of hiPSC-NPCs after each passage under CFY medium condition ($n=3$). (**G**) Schematic representation of the experimental timeline for the transplantation of hiPSC-NPCs after each

passage in CFY medium in a cisplatin-induced AKI model in NOD-SCID mice. s.c., subcutaneous injection. **(H)** BUN and S-Cre amounts in WT mice and cisplatin-induced AKI mice receiving the indicated treatments ($n=6-8$ per group). **(I and J)** HE and PAS staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (I) and the quantitative evaluation of histological findings ($n=15$, 15 HPF images from 6-8 mice per group; J). Scale bars, 200 μm . **(K)** Kaplan-Meier survival curves of cisplatin-induced AKI mice receiving the indicated treatments. The p -values were assessed by the log-rank test comparing treatment and saline control groups ($n=5$ per group). P1, passage 1; P2, passage 2; HPF, high power field. Data are represented as means $\pm$ SD in (B, D, F, H, and J). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and not significant (NS) by unpaired t -test for (B) and one-way ANOVA with Tukey-Kramer test for (H and J). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6c3-10) were used in all experiments.

Fig. 3. hiPSC-NPCs prevent kidney functional decline, fibrosis, and senescence caused by CKD. **(A)** Schematic representation of the experimental timeline for the transplantation of hiPSC-NPCs in an aristolochic acid (AA)-induced CKD model in NOD-SCID mice. i.p., intraperitoneal injection. **(B)** Time course of kidney function as indicated by BUN and S-Cre in AA-induced CKD mice receiving the indicated treatments ($n=15$ per group). **(C and D)** Masson-Trichrome (MT), anti- α -SMA and Picro-Sirius Red (SR) staining of kidney sections from WT mice and CKD mice receiving the indicated treatments at 4 months after AA administration (C) and the quantitative evaluation of the three stains ($n=20$, 20 HPF images from 15 mice per group; D). Scale bars, 800 μm for MT and anti- α -SMA staining and 100 μm for SR staining. **(E)** Gene expression of fibrosis markers in CKD mouse kidneys receiving the indicated treatments at 4 months after AA

administration ($n=4$ per group). (F) Urinary albumin (U-Alb) excretion corrected by urinary creatinine (U-Cre) in CKD mice receiving the indicated treatments at 4 months after AA administration ($n=3$ per group). (G and H) Senescence-associated β -galactosidase (SA- β -GAL) staining of kidney sections from WT mice and CKD mice receiving the indicated treatments at 4 months after AA administration (G) and the number of regions stained with SA- β -Gal ($n=20$, 20 HPF images from 15 mice per group; H). Scale bars, 50 μ m. (I) Gene expression of senescence markers in CKD mouse kidneys receiving the indicated treatments at 4 months after AA administration ($n=4$ per group). HPF, high power field. Data are represented as means $\pm$ SD in (B, D, E, F, H, and I). * $P<0.05$, ** $P<0.01$ and not significant (NS) by unpaired t -test for (B, E, F, and I) and *** $P<0.001$ by one-way ANOVA with Tukey-Kramer test for (D and H). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. 4. Conditioned medium of giant hiPSC-NPC aggregates ameliorates AKI in mice. (A) Schematic representation of the experimental protocol. i.p., intraperitoneal injection. (B) Temporal BF and fluorescent images showing the morphology and expression of OSR1 and SIX2 during the formation of a giant aggregate from mini aggregates of hiPSC-NPCs in (A). Scale bars, 1 mm. (C) Immunostaining analysis of a giant aggregate from hiPSC-NPCs after 10 days of expansion culture for NPC markers SIX2, PAX2, SALL1, and WT1 and the proliferation marker Ki67. Scale bars, 200 μ m. (D) A BF image of a kidney organoid from a giant hiPSC-NPC aggregate. Scale bar, 500 μ m. (E and F) Immunostaining analysis of kidney organoids from giant hiPSC-NPC aggregates showing PODXL⁺ glomeruli, LTL⁺CDH1⁻ proximal tubules, and LTL⁻CDH1⁺ distal tubules (E) and NPHS1⁺ or WT1⁺ glomeruli, JAGEED1⁺CDH1⁻ proximal tubules, BRN1⁺DBA⁻ Henle's loop,

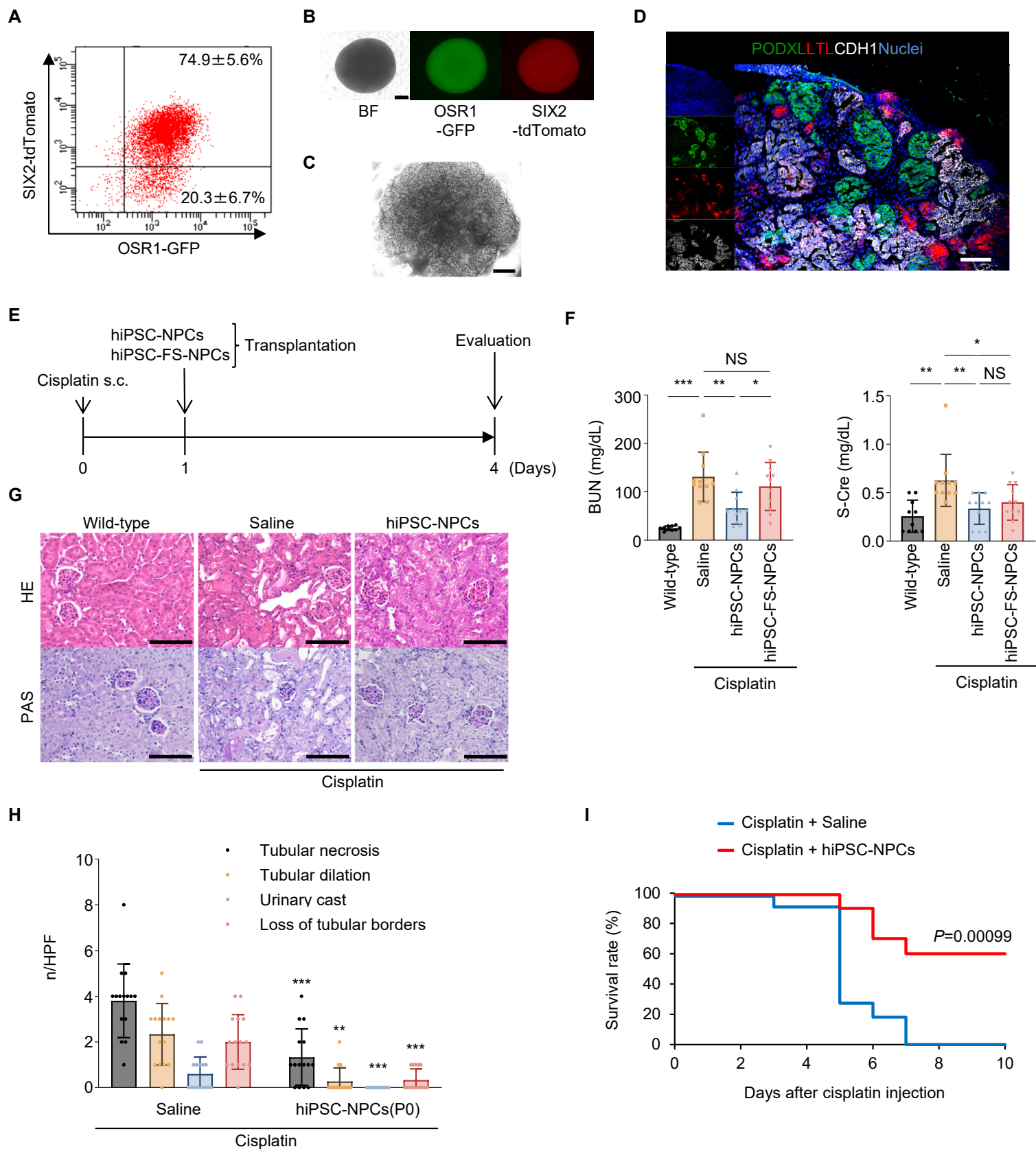
and CDH1⁺DBA⁺ or BRN1⁺DBA⁺ distal tubules (F). Scale bars, 200 μ m in (E), 100 μ m in the left and middle panels in (F), and 50 μ m in the right panel in (F). (G) Immunostaining analysis of a giant aggregate from hiPSC-NPCs after 10 days of expansion culture for the apoptotic cell marker CLEAVED CASPASE 3 (CC3). Scale bar, 200 μ m. (H) An LDH assay showing temporal changes in cytotoxicity during the formation of giant aggregates and aggregates formed in 96-wells ($n=3$, respectively). (I) BUN and S-Cre amounts in cisplatin-induced AKI mice receiving the indicated treatments ($n=11$ per group). (J and K) HE and PAS staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (J) and the quantitative evaluation of the histological findings ($n=15$, 15 HPF images from 11 mice per group; K). Scale bars, 100 μ m. (L) Kaplan-Meier survival curves of cisplatin-induced AKI mice receiving the indicated treatments ($n=7$ per group). The p -value was assessed by the log-rank test comparing CM treatment and control CFY medium groups. CFY, CFY medium; CM, conditioned medium; HPF, high power field. Data are represented as means $\pm$ SD in (H, I, and K). ** $P<0.01$ and *** $P<0.001$ by Mann-Whitney U-test for (H and K), and *** $P<0.001$, ** $P<0.01$, and * $P<0.05$ by unpaired t -test for (I). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

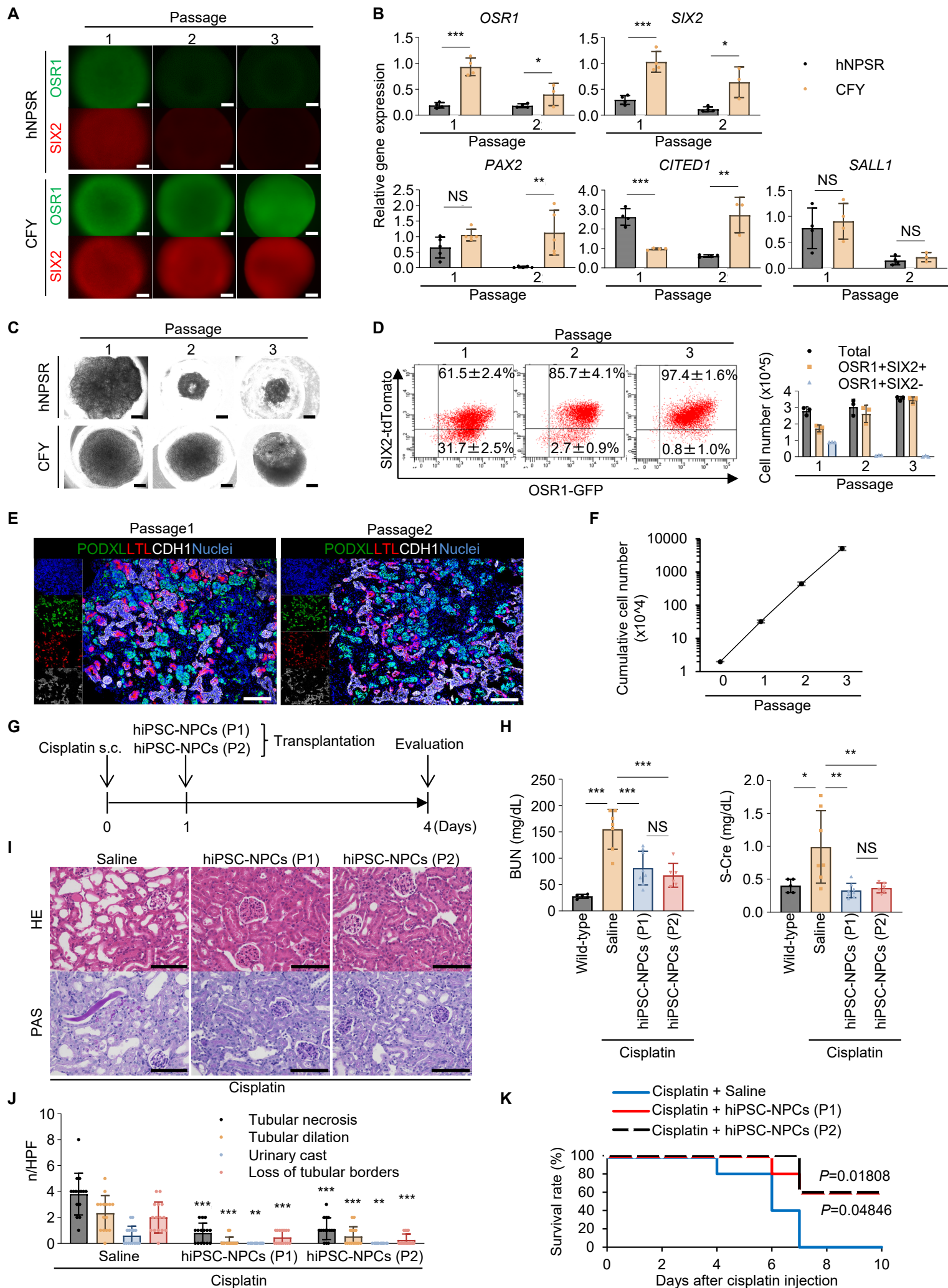
Fig. 5. Therapeutic mechanisms of hiPSC-NPCs for AKI include VEGF-A-mediated angiogenesis. (A) A volcano plot of DEGs between hiPSC-NPCs and hiPSC-FS-NPCs. Red and blue dots indicate upregulated and downregulated DEGs in hiPSC-NPCs, respectively, with statistical significance ($FDR < 0.1$) and a fold change greater than 1.0, and black dots represent non-significant DEGs. (B) Results of Enrichment (GO Biological Process) analysis between hiPSC-NPCs and hiPSC-FS-NPCs. Red terms and bars indicate GO terms related to circulatory

system development. (C) Immunostaining analysis of an hiPSC-NPC aggregate shows VEGF-A protein expression in the cytoplasm. Scale bars, 100 μ m (upper panels) and 50 μ m (lower panels). (D) VEGF-A concentrations in the CM of hiPSC-NPCs and hiPSC-FS-NPCs as measured by an ELISA assay ($n=3$ per group). (E) Schematic representation of the experimental timeline for the transplantation of hydrogelatinized human recombinant VEGF165 (hVEGF165) in a cisplatin-induced AKI model in NOD-SCID mice. s.c., subcutaneous injection. (F) BUN and S-Cre amounts in cisplatin-induced AKI mice treated with the sustained release of hVEGF165 protein from bioabsorbable hydrogels implanted under renal subcapsules ($n=3-7$). (G and H) Immunostaining images of kidney sections from cisplatin-induced AKI mice treated with the sustained release of hVEGF165 protein for CD31⁺ MVD and CC3⁺ apoptotic cells (G) and the quantitative analysis ($n=15$, 15 HPF images from 3-7 mice per group; H). Scale bars, 100 μ m. HPF, high power field. Data are represented as means $\pm$ SD in (D, F, and H). * $P<0.05$ and *** $P<0.001$ by unpaired t -test for (D, F, and H). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in (A - E).

Fig. 6. Therapeutic effects of hiPSC-NPCs on AKI and CKD models were reduced by knocking out the *VEGFA* gene. (A) Schematic representation of generating *VEGFA* knockout (KO) hiPSCs using the CRONUS system. (B) Mutational analysis of the genomic DNAs using Sanger sequencing. Red arrows indicate single nucleotide insertion. (C) VEGF-A concentrations in the culture supernatants of WT and *VEGFA* KO hiPSC-NPCs ($n=3$ per group). (D) Schematic representation of the experimental timeline for the transplantation of WT and *VEGFA* KO hiPSC-NPCs in a cisplatin-induced AKI model in NOD-SCID mice. (E) BUN and S-Cre amounts in cisplatin-induced AKI mouse groups treated with saline or the transplantation of WT or *VEGFA*

KO hiPSC-NPCs ($n=6$ per group). (F and G) HE and PAS staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (F) and the quantitative evaluation of the histological findings ($n=15$, 15 HPF images from 6 mice per group; G). Scale bars, 100 μm . (H and I) Immunostaining analysis of CD31 and CC3 of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (H) and the quantitative evaluation of the CD31⁺ MVD and CC3⁺ cell number ($n=15$, 15 HPF images from 6 mice per group; I). Scale bars, 100 μm . (J) Schematic representation of the experimental timeline for the transplantation of WT and *VEGFA* KO hiPSC-NPCs in an AA-induced CKD model in NOD-SCID mice. (K) Time course of kidney function as indicated by BUN and S-Cre amounts in AA-induced CKD mice receiving the indicated treatments ($n=6$ per group). (L and M) Masson-Trichrome (MT) and Picro-Sirius Red (SR) staining and anti-CD31 immunostaining images of kidney sections from AA-induced CKD mice receiving the indicated treatments at 3 months after AA administration (L) and the quantitative evaluation of the two stains and CD31⁺ MVD ($n=15$, 15 HPF images from 6 mice per group; M). Scale bars, 100 μm . HPF, high power field. Data are represented as means $\pm$ SD in (C, E, G, I, K, and M). *** $P<0.001$ by unpaired t -test for (C), * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ by one-way ANOVA with Tukey-Kramer test for (E, I, and M), and * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus Saline, $^{\dagger}P<0.05$ and $^{\dagger\dagger\dagger}P<0.001$ versus *VEGFA* KO hiPSC-NPCs by one-way ANOVA with Tukey-Kramer test for (G and K).





The diagram illustrates the experimental timeline in days. At the bottom, a series of upward-pointing arrows represent the induction of AA at days 0, 2, 4, 6, 8, and 10, labeled "AA i.p.". A horizontal timeline line follows, with a break between day 12 and day 30. At day 12, a downward arrow indicates "hiPSC-NPCs Transplantation". From day 30 to day 120, a bracket labeled "Evaluation" spans the period. Downward arrows at days 30, 60, 90, and 120 indicate the timing of blood sampling for evaluation. The timeline ends at day 120, labeled "(Days)".

Figure 2 consists of two line graphs. The top graph shows BUN (mg/dL) on the y-axis (0 to 150) against Months (0 to 4) on the x-axis. The bottom graph shows S-Cre (mg/dL) on the y-axis (0.0 to 1.5) against Months (0 to 4) on the x-axis. Both graphs compare Saline (blue line) and hiPSC-NPCs (red line) groups. Error bars represent standard error. Statistical significance is indicated by asterisks: * p < 0.05, ** p < 0.01.

Month	Saline BUN (mg/dL)	hiPSC-NPCs BUN (mg/dL)	Saline S-Cre (mg/dL)	hiPSC-NPCs S-Cre (mg/dL)
0	~30	~30	~0.4	~0.4
1	~85	~70	~0.6	~0.5
2	~65	~55	~0.75	~0.6
3	~70	~50	~1.0	~0.75
4	~75	~50	~1.0	~0.5

Figure 3: Histological analysis of kidney sections. The figure displays a 3x3 grid of micrographs showing kidney sections stained for MT (Masson's trichrome), α -SMA (smooth muscle actin), and SR (Sudan red). The columns represent different groups: WT (wild-type), Saline, and hiPSC-NPCs. The rows represent different staining methods. The bottom row is also labeled 'Aristolochic acid (AA)'. Scale bars are present in the bottom right of each image.

Figure 3 consists of three bar graphs showing the quantification of fibrotic area (%) for WT, Saline, and hiPSC-NPCs under AA treatment. The y-axis for all graphs is 'Fibrotic area (%)'. The x-axis categories are WT, Saline, and hiPSC-NPCs, with a bracket labeled 'AA' below them. Error bars represent SEM. Statistical significance is indicated by asterisks (***) and brackets.

- MT staining:** The y-axis ranges from 0 to 25. WT is near 0. Saline is approximately 15.5%. hiPSC-NPCs is approximately 8.5%. Significance: Saline vs WT (***), Saline vs hiPSC-NPCs (***), hiPSC-NPCs vs WT (***).
- α -SMA positive area (%):** The y-axis ranges from 0 to 30. WT is near 0. Saline is approximately 21.5%. hiPSC-NPCs is approximately 13.5%. Significance: Saline vs WT (***), Saline vs hiPSC-NPCs (***), hiPSC-NPCs vs WT (***).
- SR staining:** The y-axis ranges from 0 to 30. WT is near 0. Saline is approximately 22.5%. hiPSC-NPCs is approximately 12.5%. Significance: Saline vs WT (***), Saline vs hiPSC-NPCs (***), hiPSC-NPCs vs WT (***).

Figure 3 consists of four dot plots with error bars showing the relative gene expression of *α-Sma*, *Col4a1*, *Col1a1*, and *Fn1* in Saline and hiPSC-NPCs under AA conditions. The y-axis for all plots is 'Relative gene expression'. The x-axis for each plot has two categories: 'Saline' and 'hiPSC-NPCs', both under the 'AA' condition. In each plot, the 'Saline' group is represented by a grey bar and the 'hiPSC-NPCs' group by an orange bar. Individual data points are shown as dots. Asterisks (*) indicate a significant difference between the two groups.

Gene	Saline (AA)	hiPSC-NPCs (AA)
<i>α-Sma</i>	~1.3	~0.6*
<i>Col4a1</i>	~1.5	~0.8*
<i>Col1a1</i>	~2.3	~0.7*
<i>Fn1</i>	~2.4	~0.7*

U-Alb/U-Cre (mg/g·Cre)

Saline hipSC-NPCs

NS

Group	U-Alb/U-Cre (mg/g·Cre)
Saline	~180
hipSC-NPCs	~50

3

WT

SA- β -Gal staining

Saline

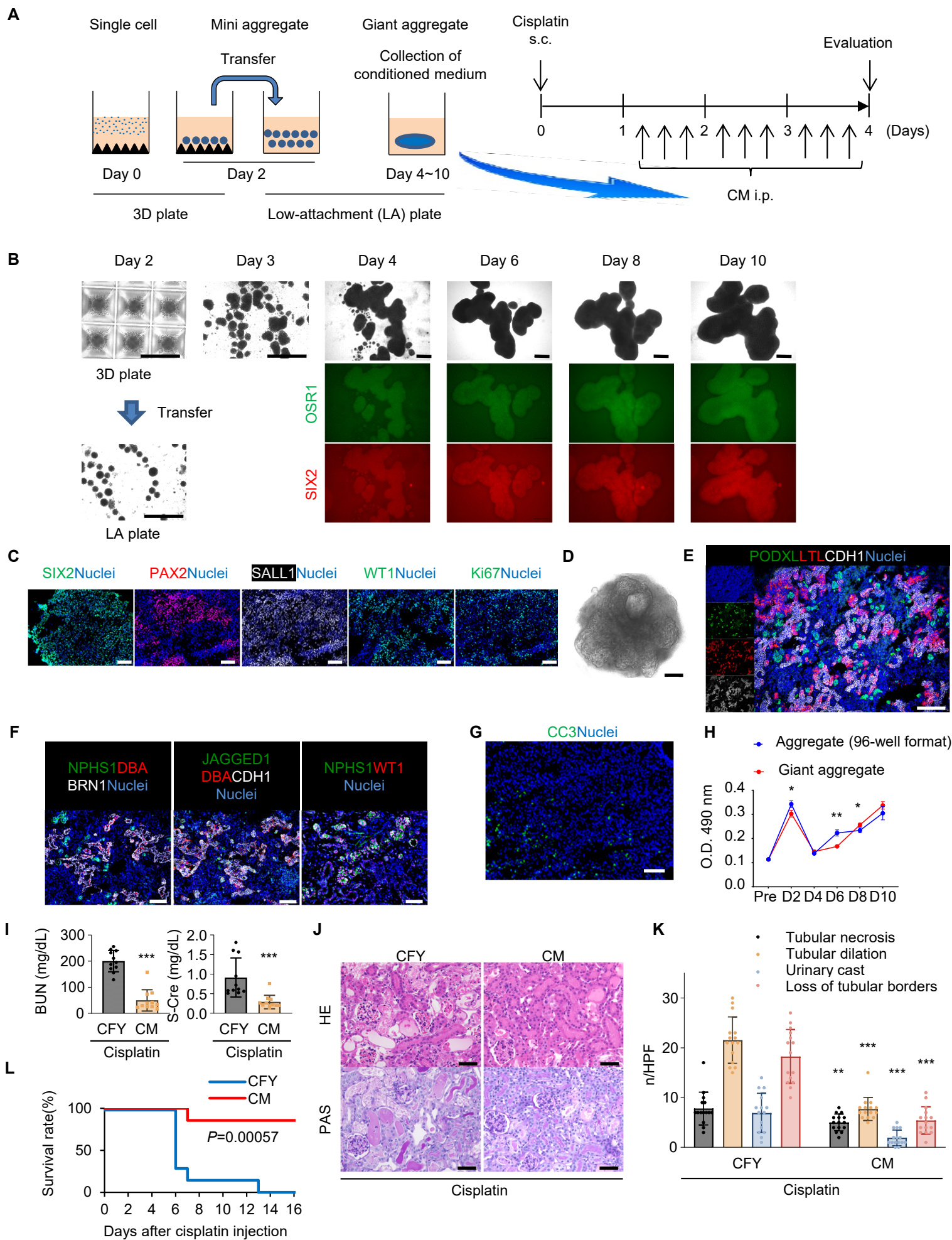
hiPSC-NPCs

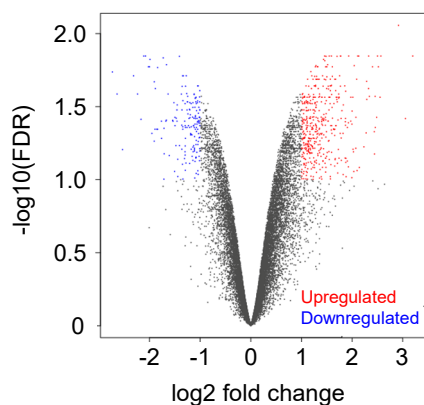
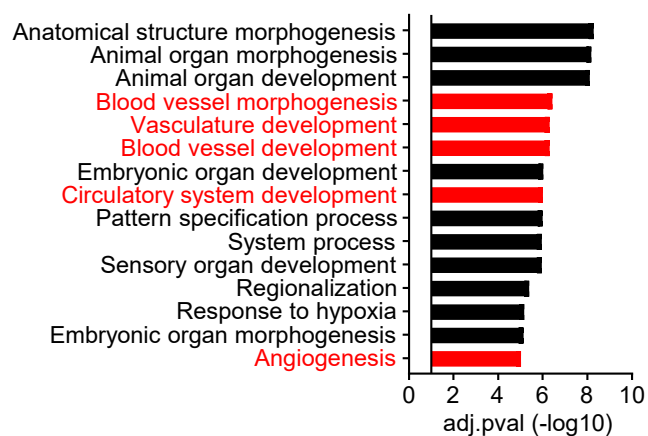
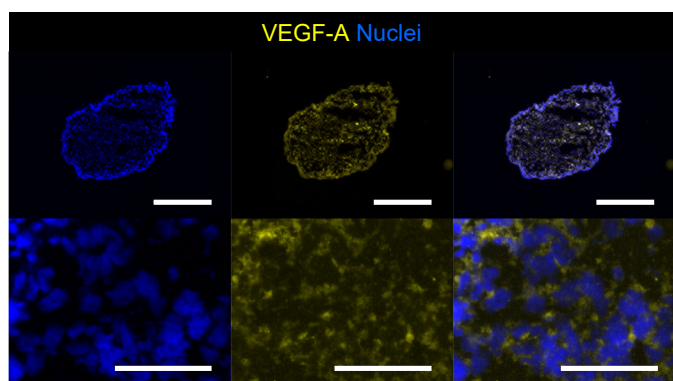
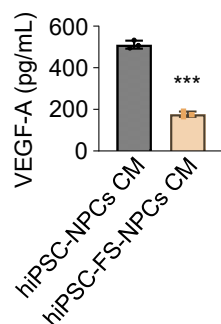
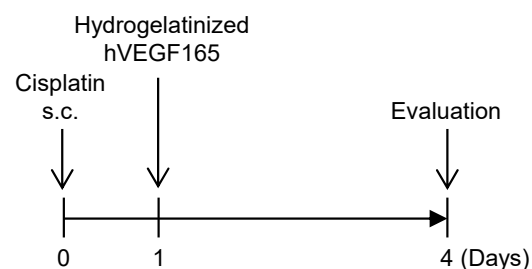
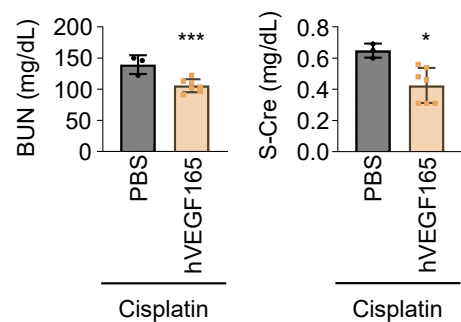
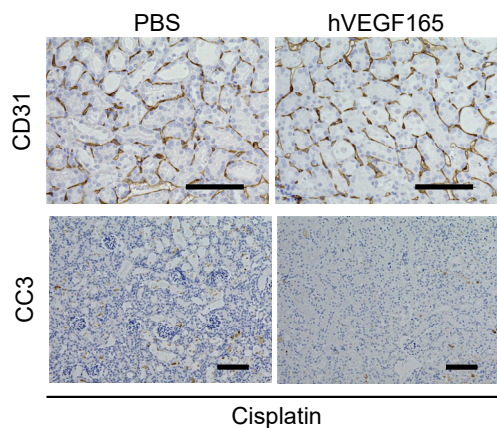
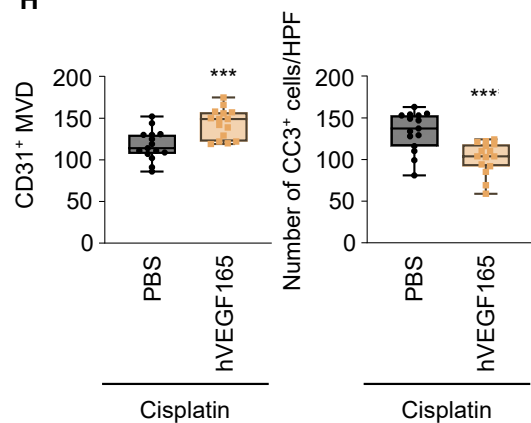
AA

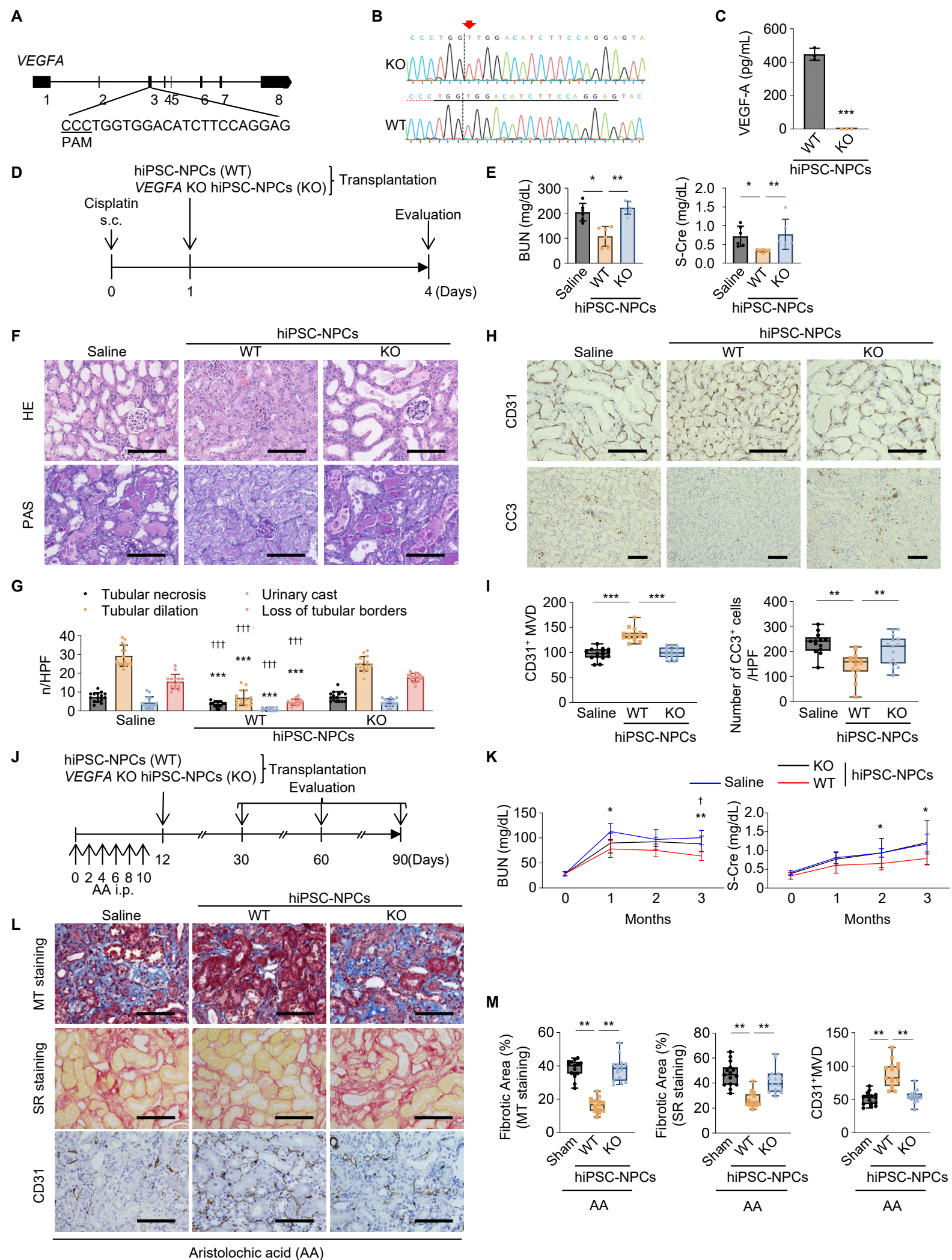
Group	n/HPF (Mean ± SD)
WT	0.5 ± 0.5
Saline	10.0 ± 4.0
hiPSC-NPCs	3.0 ± 1.0

Figure 3 consists of nine bar charts showing the relative gene expression of various genes in AA and hiPSC-NPCs. The y-axis for all charts is 'Relative gene expression'. The x-axis for each chart has two categories: 'Saline' and 'hiPSC-NPCs'. The genes are: *Mmp13*, *p16*, *p21*, *Atf3*, *Gadd45b*, *Il-6*, *PAI-1*, *Tgfb1*, and *Btg2*. In all cases, the expression of these genes is significantly lower in hiPSC-NPCs compared to Saline. Statistical significance is indicated by asterisks (*, **).

Gene	Saline	hiPSC-NPCs	Significance
<i>Mmp13</i>	~1.1	~0.2	*
<i>p16</i>	~0.6	~0.2	**
<i>p21</i>	~0.7	~0.2	*
<i>Atf3</i>	~3.0	~0.5	*
<i>Gadd45b</i>	~3.5	~1.0	*
<i>Il-6</i>	~0.5	~0.2	*
<i>PAI-1</i>	~1.3	~0.3	**
<i>Tgfb1</i>	~3.5	~1.0	*
<i>Btg2</i>	~2.5	~1.0	*



A**B****C****D****E****F****G****H**



Supplementary Materials

Materials and Methods

Cell lines

A previously generated OSR1-GFP/SIX2-tdTomato double knock-in hiPSC line (4A6C3-10) (15) was used to develop expansion cultures for hiPSC-NPCs and cell therapies and to obtain the conditioned medium (CM). A luciferase knock-in hiPSC line (201B7 luc-7) (66) was a generous gift by Dr. Y. Yoshida (CiRA, Kyoto University) and was used to confirm the long-term viability of hiPSC-NPCs transplanted into renal subcapsules. An hiPSC line with two alleles of the same human leukocyte antigen (HLA) type (Ff-I14s04) was provided by the CiRA Foundation (Japan) and used to demonstrate the versatility of the expansion culture and its potential use in cell therapies.

Cell culture

hiPSCs were maintained with feeder-free cultures using Stem Fit AK02 N medium (Ajinomoto) on 6-well cell culture plates (CellBIND #3335, Corning) coated with iMatrix-511 silk (Nippi). Cells were passaged using treatment with 0.5 mM EDTA/PBS (Thermo Fisher Scientific) for 4 min at 37°C and gentle pipetting every 4 to

6 days and routinely tested for mycoplasma contamination. After the hiPSCs were maintained on iMatrix-coated 24-well cell culture plates (CellBIND #3337, Corning) for 2 days, they were differentiated into NPCs and kidney organoids by changing the concentration of CHIR99021 from 3 μ M to 5 μ M (16). Harvesting and maintaining Six2-expressing mouse NPCs (mNPCs) from B6; 129-Six2^{tm3(EGFR/cre/ERT2)Amc/J} (JAX Stock No. 009600) mice were done as described previously (21). However, because we found it difficult to maintain mNPCs for a long time using 200 ng/mL human FGF basic (Wako), we used 300 ng/mL. Expandable human fetal kidney-derived NPCs established in a previous report (21) were maintained in human NPSR medium (300 ng/mL human FGF basic, 50 ng/mL BMP-7, 10 ng/mL human LIF, 10 μ M Y-27632, 1 μ M CHIR99021, 1 μ g/mL Heparin, 50 nM A 83-01, and 10 nM LDN193189) and used for experiments at the Salk Institute for Biological Studies. Information on growth factors, low-molecular-weight compounds, and reagents used in this study is listed in table S5.

Urinary albumin measurement

Urinary albumin concentration was determined by ELISA using a mouse Albuwell M Kit (Exocell) following the manufacturer's instructions. Urinary creatinine concentration was determined by Jaffe's reaction using a QuantiChrom Creatinine Assay

Kit (BioAssay Systems) following the manufacturer's instructions. Albumin was normalized by creatinine, and albuminuria was quantified by the albumin/creatinine ratio.

Flow cytometry

After hiPSC-NPCs generated in 2D culture and hiPSC-NPC aggregates expanded in suspension culture were treated with Accumax (Innovative Cell Technologies) for 15 min at 37°C and dissociated by gentle pipetting, dissociated cells were reacted with an anti-c-MET/HGFR antibody (FAB3582A, R&D) in 2% FBS (Wako) for 25 min on ice. Mouse IgG1 antibody (IC002A, R&D) was used as an isotype control. Cells were analyzed and sorted using a FACS ARIA II cell sorter (BD), and dead cells stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Thermo Fisher Scientific) were excluded from the analyses. Isolated cells were collected in CFY medium. Data were analyzed using FACS DIVA (BD) software and FlowJo v10.10 software. Information on antibodies used in this study is listed in table S6.

Immunostaining, RNA purification, and qRT-PCR

Whole-mount and section immunostaining were performed as described previously (16, 21). Information on antibodies used in this study is listed in table S6. Total

RNA was extracted using a miRNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendation, followed by cDNA synthesis using standard protocols for ReverTra Ace (TOYOBO). qRT-PCR was performed as described previously (16). Primers used in this study are listed in tables S7 (human) and S8 (mouse).

Bioluminescence Imaging (BLI)

Mice were examined at each time point after the left renal subcapsular transplantation of luciferase knock-in hiPSC line (201B7 luc-7)-derived NPCs for BLI analysis using an IVIS Lumina Series III (PerkinElmer). hiPSC (4A6C3-10)-NPCs, which do not express luciferase, were used as a control. Images were captured as described previously (67).

LDH leakage assay

Cytotoxicity induced by long-term cultures of giant aggregates was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. LDH activity in the CM obtained from giant aggregates was determined using a commercially available assay kit (Cytotoxicity LDH Assay Kit #347-91751, Dojindo Molecular Technologies, Inc.). The CM and reagents were mixed in a 96-well plate (Falcon), and absorbance was recorded

using an EnVision 2104 Multilabel Reader (PerkinElmer). Results were analyzed with EnVion Manager (version 1.14.3049.528).

RNA-seq data processing

Total RNA of OSR1⁺SIX2⁺, OSR1⁺SIX2⁻, and OSR1⁻SIX2⁻ cells were extracted using the miRNeasy Mini Kit and subjected to the preparation of sequencing libraries using the KAPA Stranded mRNA-seq Kit (KAPA Biosystems). Libraries were sequenced in the 100-cycle Single-Read mode of HiSeq2500. All sequence reads were extracted in FASTQ format using BCL2FACSTQ Conversion Software 1.8.4 in the CASAVA 1.8.2 pipeline. Sequence reads were mapped to hg19 reference genes using Tophat v2.0.14 and normalized and quantified using RPKMforGenes, downloaded on 10 December 2012. Gene expression was represented by log₂ (RPKM+1). Differentially expressed genes (DEGs) were extracted by comparing cells of interest with the control. DEGs coding transmembrane proteins were extracted using the GO term GO:0055085. A heatmap of relative gene expression was generated using the pheatmap 1.0.12 package in R 4.0.2.

Total RNA was extracted from hiPSC-NPCs and hiPSC-FS-NPCs using the RNeasy Mini Kit (Qiagen), and the quality of the RNA was assessed using a Bioanalyzer (Agilent) to ensure that the RNA integrity number (RIN) was above 7.0. RNA-seq library

preparation, sequencing, mapping, and gene expression analysis were performed by DNAFORM . After poly(A)⁺ enrichment by the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs), double-stranded cDNA libraries (RNA-seq libraries) were prepared using the SMARTer Stranded Total RNA Sample Prep Kit-HI Mammalian (Clontech) according to the manufacturer's instructions. RNA-seq libraries were sequenced on a NextSeq 500 instrument (Illumina) using paired-end reads (50nt for read1 and 25nt for read2). Obtained raw reads were trimmed and quality-filtered using Trim Galore! (version 0.4.4), Trimmomatic (version 0.36), and cutadapt (version 1.16) software. Trimmed reads were mapped to the human GRCh38 genome using STAR (version 2.7.2b). Reads on annotated genes were counted using featureCounts (version 1.6.1), and FPKM values were calculated from mapped reads by normalizing to total counts and transcript. DEG and Enrichment (GO Biological Process) analysis was performed using iDEP (version 0.96).

***In vitro* differentiation into kidney organoids**

As previously described, hiPSC-NPC aggregates were cultured on Transwell (Corning) in KR5 medium consisting of DMEM/F12 GlutaMAX medium (Thermo Fisher Scientific), 5% KnockOut Serum Replacement (Thermo Fisher Scientific), MEM NEAA

(GIBCO), 55 μ M 2-Mercaptoethanol (Sigma-Aldrich) and 500 U/L Penicillin-Streptomycin (Thermo Fisher Scientific) supplemented with 5 μ M CHIR99021 and 200 ng/mL FGF2 for 2 days, followed by 8 days of culture in KR5 medium without inducing factors (16, 21). Kidney organoids were used for whole-mount and section immunostaining as described below. Information on growth factors, low-molecular-weight compounds, and reagents used in this study is listed in table S5.

***In vivo* differentiation into kidney organoids**

hiPSC-NPC aggregates were treated with 5 μ M CHIR99021 and 200 ng/mL FGF2 for 1 day in suspension culture using low-attachment 96-well plates and then transplanted into left renal subcapsules of NOD-SCID mice. After four weeks, grafts and host kidneys were removed and used for section immunostaining as described below.

Inhibition of VEGF with axitinib in cis-AKI mice

The administration of axitinib (Selleck Biotech) to NOD-SCID mice was performed as previously described (37). Briefly, NOD-SCID mice were orally treated with 30 mg/kg axitinib twice daily for 14 days; on day 10 of axitinib treatment, 21 mg/kg cisplatin was subcutaneously injected, and 24 h later, hiPSC-NPCs were transplanted into

left renal subcapsules (axitinib(+)/hiPSC-NPCs(+); $n=5$). Other groups of NOD-SCID mice were treated with one of the following: oral administration of 0.5% carboxymethylcellulose and injection of PBS into left renal subcapsules (axitinib(-)/hiPSC-NPCs(-); $n=5$); oral administration of 0.5% carboxymethylcellulose and transplantation of hiPSC-NPCs into left renal subcapsules (axitinib(-)/hiPSC-NPCs(+); $n=5$); and axitinib treatment and injection of PBS into left renal subcapsules (axitinib(+)/hiPSC-NPCs(-); $n=5$). All mice were sacrificed 4 days after cisplatin injection.

Transplantation of hydrogels sustainably releasing recombinant human VEGF165 proteins into cis-AKI mice

Hydrogels that sustainably release recombinant human VEGF165 proteins were generated using a commercially available bioabsorbable material (MedGel microsphere, MedGEL CO., LTD). Hydrogelatinized VEGF165 (PeproTech Inc.; 1 μ g/10 μ L) was transplanted into left renal subcapsules of NOD-SCID mice 1 day after cisplatin administration ($n=7$). For the sham group, MedGel microsphere soaked in PBS(-) was used as a control ($n=3$).

Histological analyses of renal microvascular network, apoptosis, and fibrosis in cis-

AKI and AA-CKD mice treated with VEGF-A

Microvessel density (MVD) was determined by quantifying the number of CD31⁺ vascular structures, whereas the number of cleaved caspase-3 (CC3)⁺ cells was used to quantify apoptosis. Fibrotic areas were quantified by measuring the areas positive for Masson-Trichrome (MT) and Picro-Sirius Red (SR) staining. Multiple histological sites were assessed to compile a comprehensive view of the tissue response using a BZ-X Analyzer (KEYENCE, version 1.4.1.1). Information on antibodies used in this study is listed in table S6.

Quantification of VEGF proteins in conditioned media using enzyme-linked immunosorbent assay (ELISA)

Concentrations of human VEGF protein in the CM of hiPSC-NPC aggregates and mouse serum were determined using the LBIS Human VEGF ELISA Kit (FUJIFILM Wako Shibayagi Corp.) following the manufacturer's instructions. To detect human VEGF in mouse serum, serum samples were concentrated using Amicon Ultra-0.5 mL centrifugal filters (Merck Millipore Ltd.) according to the manufacturer's instructions. Following concentration, samples were subjected to ELISA for VEGF quantification.

Generation of *VEGF-A* knockout hiPSC lines

The CRISPR-Cas9 regulated by transcription and nuclear shuttling (CRONUS) system (39) was employed to generate *VEGFA* knockout hiPSC lines from 4A6C3-10 (15). Briefly, a CRONUS *piggyBac* vector containing the TetO-Cas9-GR-IRES-mCherry construct (Addgene ID 100596) and the gRNA-RFP-expressing *piggyBac* vector pPV-H1-ccdB-mEF1 α -RiH (Addgene ID 100598), which can be selected using puromycin and hygromycin, respectively, were sequentially introduced into 4A6C3-10 cells by lipofection with the aid of the *piggyBac* transposase (PBase) vector pHL-EF1 α -hcPBase-A. The gRNA sequence targeting the *VEGFA* exon 3 site was cloned into the pPV-H1-ccdB-mEF1 α -RiH vector using the following sequence: 5'-GAGACACACTTGGGATCCGTCCTGGAAGATGTCCACCAGTTTTAGAGCTAGAAATAGCA -3' (target sites are underlined). After transfection with the CRONUS *piggyBac* vectors, cells were plated onto a 6-well iMatrix-511 silk-coated plate in StemFit AK02N medium supplemented with 10 μ M Y-27632. Drug selection was performed using 1 μ g/mL puromycin (Nacalai Tesque) and 200 μ g/mL hygromycin B (Nacalai Tesque) for 5 days. The expression of mRFP1 protein confirmed gRNA expression, with mRFP1⁺ colonies picked and expanded to obtain CRONUS 4A6C3-10 cell lines. Subsequently, cells were treated with 2 μ M dexamethasone (Wako) and 1 μ M doxycycline (LTK Labs)

to induce the expression of Cas9 protein and facilitate its translocation into the nuclei. Single cells were isolated using flow cytometry, and clones were expanded in 24-well culture plates (Corning) coated with iMatrix-511 (Nippi). Homozygous *VEGFA* knockout hiPSC lines were carefully selected from the same batch and genotyped using Sanger sequencing. Primer sequences used in this study are listed in table S9.

Immunoprecipitation and western blot analysis

Kidneys were homogenized in RIPA buffer (Nacalai Tesque, 16488-34) containing protease inhibitors (Sigma-Aldrich, P8340), phosphatase inhibitor cocktail 2 (Sigma-Aldrich, P5726), and phosphatase inhibitor cocktail 3 (Sigma-Aldrich, P0044). For immunoprecipitation, 1 mg of total protein extract was diluted to 1 mL and mixed with 10 μ L of VEGFR2 antibody (Cell Signaling, #2479, 1:500 dilution) pre-bound to 100 μ L of Protein A-agarose beads (Santa Cruz Biotechnology, sc-2001). The mixture was incubated at 4°C overnight. Beads were washed thrice with lysis buffer and then resuspended and boiled in sample buffer solution (Nacalai Tesque, 30566-22). Immunoprecipitated proteins were separated on 4-15% Mini-PROTEAN TGX precast gels (Bio-Rad, #4561083) and transferred onto nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad, #1704159).

Membranes were blocked with 3% BSA in TBST (1X Tris-buffered saline, 0.1% Tween-20) for 2 hours at room temperature and then probed overnight at 4°C with primary antibodies against phosphorylated VEGFR2 (Cell Signaling, #2478, 1:500 dilution) and VEGFR2 (Cell Signaling, #2479, 1:500 dilution). After washing thrice with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, #7074, 1:1,000 dilution) for 2 hours at room temperature. Protein bands were visualized using the Amersham ECL Western Blotting Detection Reagent (Cytiva), and images were captured and analyzed using an ImageQuant 800 imager.

Blood counting and biochemical examination related to table S2

Blood samples were collected from five control mice and five NOD-SCID mice 3 months after hiPSC-NPC transplantation, respectively. Blood samples were assayed using VETSCAN5 HM5 (Zoetis) for blood counting and Piccolo Xpress (Abbott) for biochemical examination to observe systemic conditions.

Histological analyses of major tissues after hiPSC-NPC transplantation related to table S3

The retina (eyeball), liver, spleen, heart, and lung of NOD-SCID mice 3 months after hiPSC-NPC transplantation and their control mice were collected and histologically evaluated by a third-party institution, CMIC Holdings Co., Ltd. Tissue sections of 3- μ m thickness collected from each organ were examined in detail after HE staining and histological findings were qualitatively evaluated.

Nano LC-MS/MS-based proteome analysis

Cell supernatants were collected to which equal volumes of 2X PTS buffer containing a protease inhibitor mixture (Cat#P8340, Sigma-Aldrich) (24 mM sodium deoxycholate (SDC, #190-08313), 24 mM sodium lauroyl sarcosinate (SLS, #192-10382), and 200 mM Tris-HCl (pH 9.0)) were added to make protein samples. Protein samples were processed and analyzed by diaPASEF (68) and DIA-NN (69) as previously reported (70). Protein-protein interaction analysis was performed by using STRING (ver.11.5.). An enrichment map was visualized by using Cytoscape (ver.3.10.2) with stringent pathway similarity scores (Jaccard and overlap combined coefficient 0.6) and manually curated for the most representative groups of similar pathways and processes. Singleton pathways that were redundant with larger groups of pathways were discarded. For gene ontology analysis (biological process), proteins were classified into annotated groups

using AutoAnnotate (ver.1.5.1). A list of unique secreted proteins derived from giant-CM and Network coordinates are shown in tables S4 and data file S5, respectively.

Supplementary Figures

Fig. S1. Expanded human fetal kidney-derived NPCs have therapeutic effects on cisplatin-induced AKI in mice. (A) Schematic representation of the experimental timeline for the transplantation of FS-hfNPC-NPCs in a cisplatin-induced AKI model in NOD-SCID mice. s.c, subcutaneous injection. (B) Blood urea nitrogen (BUN) and serum creatinine (S-Cre) amounts in cisplatin-induced AKI mice receiving the indicated treatments ($n=5$ per group). (C and D) Hematoxylin and eosin (HE) and Periodic acid-Schiff (PAS) staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (C) and the quantitative evaluation of the histological findings ($n=15$, 15 HPF images from 5 mice per group; D). Scale bar, 50 μm . FS-hfNPCs, fate-specified human fetal kidney-derived nephron progenitor cells. HPF, high power field. Data are represented as means $\pm$ SD in (B and D). *** $P<0.001$ by one-way ANOVA with Tukey test for (B and D).

Fig. S2. Suspension culture increases the differentiation efficiency and enables the expansion of hiPSC-NPCs. (A) Flow cytometry analysis of OSR1- and SIX2-expressing cells (left panel) and the percentage of OSR1⁺SIX2⁺ cells (right panel) in hiPSC-NPCs after 2 days of monolayer (blue line) or suspension culture (orange line) ($n=7$ per group). (B) Changes in gene expression of NPC markers in hiPSC-NPCs after each passage in CFY medium ($n=3$). (C) Immunostaining analysis of hiPSC-NPCs after two passages in CFY medium for NPC markers SIX2, SALL1, PAX2, HOXD11, and WT1 and the proliferation marker Ki67. Scale bars, 200 μ m. Data are represented as means $\pm$ SD in (A and B). * $P<0.05$ by unpaired t -test for (A). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S3. Expanded hiPSC-NPCs differentiate into kidney structures with vascularization after renal subcapsular transplantation *in vivo*. (A and B) Immunostaining analysis of kidney organoids derived from hiPSC-NPCs after expansion culture with 1 (A) and 2 passages (B) for podocyte markers NPHS1 and WT1, proximal tubule marker JAGGED1, Henle's loop and distal tubule marker BRN1, and distal tubule

markers DBA and CDH1. Organoids show NPHS1⁺WT1⁺ glomeruli, JAGEED1⁺CDH1⁻ proximal tubules, BRN1⁺DBA⁻ Henle's loop, and CDH1⁺DBA⁺ or BRN1⁺DBA⁺ distal tubules. Scale bars, 100 μ m. (C) An image of the kidney before removal from the abdominal cavity of a NOD-SCID mouse at 4 weeks after the renal subcapsular transplantation of hiPSC-NPCs expanded and treated with CHIR99021 and FGF2 for 1 day. White arrows indicate hiPSC-NPC grafts integrated with host blood vessels. (D and E) Immunostaining analysis of hiPSC-NPC-derived kidney structures in grafts from (C) for NPHS1, DBA, and BRN1 (D) and for PODXL, LTL, and CDH1 (E). Scale bars, 50 μ m in (D) and 200 μ m in (E). (F) Immunostaining analysis of vascularized glomeruli in hiPSC-NPC-derived kidney structures from (C) for a vascular endothelial marker, CD31, specific to mice (mCD31) and two podocyte markers, PODXL and WT1. Scale bar, 50 μ m. hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S4. CHIR99021, FGF9, and Y-27632 are essential factors for the expansion culture of hiPSC-NPCs. (A) Bright-field (BF) images showing temporal morphological changes of hiPSC-NPC aggregates in suspension cultures with three different cell numbers during initial seeding. Scale bar, 200 μ m. (B and C) Temporal changes of the

proliferation rate ($n=3$ per group; B) and cell number per aggregate ($n=3$ per group; C) with three different cell numbers during initial seeding. (D) BF and fluorescent images showing the morphology and expression of OSR1 and SIX2 in hiPSC-NPC aggregates cultured in CFY medium without the indicated components for 8 days. An aggregate cultured in complete CFY medium (Full) was used as a control. C, CHIR99021; F, FGF9; Y, Y-27632. Scale bars, 200 μm . (E) Graphs showing the number of total (gray) and OSR1⁺SIX2⁺ cells (pale orange) in aggregates cultured in CFY medium without the indicated components for 8 days and evaluated by flow cytometry ($n=3$ per group). Aggregates cultured in complete CFY medium (Full) were used as a control. (F) Cumulative cell number of hiPSC-NPCs after each passage under CFY medium conditions, with data from validation experiments across independent laboratories demonstrating the reproducibility of the expansion method ($n=3$). (G) BF images of kidney organoids derived from hiPSC-NPCs after each passage in CFY medium, as captured in validation experiments across independent laboratories demonstrating reproducibility. Scale bars, 500 μm . Data are represented as means $\pm$ SD in (B, C, E, and F). §§ $P<0.01$, §§§ $P<0.001$ ($0.2 \times 10^5/\text{well}$ vs $0.6 \times 10^5/\text{well}$), ††† $P<0.001$ ($0.2 \times 10^5/\text{well}$ vs $1.5 \times 10^5/\text{well}$), and * $P<0.05$, ** $P<0.01$, *** $P<0.001$ ($0.6 \times 10^5/\text{well}$ vs $1.5 \times 10^5/\text{well}$) by one-way ANOVA with Tukey-Kramer test for (B and C), and *** $P<0.001$

(total cells) and $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (OSR1⁺SIX2⁺ cells) by unpaired *t*-test for (E).

hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S5. c-MET specifically expressed in OSR1⁺SIX2⁺ cells enables purification of

hiPSC-NPCs. (A) A heatmap showing the expression of candidate NPC markers. The left panel indicates the expression fold change (FC) of OSR1⁺SIX2⁺ cells to OSR1⁻SIX2⁻ cells; other panels show the expression amounts of the top 30 transmembrane genes (upper) and previously reported NPC markers (lower) differentially expressed among OSR1⁻SIX2⁻, OSR1⁺SIX2⁻ and OSR1⁺SIX2⁺ cells. (B) Flow cytometry analysis of c-MET⁻, OSR1⁻, and SIX2-expressing hiPSC-NPCs (*n*=8). Red and blue numbers in the right panel indicate percentages of OSR1⁺SIX2⁺ and OSR1⁺SIX2⁻ cells and OSR1⁺SIX2⁺ cells among c-MET⁺ cells, respectively. (C) BF and fluorescent images showing the morphology and expression of OSR1 and SIX2 in an aggregate formed from c-MET-purified hiPSC-NPCs. Scale bar, 100 μ m. (D) Differences in NPC marker expression between purified c-MET⁺ and c-MET⁻ hiPSC-NPC populations (*n*=5 per group). (E) Whole-mount immunostaining analysis of a kidney organoid derived from c-MET-purified hiPSC-NPCs for PODXL, LTL, and CDH1. Scale bar, 100 μ m. Data are

represented as means $\pm$ SD in (D). ** P <0.01 and *** P <0.001 by unpaired t -test for (D).

hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S6. c-MET-purified hiPSC-NPCs can be expanded and show therapeutic effects

on AKI in mice. (A) Flow cytometry analysis of OSR1⁺SIX2⁺, OSR1⁺SIX2⁻, and c-MET⁺ cells after each passage of c-MET-purified hiPSC-NPCs cultured in CFY medium ($n=3$ per group). (B) Whole-mount immunostaining analysis of a kidney organoid derived from hiPSC-NPCs after purification with c-MET and one passage in CFY medium for PODXL, LTL, and CDH1. Scale bar, 100 μ m. (C) Cumulative cell number of c-MET-purified hiPSC-NPCs after each passage in CFY medium ($n=3$). (D) Schematic representation of the experimental timeline for the transplantation of c-MET⁺ hiPSC-NPCs (P2) in a cisplatin-induced AKI model in NOD-SCID mice. s.c, subcutaneous injection. (E) BUN and S-Cre amounts in wild-type (WT) mice and cisplatin-induced AKI mice receiving the indicated treatments ($n=8$ per group). (F and G) HE and PAS staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (F) and the quantitative evaluation of the histological findings ($n=15$, 15 HPF images from 8 mice per group; G). Scale bars, 200 μ m. (H) Kaplan-Meier survival curves

of cisplatin-induced AKI mice receiving the indicated treatments. The *p*-value was assessed by the log-rank test comparing hiPSC-NPC transplantation and saline control groups (*n*=6 per group). P2, passage 2; HPF, high power field. Data are represented as means $\pm$ SD in (A, C, E, and G). **P*<0.05, ***P*<0.01, ****P*<0.001, and not significant (NS) by one-way ANOVA with Tukey-Kramer test for (E) and **P*<0.05, ***P*<0.01, and ****P*<0.001 by unpaired *t*-test for (G). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line were (4A6C3-10) used in all experiments.

Fig. S7. c-MET-purified hiPSC-NPCs derived from a healthy volunteer with homologous human leukocyte antigen (HLA) haplotypes ameliorate AKI in mice.

(A) Flow cytometry analysis of c-MET-expressing HLA homologous (homo) iPSC-derived NPCs (HLA-hNPCs; *n*=4). (B) Immunostaining analysis of an aggregate of HLA-hNPCs cultured in CFY medium for 1 day for the NPC marker SIX2 and the percentage of SIX2⁺ cells (*n*=4). Scale bar, 100 μ m. (C) BF images of kidney organoids derived from bulk and sorted c-MET⁻ and c-MET⁺ HLA-hNPCs. Scale bars, 500 μ m. (D) Whole-mount immunostaining analysis of a kidney organoid generated from HLA-hNPCs after purification with c-MET and 2 passages of expansion in CFY medium for PODXL, LTL,

and CDH1. Scale bar, 100 μ m. (E) BUN and S-Cre amounts in WT mice and cisplatin-induced AKI mice receiving the indicated treatments ($n=3$ per group). (F and G) HE and PAS staining of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (F) and the quantitative evaluation of the histological findings ($n=15$, 15 HPF images from 3 mice per group; G). Scale bars, 200 μ m. P2, passage 2; HPF, high power field. Data are represented as means $\pm$ SD in (B, E, and G). * $P<0.05$ and not significant (NS) by one-way ANOVA with Tukey-Kramer test for (E) and $§§§P<0.001$ (saline vs c-MET⁻), $†††P<0.001$ (saline vs c-MET⁺), and $***P<0.001$ (c-MET⁻ vs c-MET⁺) by one-way ANOVA with Tukey-Kramer test for (G).

Fig. S8. mNPCs and hiPSC-NPCs show therapeutic effects on aristolochic acid (AA)-induced CKD in mice. (A) Schematic representation of the experimental timeline for the transplantation of mNPCs in an AA-induced CKD model in NOD-SCID mice. i.p., intraperitoneal injection. (B) Time course of the kidney function as indicated by S-Cre in AA-induced CKD mice receiving the indicated treatments. mNPCs, mouse embryonic kidney-derived NPCs expanded under mouse NPSR medium condition; 1/2 mNPCs, half the number of mNPCs; FS-mNPCs, mNPCs induced into kidney organoids by CHIR99021 and FGF2 for 2 days ($n=5$ per group). (C and D) Masson-Trichrome (MT),

anti- α -SMA, and Picro-Sirius Red (SR) staining of kidney sections from WT mice and CKD mice receiving the indicated treatments at 4 months after AA administration (C) and the quantitative evaluation of the three stains ($n=20$, 20 HPF images from 5 mice per group; D). Scale bars, 800 μ m for MT and α -SMA staining and 100 μ m for SR staining, respectively. (E) Body weights of CKD mice receiving the indicated treatments ($n=15$ per group). (F and G) MT staining of fibrotic regions of glomeruli with Bowman's capsule in CKD mice receiving the indicated treatments at 4 months after AA administration (F) and the percentage of glomerular fibrosis areas ($n=20$, 20 HPF images from 15 mice with saline and hiPSC-NPC transplants and 5 mice with mNPC transplants per group; G). Data are represented as means $\pm$ SD in (B, D, E, and G). *** $P<0.001$ by one-way ANOVA with Tukey-Kramer test for (D and G) and not significant (NS) by unpaired t -test for (E). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in (E – G).

Fig. S9. Transplanted hiPSC-NPCs remained engrafted for an extended period with retained NPC marker expression without migration or integration into the host kidney parenchyma. (A) Images of both kidneys of a mouse transplanted with hiPSC-

NPCs under the left renal capsule at 4 months after AA administration. **(B)** HE staining of the kidney at 4 months after the renal subcapsular transplantation of hiPSC-NPCs. Scale bar, 200 μ m. **(C)** Immunostaining analysis of hiPSC-NPC-derived grafts at 4 months after renal subcapsular transplantation in CKD mice showing human PODXL^+ (hPODXL) glomeruli, $\text{LTL}^+\text{CDH1}^-$ proximal tubules, and $\text{LTL}^-\text{CDH1}^+$ distal tubules (left panel) and mouse PODXL^+ (mPODXL) glomeruli, $\text{BRN1}^+\text{DBA}^-$ Henle's loop, and $\text{BRN1}^+\text{DBA}^+$ distal tubules (right panel). Scale bars, 100 μ m. **(D)** Immunostaining analysis of hiPSC-NPC-derived grafts at 4 months after renal subcapsular transplantation in CKD mice for a vascular endothelial marker, including mouse-specific CD31 (mCD31), human-specific CD31 (hCD31), CD31 common to both mouse and human (hmCD31), and human nuclei (HuNu). Scale bar, 100 μ m. **(E)** Immunostaining analysis of hiPSC-NPC-derived grafts at 4 months after renal subcapsular transplantation in CKD mice for NPC markers OSR1 (GFP), SIX2, PAX2, and SALL1, an NPC purification marker c-MET, the proliferation marker Ki67, and human nuclei (HuNu). Scale bars, 100 μ m. hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S10. Enhanced therapeutic effects of c-MET⁺ cell transplantation compared to

c-MET⁻ cells in CKD model mice. (A) Schematic representation of the experimental timeline for the transplantation of c-MET⁻ HLA homo iPSC-derived NPCs (HLA-hNPCs) and c-MET⁺ HLA-hNPCs in an AA-induced CKD model in NOD-SCID mice. (B and C) Time course of kidney function as indicated by BUN and S-Cre (B) and body weight (BW) (C) in AA-induced CKD mice receiving the indicated treatments ($n=7$ per group). (D and E) Masson-Trichrome (MT), anti- α -SMA, and Picro-Sirius Red (SR) staining of kidney sections from WT mice and CKD mice receiving the indicated treatments at 3 months after AA administration (D) and the quantitative evaluation of the three stains ($n=15$, 15 HPF images from 3 mice per group; E). Scale bars, 100 μ m. Data are represented as means $\pm$ SD in (B, C, and E). * $P<0.05$, ** $P<0.01$ (saline vs c-MET⁺), $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ (c-MET⁻ vs c-MET⁺) and not significant (NS) by one-way ANOVA with Tukey-Kramer test for (B and C) and *** $P<0.001$ and NS by one-way ANOVA with Tukey-Kramer test for (E).

Fig. S11. hiPSC-NPCs transplanted under renal capsules survive for a long time. (A)

Images showing renal subcapsular (left panel) and subcutaneous transplantations of hiPSC-NPCs (right panel). hiPSC-NPCs were transplanted into the left renal subcapsule (red arrow) or a subcutaneous pocket created on the right shoulder (black arrow). (B)

Time course of luciferase imaging of mice after the right subcutaneous transplantation (SC) and left renal subcapsular transplantation (RS) of NPCs derived from hiPSCs constitutively expressing luciferase.

Fig. S12. hiPSC-NPCs alleviate damage to both the transplanted and contralateral kidneys in AKI and CKD mice. (A) Quantitative evaluation of histological findings by HE and PAS staining of left and right kidney sections from cisplatin-induced AKI mice receiving the indicated treatments ($n=15$, 15 HPF images from 6-8 mice per group). (B) Quantitative evaluation of fibrotic areas by MT staining of left and right kidney sections from WT and CKD mice receiving the indicated treatments at 4 months after AA administration ($n=20$, 20 HPF images from 15 mice per group). P1, passage 1; P2, passage 2; HPF, high power field. Data are represented as means $\pm$ SD. *** $P<0.001$ and not significant (NS) by one-way ANOVA with Tukey-Kramer test. hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S13. Cryopreserved conditioned medium (CM) from giant hiPSC-NPC aggregates ameliorates AKI in mice. (A) BUN and S-Cre amounts in cisplatin-induced

AKI mice receiving the indicated treatments ($n=5$ per group). (B) Schematic representation of the experimental protocol. LA plate, low-attachment plate. i.p., intraperitoneal injection. (C) BUN and S-Cre amounts in WT mice and cisplatin-induced AKI mice receiving the indicated treatments ($n=4$ per group). (D) Kaplan-Meier survival curves of cisplatin-induced AKI mice receiving the indicated treatments. The p -value was assessed by the log-rank test comparing cryopreserved CM treatment and control CFY medium groups ($n=4$ per group). CFY, CFY medium; CM, conditioned medium. Data are represented as means $\pm$ SD in (A and C). Not significant (NS) by unpaired t -test for (A) and $***P<0.001$ and NS by one-way ANOVA with Tukey test for (C). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S14. Transplantation of hiPSC-NPCs into mice treated with a VEGF receptor inhibitor axitinib resulted in a diminished therapeutic effect on AKI. (A) Schematic representation of the experimental timeline for the transplantation of hiPSC-NPCs in a cisplatin-induced AKI model in NOD-SCID mice following axitinib administration. s.c., subcutaneous injection. (B) BUN and S-Cre amounts in cisplatin-induced AKI mice treated with the indicated combinations of axitinib and hiPSC-NPCs ($n=5$ per group). (C)

and **D**) Immunostaining images of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (C) and the quantitative analysis (D) of CD31⁺ MVD ($n=15$, 15 HPF images from 5 mice per group). Scale bars, 100 μ m. (**E** and **F**) Immunostaining images of kidney sections from cisplatin-induced AKI mice receiving the indicated treatments (E) and the quantitative analysis (F) of CC3⁺ apoptotic cells ($n=15$, 15 HPF images from 5 mice per group). Scale bars, 100 μ m. HPF, high power field. Data are represented as means $\pm$ SD in (B). The box defines the interquartile range (IQR) between the first (Q1) and third (Q3) quartiles, with the median quartile indicated by a line. The whiskers extend to the minimum and maximum values, displaying all data points in the plot (D and F). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and not significant (NS) by one-way ANOVA with Tukey-Kramer test for (B, D, and F). hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S15. Transplantation of hiPSC-NPCs enhances bilateral renal repair by VEGF-

A secretion. (**A** and **B**) Western blot analysis (A) and quantification (B) of renal VEGFR2 phosphorylation in cisplatin-induced AKI model mice receiving PBS administration ($n=3$) and hiPSC-NPC transplantation ($n=6$). (**C**) The concentration of human VEGF-A

in sera of mice receiving PBS administration and hiPSC-NPC transplantation ($n=5$ per group). (D) Immunostaining images of left and right kidney sections from cisplatin-induced AKI mice receiving the indicated treatments and the quantitative analysis of CC3⁺ apoptotic cells and CD31⁺ MVD ($n=15$, 15 HPF images from 5 mice per group). Scale bars, 100 μ m. (E) Immunostaining images of left and right kidney sections from AA-induced CKD mice receiving the indicated treatments and the quantitative analysis of CD31⁺ MVD ($n=15$, 15 HPF images from 5 mice per group). Scale bars, 100 μ m. Data are represented as means $\pm$ SD in (B and C). The box defines the interquartile range (IQR) between the first (Q1) and third (Q3) quartiles, with the median quartile indicated by a line. The whiskers extend to the minimum and maximum values, displaying all data points in the plot (D and E). * $P<0.05$ and *** $P<0.001$ by unpaired t -test for (B and C) and * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ by one-way ANOVA with Tukey-Kramer test for (D and E). HPF, high power field. hiPSC-NPCs differentiated from an OSR1-GFP/SIX2-tdTomato double knock-in reporter hiPSC line (4A6C3-10) were used in all experiments.

Fig. S16. CM from giant hiPSC-NPC aggregates ameliorates AKI in mice by VEGF.

(A) The concentration of VEGF-A protein in giant hiPSC-NPC aggregate-derived CM (giant-CM) treated with various concentrations of bevacizumab was determined by

ELISA ($n=3$ per concentration of bevacizumab). (B) BUN and S-Cre amounts in cisplatin-induced AKI mice receiving the indicated treatments ($n=3$ per group). Bevacizumab was used at a concentration of 10,000 ng/mL. (C) BUN and S-Cre amounts in cisplatin-induced AKI mice receiving the indicated treatments ($n=5$ per group). (D) Venn diagram showing the number of secreted proteins detected by mass spectrometry for CM collected from hiPSC-NPC aggregates formed in 96-well plates (96-well-CM) and giant-CM. (E) The concentration of VEGF-A in 96-well- and giant-CMs. (F) Protein-protein interaction (PPI) network for secreted proteins detected only in giant-CM and VEGF-A. Network nodes and edges represent proteins and PPI, respectively. Red nodes indicate secreted proteins related to blood vessel development. (G) Enrichment map for secreted proteins detected only in giant-CM and VEGF-A. The enrichment network shows significantly associated (p -value <0.05) gene ontology (GO)-biological process (BP) gene sets (nodes). Data are represented as means $\pm$ SD in (A, B, C, and E). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and not significant (NS) by one-way ANOVA with Tukey test for (B and C) and * $P<0.05$ by unpaired t -test for (E).

Table S1: Previously reported expansion culture methods for hiPSC-NPCs.

		Li et al., 2016 (NPSR)	Brown et al., 2015 (NPEM)	Tanigawa et al., 2016
Culture period		Human fetal kidney-derived NPC (9-17 weeks); more than 7 months, more than 50 passages; hiPSC-NPC more than 2 months	2 passages	P0 (8 days without passage)
Culture condition	Human NPC sources and purification	Purified human fetal kidney-derived NGFR(+)/EPCAM(-) NPC; Purified SIX2-GFP(+) hiPSC-NPC	Human ESC-NPC (unpurified)	Human iPSC-NPC (unpurified)
	Number of hPSC lines	1 line; other hiPSC lines cannot be used for expansion culture	1 line	1 line
	Protocols	Taguchi et al., 2014	Takasato et al., 2014	Taguchi et al., 2014
	Format	3D	2D (Matrigel coating)	2D (Matrigel coating)
	Basal medium	DMEM/F12	APEL	DMEM/F12
	Specific factors	Factor	Factor	Factor
		Final conc.	Final conc.	Final conc.
		BMP7	BMP7	BMP7
		Heparin	Heparin	TNF- α
		FGF2	FGF9	FGF2/9
		Y-27632	Y-27632	Y-27632
		CHIR99021	CHIR99021	CHIR99021
		LDN193189	LDN193189	DAPT
		human LIF	BMP4	human LIF
Nephrogenic potential	Glomeruli	✓		✓
	Proximal tubules	✓	✓	✓
	Loop of Henle	✓		
	Distal tubules	✓	✓	✓
	In vivo	Differentiation into kidney structures with vascularization and urine production capacity by mesenteric transplantation of mice		
AKI	Cell sources and therapeutic potential	Human fetal kidney-derived NPCs; effective		
	Conditioned medium			
CKD	Cell sources and therapeutic potential			
Forming large NPC				
Cell proliferation		Split at 1:15 for each passage	Split at 1:8 for 2 passages (64-fold)	No passage (3.5-fold)

		Tanigawa et al., 2019	Our culture method (this article)
Culture period		P1 (14 days, 1 passage)	P2 (21 days, 2 passages)
Culture condition	Human NPC sources and purification	Purified ITGA8(+)/PDGFRA(-)/SIX2-GFP(+) hiPSC-NPC	Unpurified hiPSC-NPC, Purified c-MET(+) hiPSC-NPC
	Number of hPSC lines	3 lines	3 lines
	Protocols	Taguchi et al., 2014; Taguchi and Nishinakamura 2017	Tsujimoto et al., 2020
	Format	3D	3D
	Basal medium	DMEM/F12	DMEM/F12
	Specific factors	Factor	Factor
		Final conc.	Final conc.
		Activin A	
		TNF- α	
		FGF9	FGF9
		Y-27632	Y-27632
		CHIR99021	CHIR99021
		DAPT	
		human LIF	
Nephrogenic potential	Glomeruli	✓	✓
	Proximal tubules	✓	✓
	Loop of Henle		✓
	Distal tubules	✓	✓
	In vivo	Differentiation of P0 unpurified hiPSC-NPCs into kidney structures by subcapsular transplantation of mice	Differentiation of P1 unpurified hiPSC-NPCs into kidney structures by subcapsular transplantation of mice
AKI	Cell sources and therapeutic potential		Unpurified P1 and P2 hiPSC-NPCs: effective; Purified P2 hiPSC-NPCs: effective
	Conditioned medium		Supernatant derived from unpurified P1 hiPSC-NPCs: effective
CKD	Cell sources and therapeutic potential		Unpurified P1 hiPSC-NPCs: effective
Forming large NPC			Unpurified P1 hiPSC-NPCs: more than 5 mm in size
Cell proliferation		1 passage (25-fold)	Unpurified hiPSC-NPCs: 2 passages (78-fold); Purified hiPSC-NPCs: 2 passages (143-fold)

Table S2: Blood counting and biochemical examination of mice transplanted hiPSC-NPCs.

		CTL (n=5)	hiPSC-NPCs (n=5)	statistics
		average $\pm$ 2SD	average $\pm$ 2SD	(<i>t</i> -test)
Blood counting	WBC (/ μ L)	2,800 $\pm$ 695	2,880 $\pm$ 1021	NS
	lym (%)	16.4 $\pm$ 3.1	15.5 $\pm$ 1.1	NS
	Mono (%)	7.1 $\pm$ 5.5	6.7 $\pm$ 4.8	NS
	Neutro (%)	76.6 $\pm$ 7.1	79.8 $\pm$ 4.0	NS
	Hb (g/dL)	15.7 $\pm$ 1.1	15.2 $\pm$ 1.2	NS
	Hct (%)	45.4 $\pm$ 2.4	44.2 $\pm$ 2.5	NS
	MCV (fL)	46.2 $\pm$ 0.8	46.8 $\pm$ 1.2	NS
	MCH (pg)	16.0 $\pm$ 0.5	16.3 $\pm$ 0.6	NS
	MCHC (%)	34.5 $\pm$ 0.5	34.8 $\pm$ 1.9	NS
	Plt ($\times 10^3$ / μ L)	603 $\pm$ 166	628 $\pm$ 157	NS
Biochemical examination	Glu (mg/dL)	124 $\pm$ 11	106 $\pm$ 7	<i>P</i> < 0.05
	BUN (mg/dL)	25 $\pm$ 3	28 $\pm$ 1	<i>P</i> < 0.05
	S-Cre (mg/dL)	<0.2	<0.2	NS
	T-bil (mg/dL)	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0	NS
	Alb (g/dL)	2.1 $\pm$ 0.2	2.0 $\pm$ 0.1	NS
	ALT (U/L)	26 $\pm$ 7	26 $\pm$ 5	NS
	AST (U/L)	97 $\pm$ 20	113 $\pm$ 38	NS
	CK (U/L)	787 $\pm$ 360	618 $\pm$ 288	NS
	Amy (U/L)	1,148 $\pm$ 272	1,030 $\pm$ 37	NS
	Na (mmol/L)	154 $\pm$ 6	154 $\pm$ 6	NS
	K (mmol/L)	8.2 $\pm$ 0.3	8.2 $\pm$ 0.3	NS
	Ca (mg/dL)	10.6 $\pm$ 0.8	10.5 $\pm$ 0.2	NS
	CRP (mg/dL)	<0.5	<0.5	NS
		Breeder's test (n=8)	CTL (n=5)	statistics
		average $\pm$ 2SD	average $\pm$ 2SD	(<i>t</i> -test)
Biochemical examination	Glu (mg/dL)	83 $\pm$ 18	124 $\pm$ 11	<i>P</i> < 0.01
	BUN (mg/dL)	25 $\pm$ 4	25 $\pm$ 3	NS
		Breeder's test (n=8)	hiPSC-NPCs (n=5)	statistics
		average $\pm$ 2SD	average $\pm$ 2SD	(<i>t</i> -test)
Biochemical examination	Glu (mg/dL)	83 $\pm$ 18	106 $\pm$ 7	<i>P</i> < 0.05
	BUN (mg/dL)	25 $\pm$ 4	28 $\pm$ 1	NS

Table S3: Histological examination of major tissues in mice transplanted with hiPSC-NPCs.

Group		CTL	hiPSC-NPCs
Retina (eye ball)	Abnormal angiogenesis, retina	0	0
	Abnormal vascular proliferation, r	0	0
	Vasculitis, retina	0	0
	Other findings	0	0
Liver	Abnormal angiogenesis	0	0
	Abnormal vascular proliferation	0	0
	Vasculitis	0	0
	Other findings	0	0
Spleen	Abnormal angiogenesis	0	0
	Abnormal vascular proliferation	0	0
	Vasculitis	0	0
	increased	2	2
	Other findings	0	0
Heart	Abnormal angiogenesis	0	0
	Abnormal vascular proliferation	0	0
	Vasculitis	0	0
	Other findings	0	0
Lung	Abnormal angiogenesis	0	0
	Abnormal vascular proliferation	0	0
	Vasculitis	0	0
	Foam cell, alveolus	1	2
	Other findings	0	0
Grade 0: No change, 1: Very slight, 2: Slight, 3: Moderate			

Table S4: List of unique secreted proteins in giant aggregate-derived CM.

ADAMTS1	COL5A1	FABP5	IDE	MPEG1	PCDH15	SEMA3D
ADAMTS15	COL9A2	FBLN7	IGF1	MUC5AC	PLA2G3	SERPINA5
ANG	COLEC10	FGFBP3	IGFBP6	NAMPT	POSTN	SERPINB2
ANGPTL2	CPB1	FGG	IGFBPL1	NAXE	PRSS23	SERPINE1
ANXA1	CPQ	FLRT2	IL1RAP	NBL1	PRSS3	SERPINI1
BTBD17	CPXM1	FST	INS	NECTIN1	PSAP	SFN
C3	CRHBP	GAL	ISG15	NID1	PTN	SMOC1
C7	CRISPLD1	GASK1A	ISLR	NPTX2	PTPRZ1	SRPX2
CA11	CSF2RA	GDF11	LAMA2	NPW	RNASET2	TFRC
CCN2	CTHRC1	GGH	LGALS7, LGAL	NRCAM	RNPEP	TGFB1
CD163	CTSL	GNRH1	LRG1	NRP2	S100A4	TGFBR3
COL11A1	CYB5D2	GPC2	LRRC17	NUCB1	SBSN	TRH
COL15A1	DKK3	GPC6	MANF	OLFM2	SCG5	VEGFD
COL16A1	DRAXIN	GRN	MATN3	OLFML2B	SCUBE3	VGF
COL21A1	ESM1	HABP2	MFAP4	OLFML3	SDCBP	WFIKKN1
COL24A1	EYS	HAPLN1	MGAT5	OVCH1	SEMA3C	WNT10B

Table S5: Growth factors, low-molecular-weight compounds, and reagents used in this study.

Growth factor/chemical/reagent	Company	Cat.No.	Final concentrations
CHIR99021	Axon	AXN-AXON 1386-25	1 μ M, 5 μ M
BMP-7	R&D	354-BP	1 ng/mL
Human FGF basic	Wako	060-04543	300 ng/mL, 100 ng/mL
Human LIF	Millipore	LIF1050	10 ng/mL
Mouse LIF	Millipore	ESG1107	1000 units/mL
Heparin	Sigma-Aldrich	H3393	1 μ g/mL
Y-27632	Wako	034-24-24	10 μ M, 30 μ M
A 83-01	Wako	035-24113	50 nM, 10 μ M,
LDN193189	Wako	124-06011	10 nM
BMP-4	Pepro Tech	AF-120-05ET	1 ng/mL
Retinoic Acid	Sigma-Aldrich	R2625	10 nM, 100 nM
Activin A	R&D	338-AC-050	10 ng/mL
Human FGF 9	Pepro Tech	100-23	200 ng/mL
NOGGIN	Pepro Tech	120-10C	25 ng/mL
Accumax	Innovative Cell Technologies	AM105	1x
B-27 Supplement minus vitamin A (50X)	Thermo Fisher Scientific	12587001	1x
DMEM/F-12 GlutaMAX	Thermo Fisher Scientific	10565042	1x
iMatrix-511 silk	Nippi	892021	0.5 μ g/cm ²
KnockOut Serum Replacement	Thermo Fisher Scientific	A3181502	5%
TryPLE select CTS	GIBCO	A12859-01	2x
0.5 M EDTA	Thermo Fisher Scientific	15575020	0.25 mM
Stem Fit AK02N	Ajinomoto Healthy Supply	AK02N	1x
ITS Liquid Media Supplement (100X)	GIBCO	51500-056	1x
MEM NEAA (100X)	GIBCO	11140050	1x
2-Mercaptoethanol (55mM)	Sigma-Aldrich	M3148	55 μ M
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122	100x
Aristolochic acid	Sigma-Aldrich	A5512	2 mg/kg
Cisplatin	Wako	033-20091	16 mg/kg (ip), 21 mg/kg (sc)
DMSO	Sigma-Aldrich	D2660-100ML	
MedGel microsphere	MedGEL CO., LTD	PI5-95MS	1 mg/10 μ L
human recombinant VEGF165 protein	Pepro Tech		1 μ g/10 μ L
Axitinib	Selleck Biotech		30 mg/kg (po)
Bevacizumab	Selleck Biotech	A2006-5MG	10,000 ng/kg (ip)

Table S6: Antibodies and an ELISA kit used in this study.

Antibody	Company	Cat.No.	Dilution	RRID
PODOCALYXIN (human)	R&D	AF1658	x100	AB_354920
PODOCALYXIN (mouse)	R&D	MAB1556	x100	AB_2166010
LTL	Vector laboratories	B-1325	x200	AB_2336558
CDH1	Cell Signaling Technology	3195S	x100	AB_2291471
SIX2	Proteintech	11562-1-AP	x50	AB_2189084
SALL1	Perseus Proteomics	PP-K9814-00	x50	AB_2183228
PAX2	R&D	AF3364	x100	AB_10889828
HOXD11	Abcam	ab55255	x100	AB_942003
JAGGED1	Santa Cruz	SC-6011	x100	AB_649689
WT1	Abcam	ab89901	x100	AB_2043201
Ki67	Abcam	ab15580	x50	AB_443209
NPHS1	R&D	AF4269	x100	AB_2154851
DBA	Vector laboratories	B-1035	x200	AB_2314288
BRN1(POU3F3)	Novus Biologicals	NBP2-57011	x200	AB_3065278
CD31 (PECAM-1)	Dianova	DIA-310	x20	AB_2631039
CD31 (PECAM-1)(mouse and human)	R&D	AF3628	x100	AB_2161028
CD31 (PECAM-1)(mouse)	Cell Signaling Technology	77699	x100	AB_2722705
CD31 (PECAM-1) (human)	Biologend	303102	x100	AB_314328
Cleaved CASP3	Cell Signaling Technology	9661S	x100 - x200	AB_2341188
HuNu (STEM101)	Takara	Y40400	x100	AB_2895096
α -SMA	Sigma-Aldrich	A2547	x400	AB_476701
c-MET/HGFR	Abcam	ab51067	x50	AB_880695
c-MET/HGFR (APC-conjugated)	R&D	FAB3582A	10 μ L/10 ⁶ cells for FACS	AB_1151927
Control IgG1 (APC-conjugated)	R&D	IC002A	10 μ L/10 ⁶ cells for FACS	AB_357239
DAPI	Thermo Fisher Scientific	D1306	0.1 ng/mL	AB_2629482
GFP	Cell Signaling Technology	2956	x20	AB_1196615
SA- β GAL	Cell Biolabs	CBA-230	Assay kit	
VEGF-A	Abcam	AB39250	x100	AB_778794
VEGFR2	Cell Signaling Technology	2479	x500	AB_2212507
phospho-VEGFR2	Cell Signaling Technology	2478	x500	AB_331377
ELISA	Company	Cat.No.	Dilution	RRID
LBIS Human VEGF ELISA kit	Fujifilm-Wako	631-40831	Assay kit	AB_3095015

IHC; immunohistochemistry, IF; immunofluorescence, FCM; flow cytometry

Table S7: qRT-PCR primer sequences used in this study, related to Figures 2, S2, and S5.

qRT-PCR primers (Human)		
Gene name	Forward primer	Reverse primer
OSR1	GCTGTCCACAAGACGCTACA	CCAGAGTCAGGCTTCTGGTC
SIX2	AGGAAAGGGAGAACAACGAGAA	GGGCTGGATGATGAGTGGT
PAX2	ACAAACGACAGAACCCGACTATG	GGCGTTGGGTGGAAAGG
CITED1	AGGATGCCAACCAAGAGATG	TGGTTCCATTTGAGGCTACC
SALL1	AGCGAAGCCTCAACATTTCCAATCC	AATTCAAAGAACTCGGCACAGCACC
EYA1	CTACTCAGCTCATCCCAGCATT	CATACACCACTTTTCTTCCAAACCT
WT1	AGAAACGCCCTTCATGTGT	AGAAACCTTCGTTACAGTCCTT
HOXA11	ACAGGCTTTTCGACCAGTTTTTC	CCTTCTCGGCGCTCTTGTC
HOXD11	CGGGCTGCGCCTACTATGT	AGGACGACGGTTGGGAAAG
GDNF	CCAGAGGAAAAGGTCGGAGA	CATAGCCCAGACCCAAGTCA
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG

Table S8: qRT-PCR primer sequences used in this study, related to Figure 3.

qRT-PCR primers (Mouse)		
Gene name	Forward primer	Reverse primer
α -SMA	AAGAGCATCCGACACTGCTG	AATAGCCACGCTCAGTCAGG
Col4a1	GCAACGGTACAAAGGGAGAGAG	CTTCATTCTGGTAACCCTGGTG
Col1a1	CCCGCCGATGTCGCTAT	GCTACGCTGTTCTTGCAGTGAT
Fibronectin	GATGCACCGATTGTCAACAG	ACTCTGATCAGCATGGACCA
p16	CGTGAACATGTTGTTGAGGC	GCAGAAGAGCTGCTACGTGA
p21	CGGTGTCAGAGTCTAGGGGA	ATCACCAGGATTGGACATGG
Atf3	CTCTGGCCGTTCTCTGGA	GGTCGCACTGACTTCTGAGG
Gadd45b	CGGCCAACTGATGAATGT	TCTGCAGAGCGATATCATCC
Mmp13	TGATGAAACCTGGACAAGCA	GGTCCTTGGAGTGATCCAGA
TGF- β	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
Btg2	GCGAGCAGAGACTCAAGGTT	TAGCCAGAACCTTTGGATGG
IL6	TGATGCACTTGCGAGAAAACA	ACCAGAGGAAATTTCAATAGGC
PAI-1	CCTCATCCTGGGCCTGGTTCTGGTCT	GGTTTTCCCCGCTGTGGTCATCTGC
GAPDH	TGAAGGTCGGTGTGAACGG	CGTGAGTGGAGTCATACTGGA

Table S9: A primer pair for mutational analysis used in this study, related to Figure

6.

Primers (Human)		
Gene name	Forward primer	Reverse primer
VEGF-A	CAGACTGTCCTCTGGCATCG	CAGGAGCCAGACAAGGCAAA

Data files

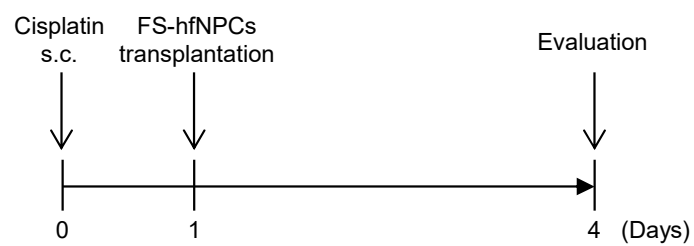
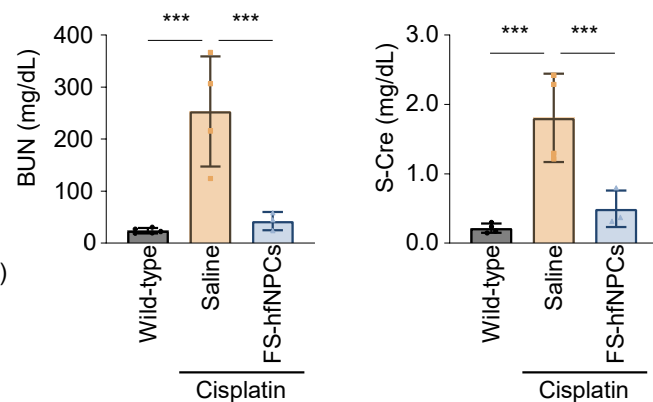
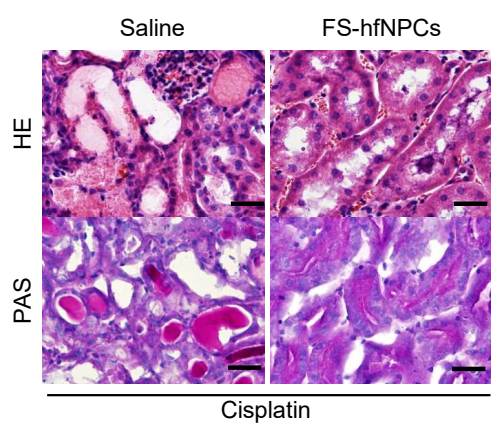
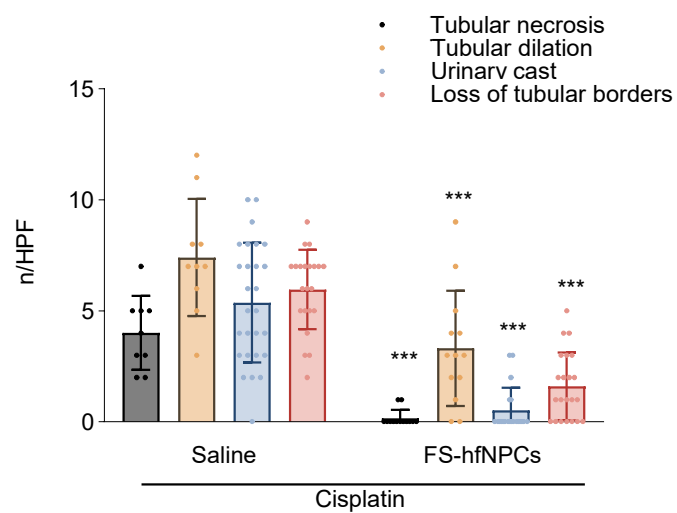
Data file S1. (separate file) Raw data for figures with $n < 20$

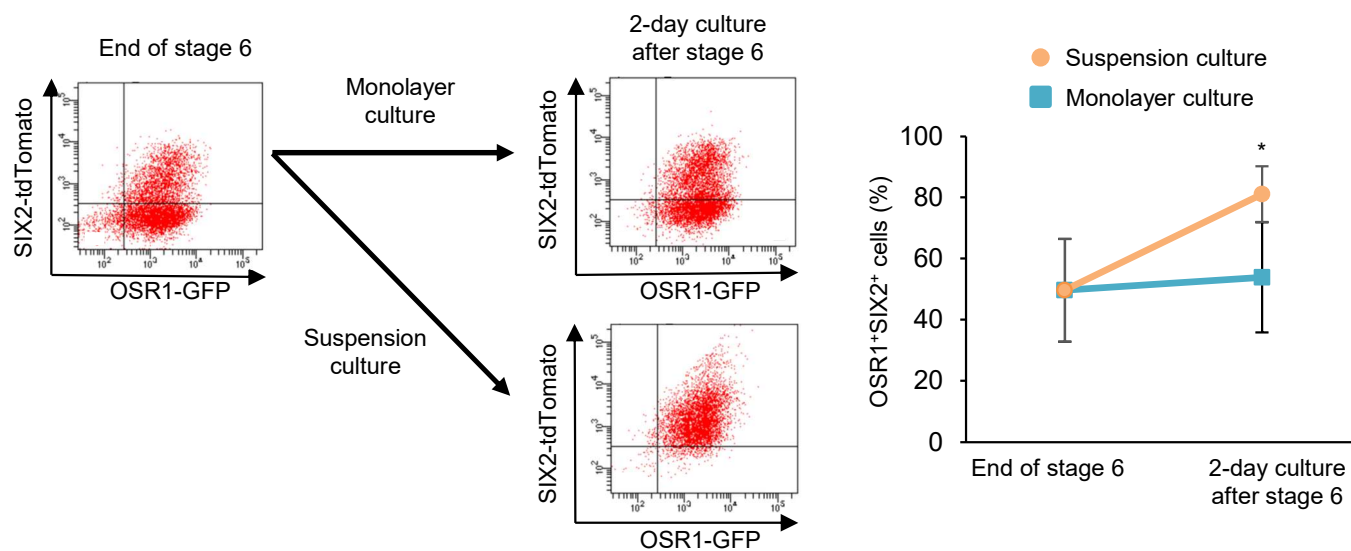
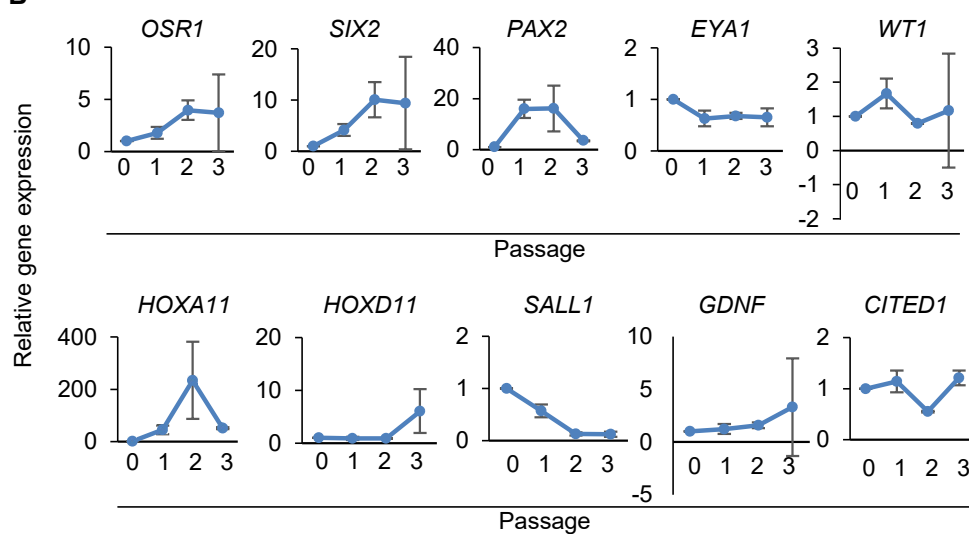
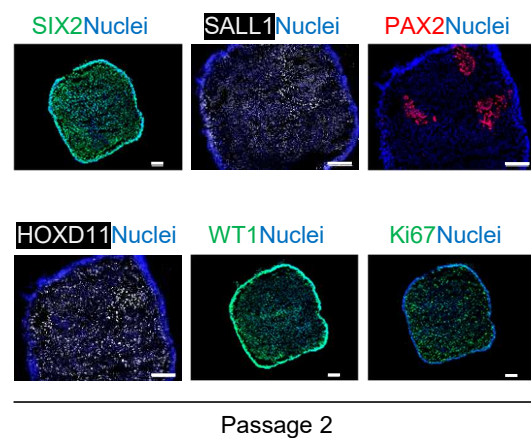
Data file S2. (separate file) Excel file containing the raw data of differentially expressed genes (DEGs) from each RNA-seq analysis, related to Figure 5.

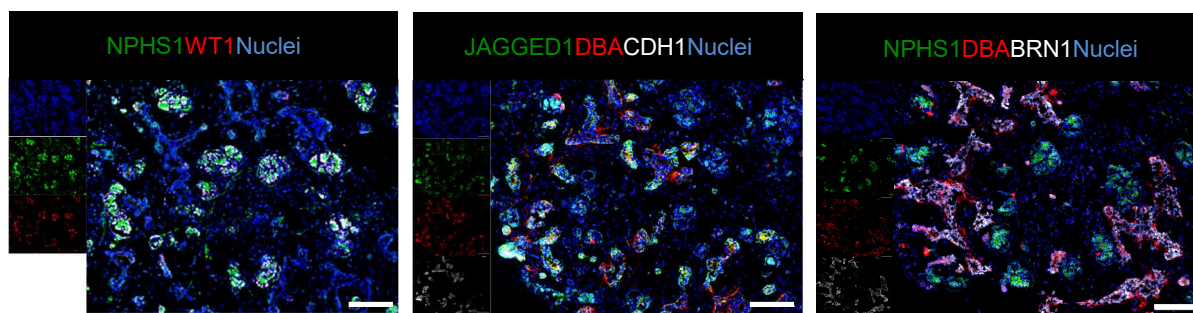
Data file S3 (separate file) Excel file containing the raw data of gene ontology (GO) analysis of DEGs shown in Data file S2, related to Figure 5.

Data file S4. (separate file) Excel file containing the raw data of the list of secreted proteins encoded by DEGs shown in Data file S2, related to Figure 5.

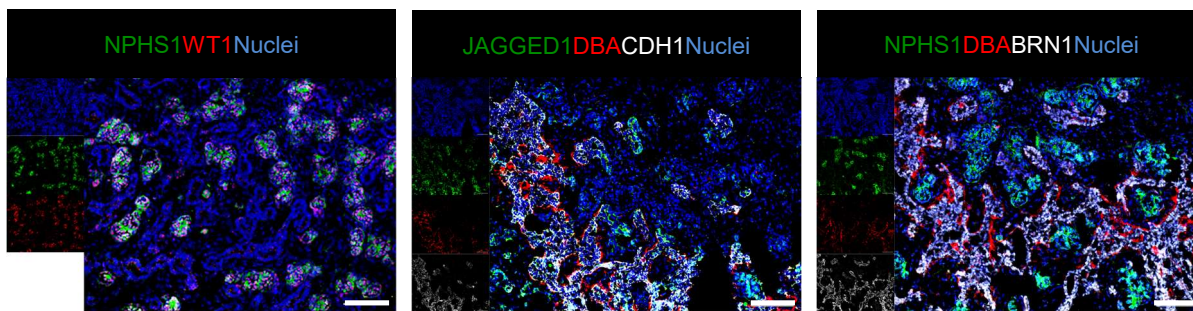
Data file S5. (separate file) Excel file containing the raw data of Network coordinates of unique secreted proteins and VEGF-A in giant-CM, related to figure S16.

A**B****C****D**

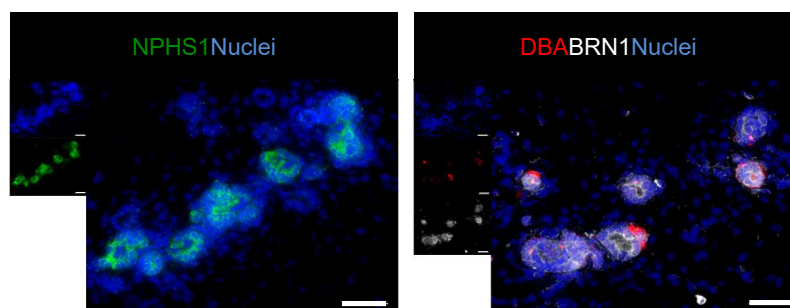
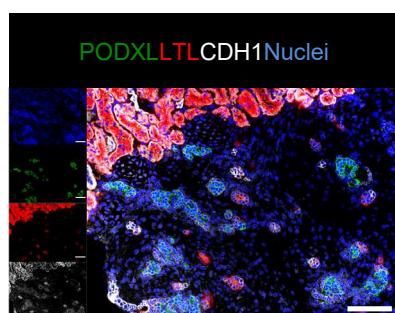
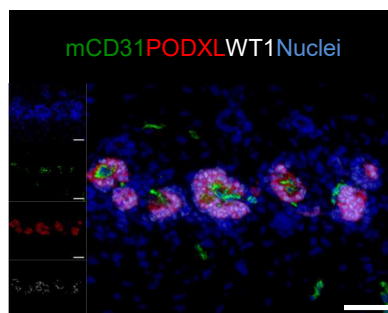
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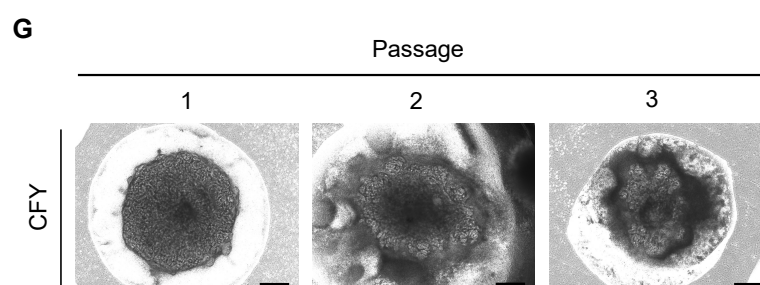
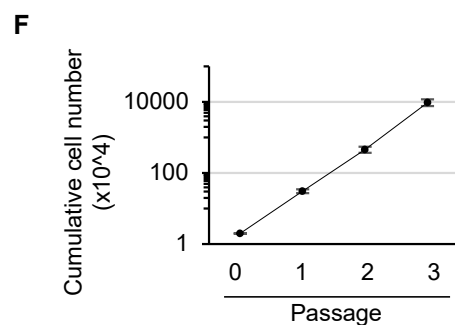
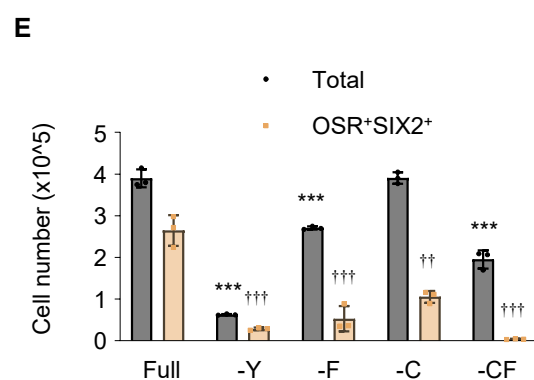
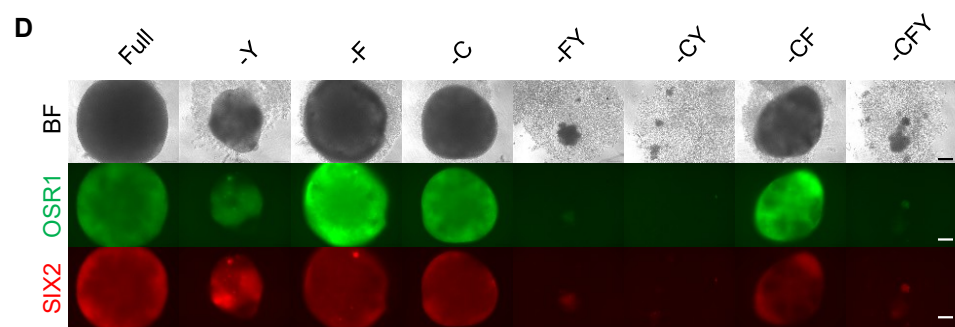
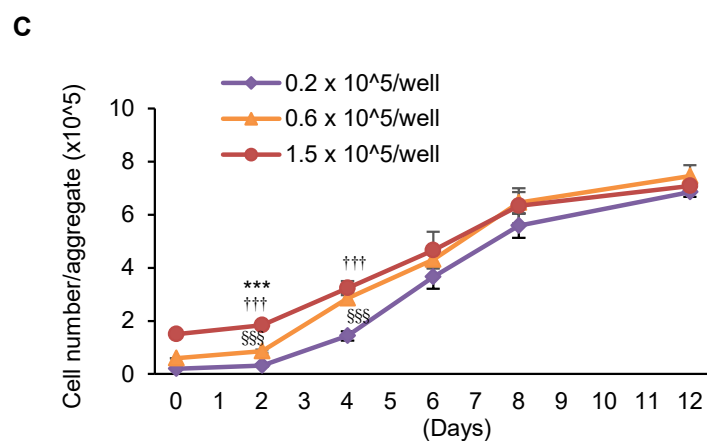
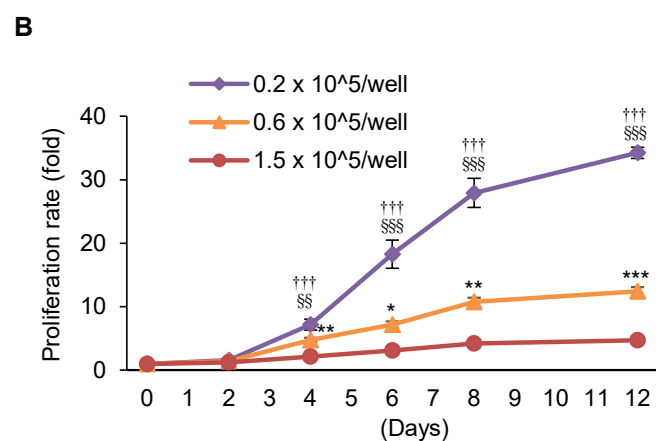
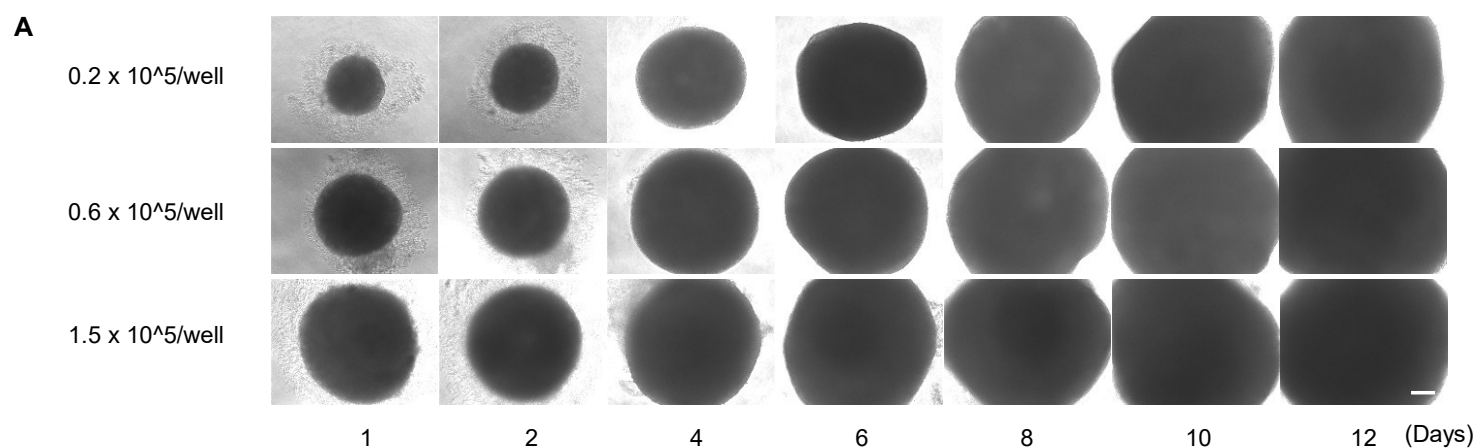
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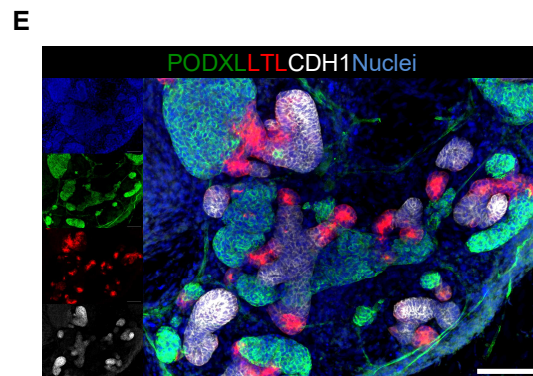
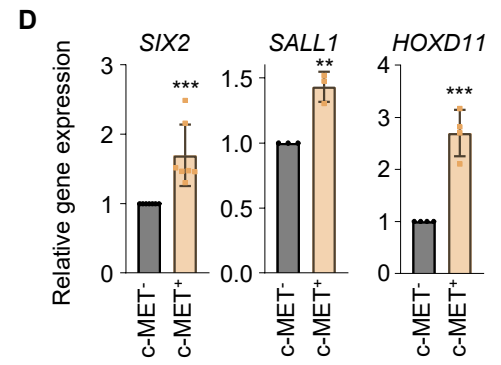
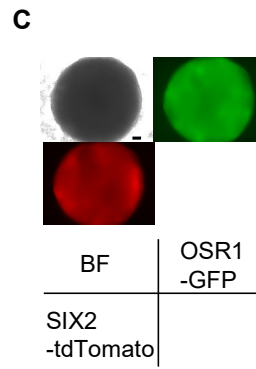
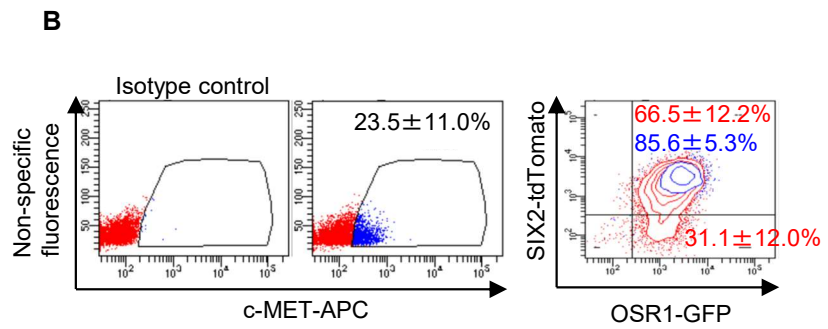
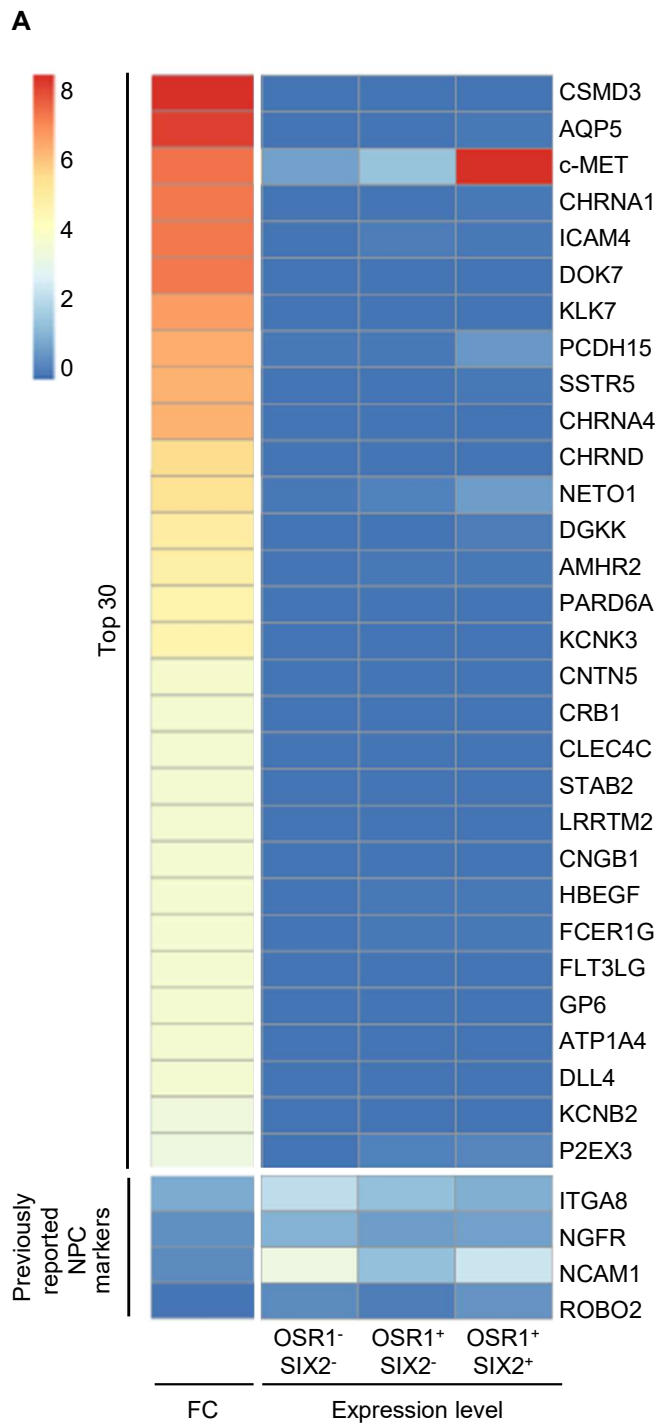
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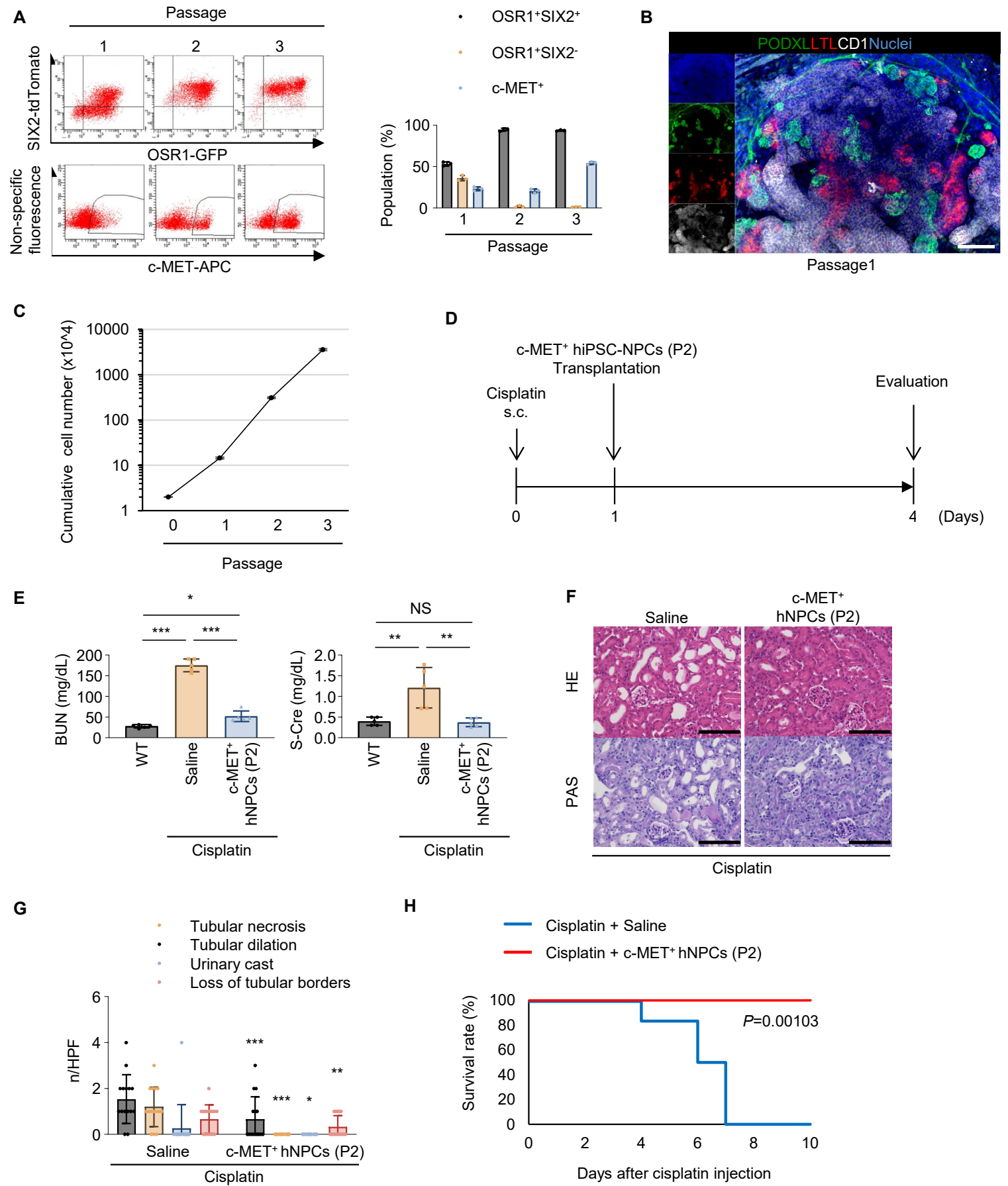
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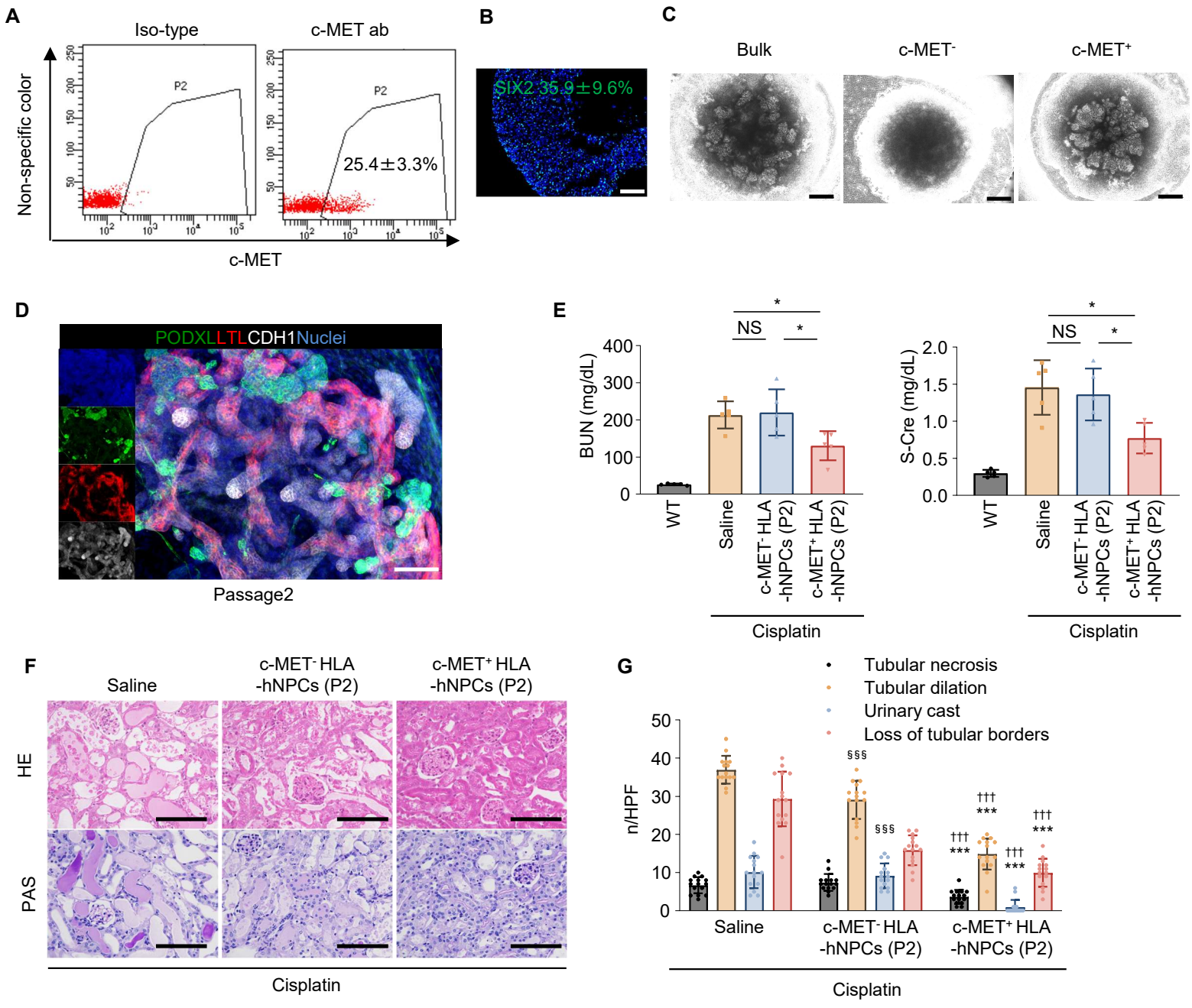
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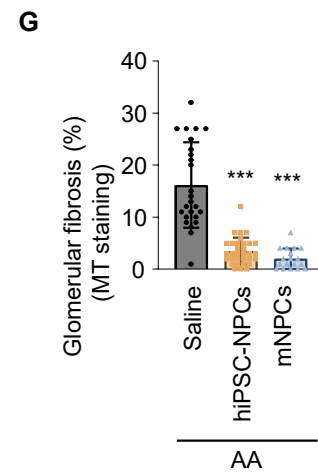
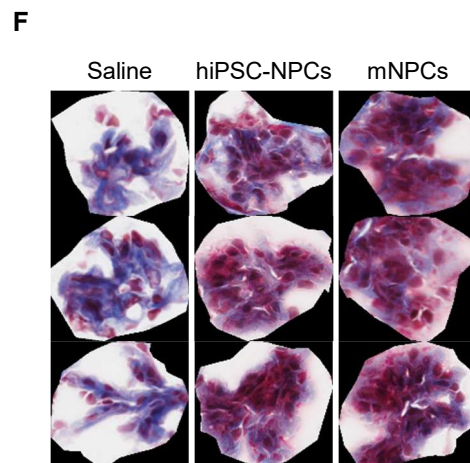
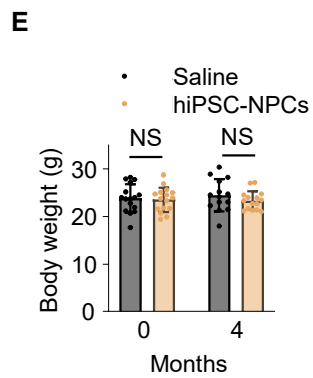
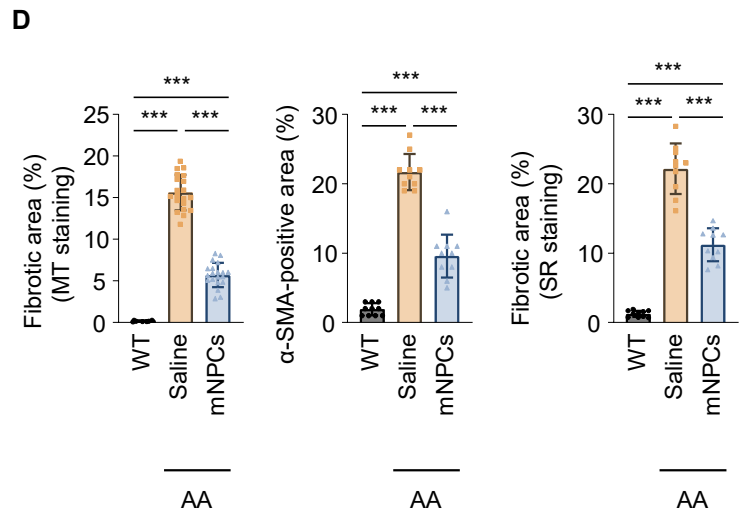
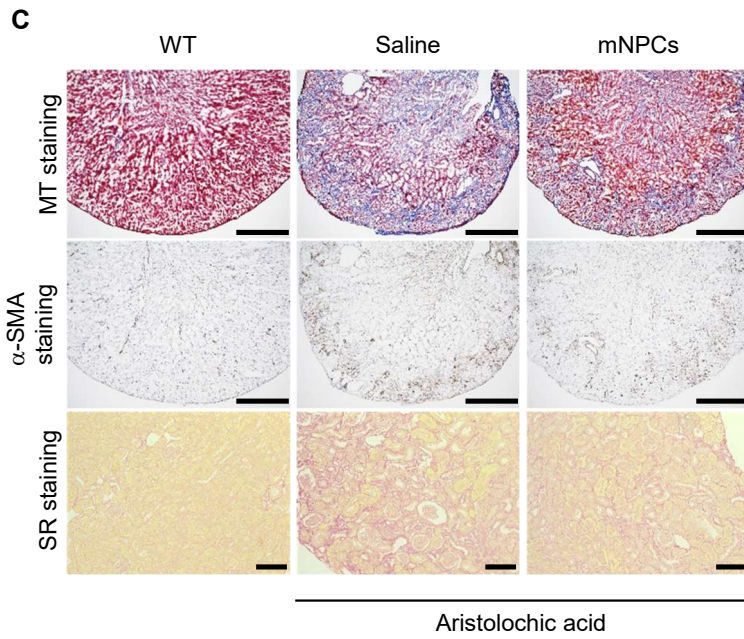
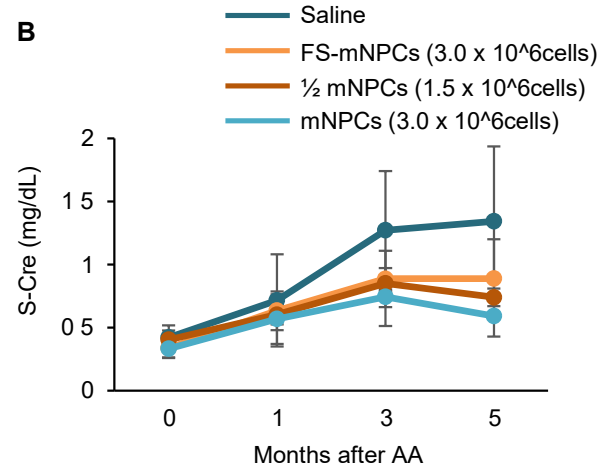
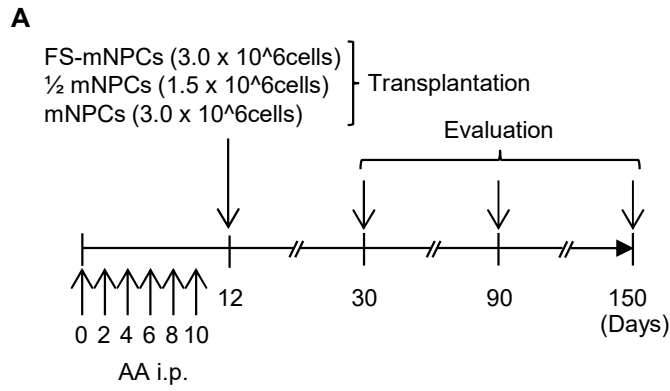
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(+)**D****E****F**

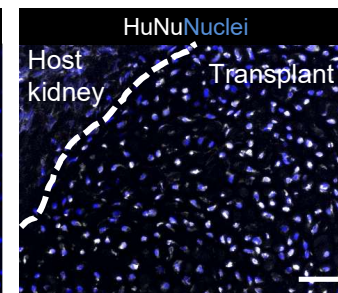
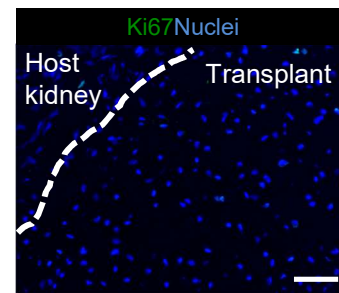
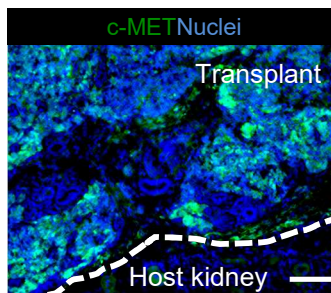
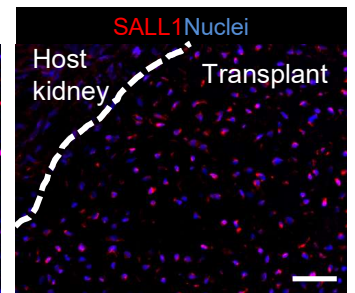
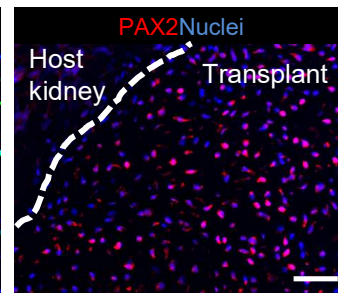
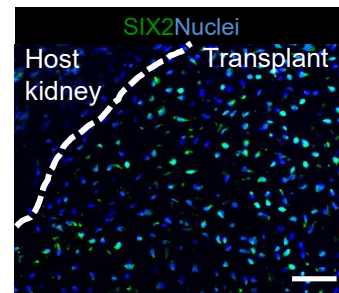
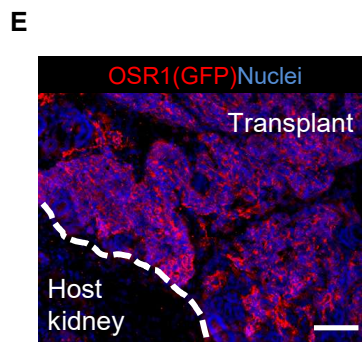
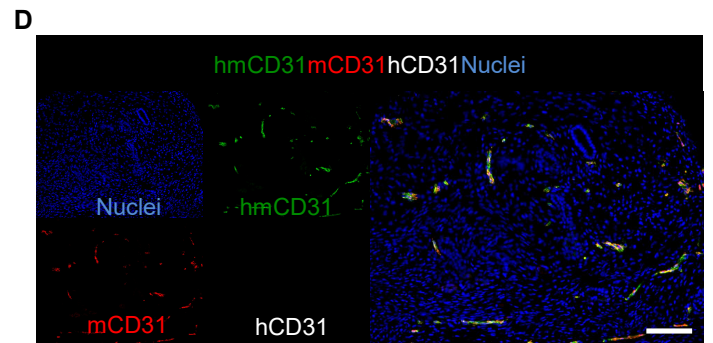
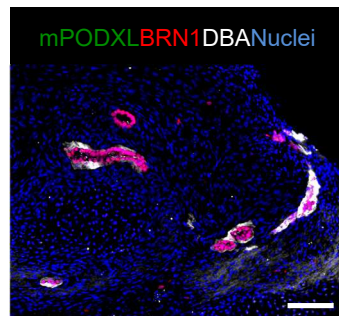
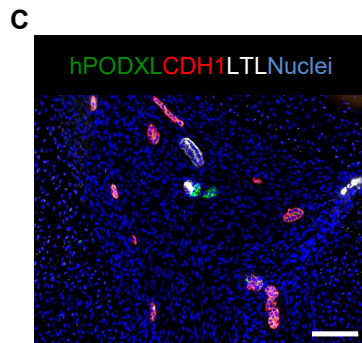
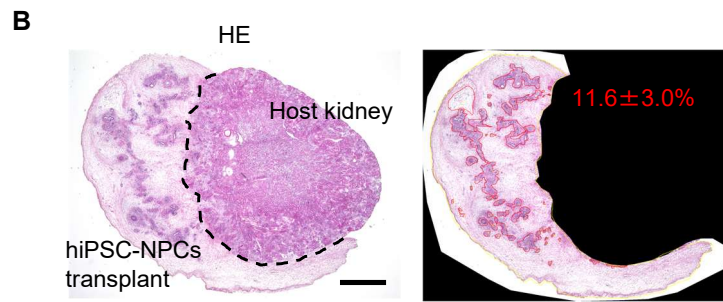
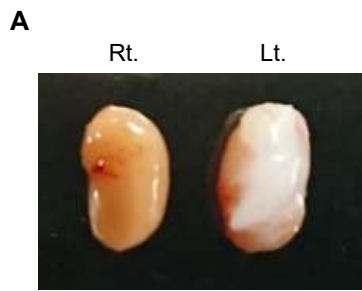


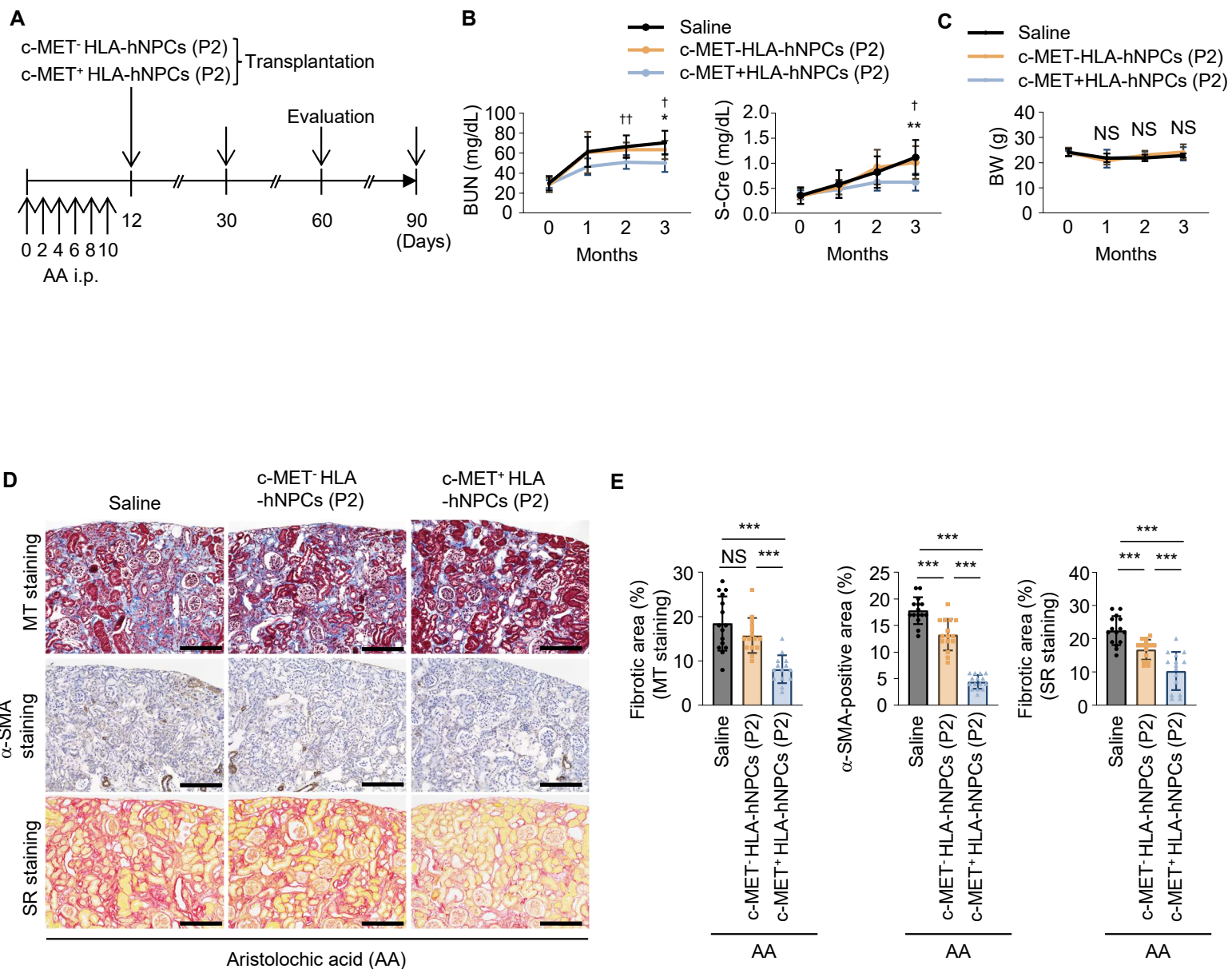






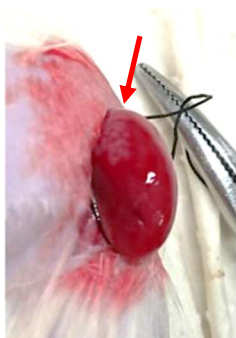




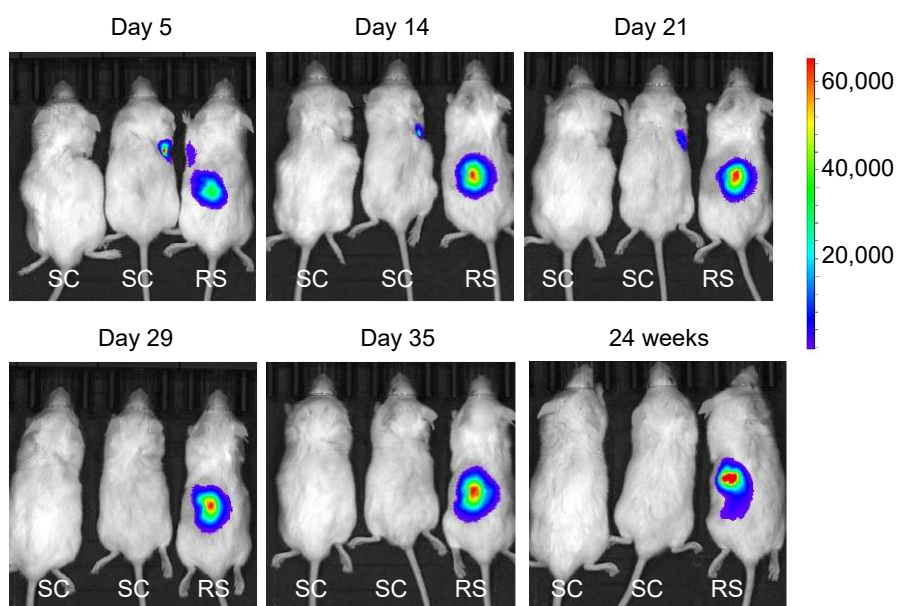


A

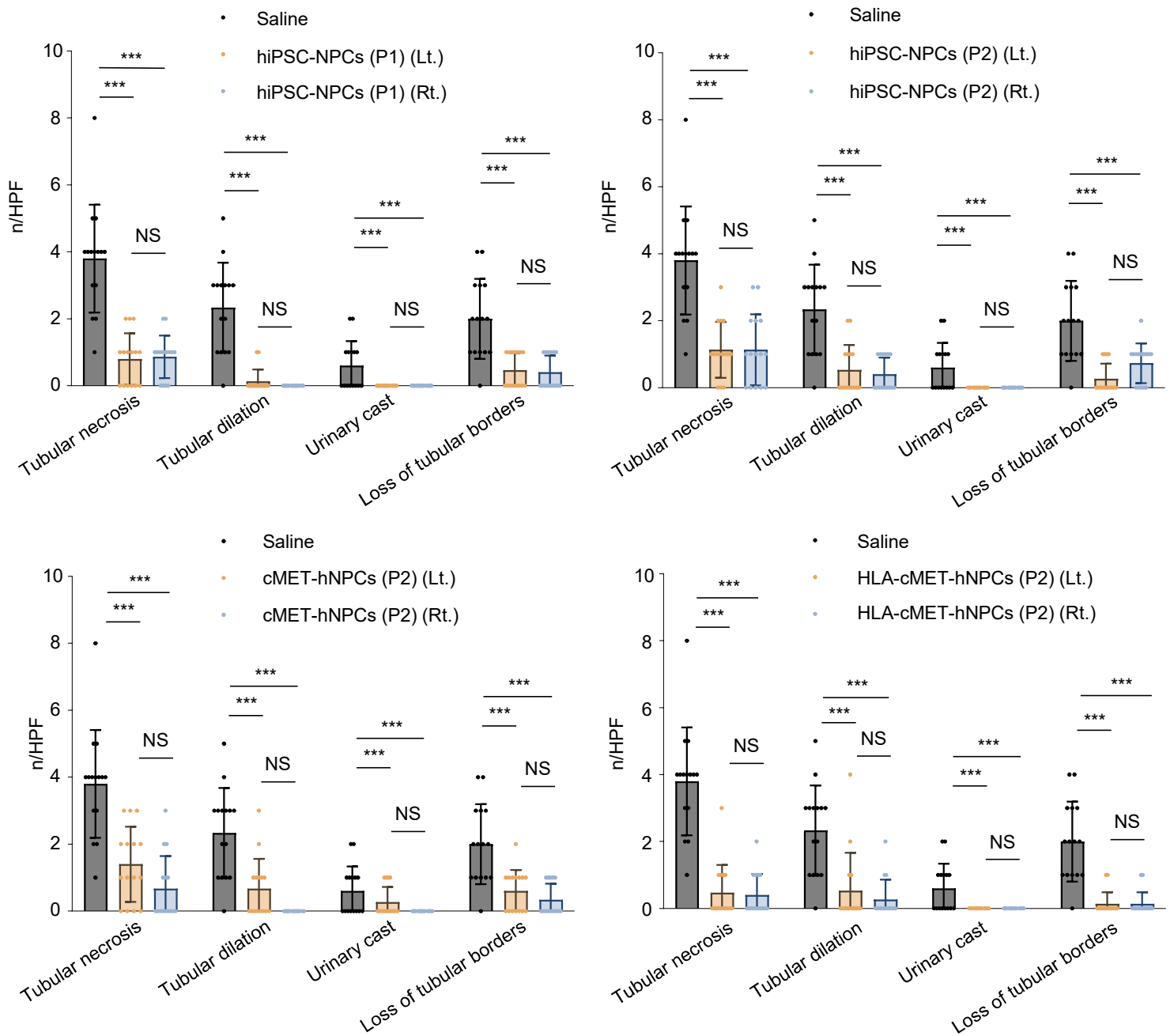
Renal
subcapsular
transplantation



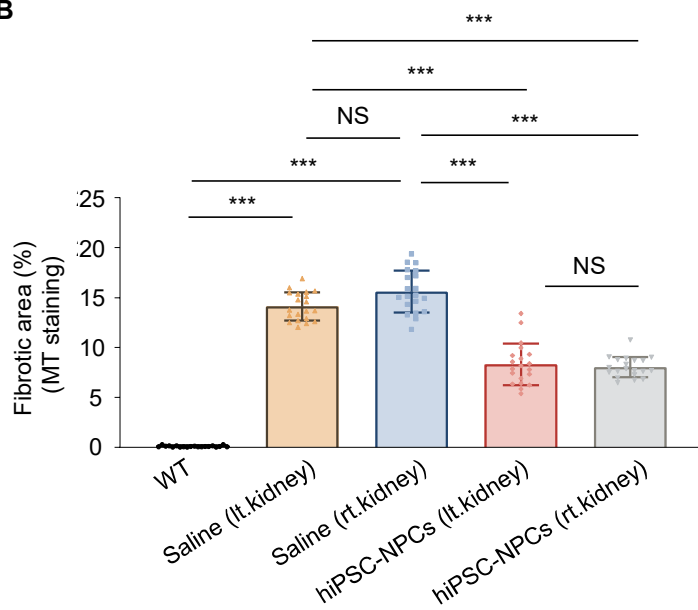
Subcutaneous
transplantation

**B**

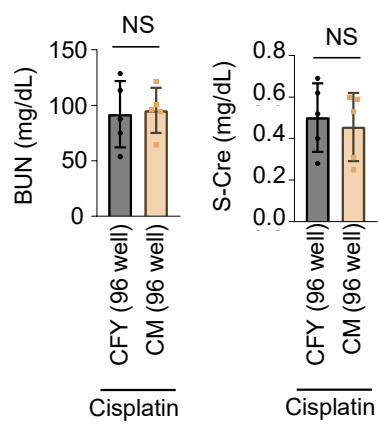
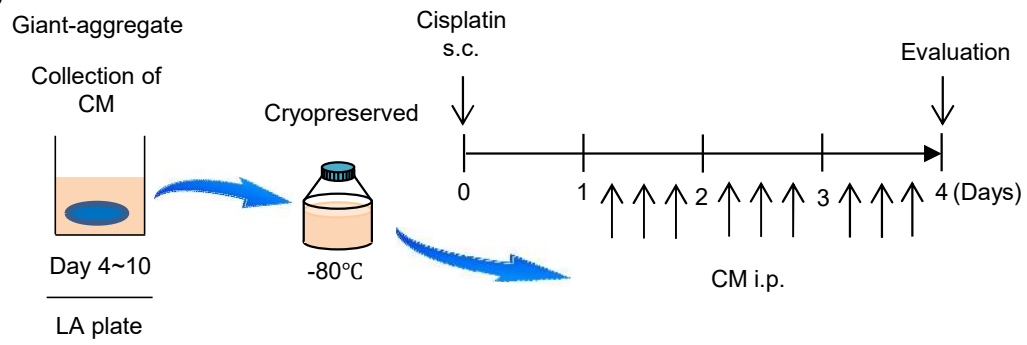
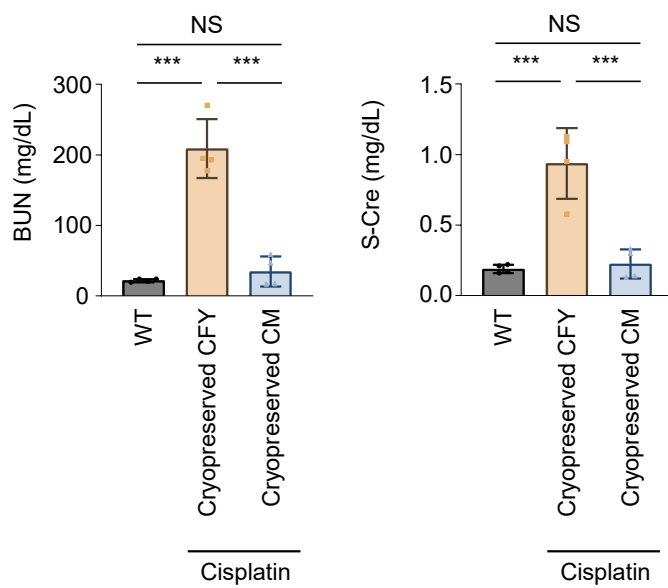
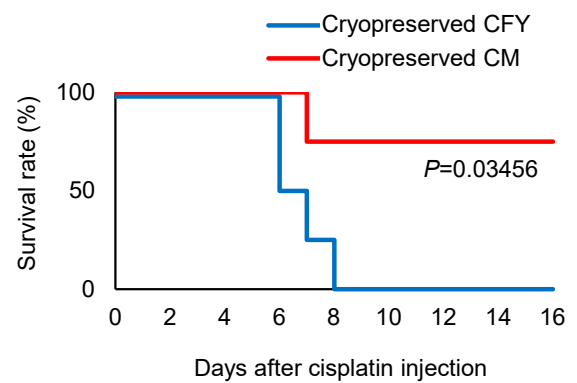
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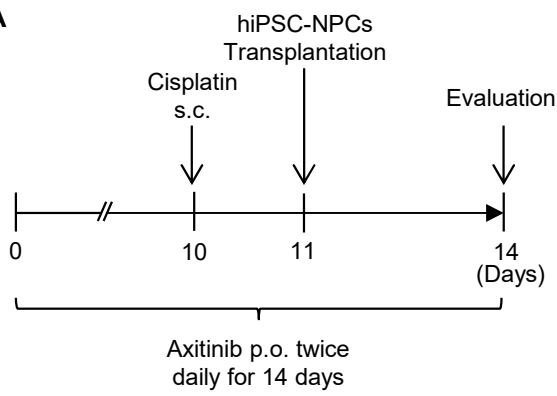
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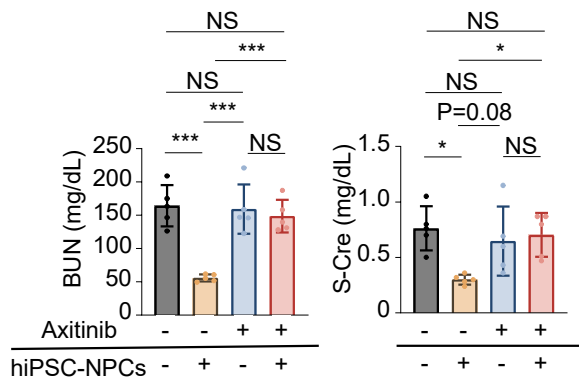
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A**B****C****D**

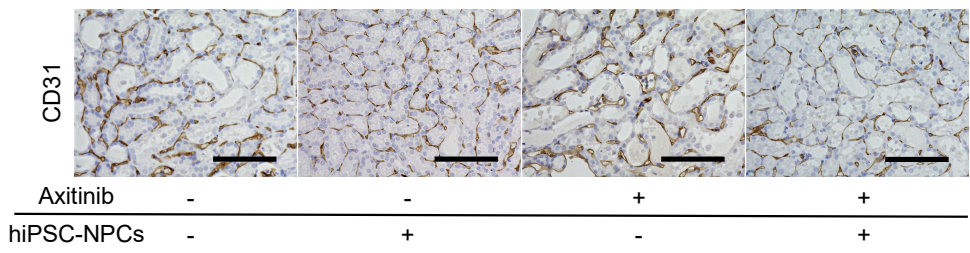
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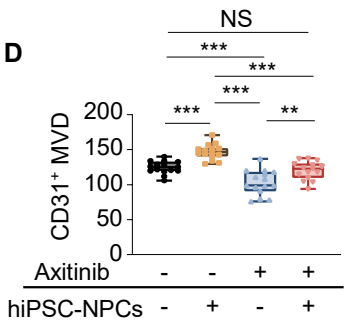
B



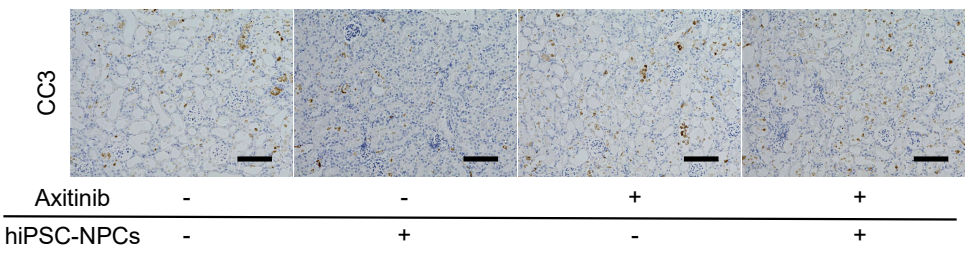
C



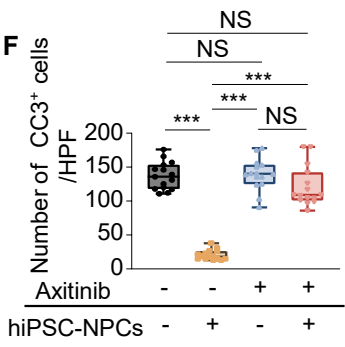
D

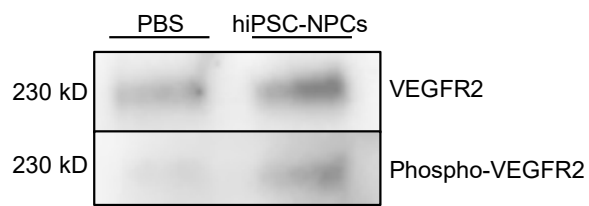
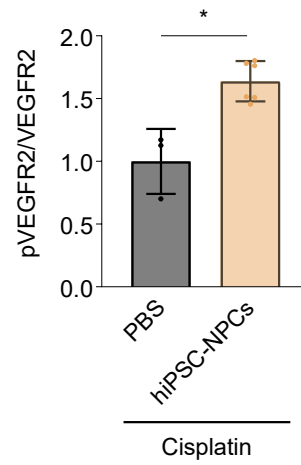
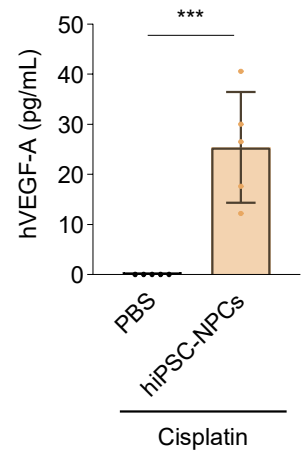
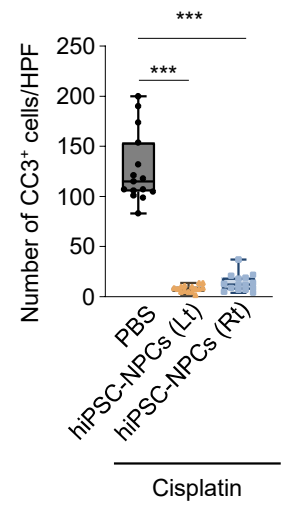
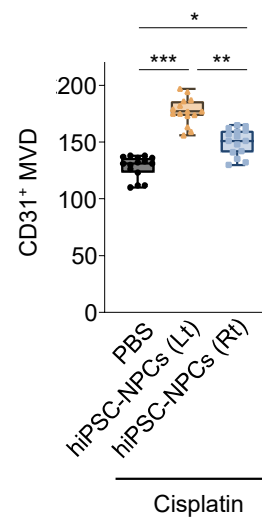
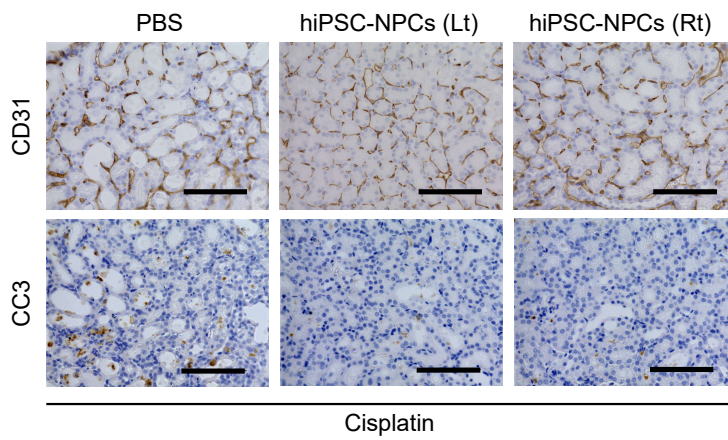


E



F



A**B****C****D****E**