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Does old-to-young kidney transplantation rejuvenate old donor kidneys?

Mayumi Takahashi-Kobayashi[1,3,4], Kunio Kawanishi[2], Joichi Usui[1], Satoshi Yamazaki[3,5], Surya V. Seshan[6] and Kunihiro Yamagata[1]

[1]Department of Nephrology, [2]Department of Experimental Pathology, [3]Laboratory of Stem Cell Therapy, Institute of Medicine, University of Tsukuba, Tsukuba, Ibaraki, [4]Research Fellow of Japan Society for the Promotion of Science, [5]Present address: Division of Cell Regulation, Center of Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan and [6]Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA

Summary. Background. The number of older organ donors is increasing due to the aging population. Aged kidneys often face problems such as delayed graft function but previous murine experiments suggested the possibilities of rejuvenation, for example, in a parabiosis setting between old and young mice. To investigate kidney-graft rejuvenation, we compared an old-to-young (O-Y) patient transplantation group and a transplantation group with donors/recipients of approx. the same age (SA) with the renal senescence marker p16 in kidney biopsy samples at baseline and one year post-transplantation.

Methods. We retrospectively analyzed our hospital's 32 cases of living-donor ABO-compatible transplants performed between 2013-2020. Both the baseline and one-year biopsy (n=9) or only the baseline biopsy (n=32) were analyzed. We divided the nine cases into an O-Y group (donors' median age 68 yrs, recipients 41, difference -27) and an SA group (donors' median age 53 yrs, recipients 51.5, difference -3.5). p16 was stained with the clones JC8 and E6H4 to determine the precise p16-positive rate.

Results. The 32 baseline biopsies' p16-positive rate was weakly related to donor age, suggesting that the p16-positive rate can help evaluate kidney senescence. The (n=5) O-Y group's p16-positive rates were at baseline 0.08 and one year 0.12; the (n=4) SA group's rate was 0.03 at both baseline and one year.

Conclusions. No kidney rejuvenation was observed, even when old donor kidneys went to young recipients.

Key words: Kidney transplantation, Renal senescence, p16, Rejuvenation

Corresponding Author: Dr. Joichi Usui, Department of Nephrology, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. e-mail: j-usui@md.tsukuba.ac.jp
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Introduction

The increase in the aging of populations worldwide has presented many public health problems. The use of organs from older donors (so-called marginal donors) in transplantation has also been increasing, (Tanabe, 2015) which reflects the shortage of donor organs. The recipients of a kidney from an aged donor often experience a poor graft outcome, resulting from events such as delayed graft function (DGF) and acute rejection (Tanabe, 2015). If an aged kidney could be rejuvenated, this would not only help improve the allograft function of the aged organ itself, but it would also help resolve the worldwide insufficiency of donor organs. In the present study, we focused on the age disparity in kidney transplantation, especially on the question of whether old-to-young (O-Y) patient kidney transplantation may have the possibility of rejuvenating aged donor kidneys.

The protein p16 is one of the most well-established markers of senescent cells. It is also known as cyclin-dependent kinase inhibitor 2A, meaning that p16 is an inhibitor of the cell cycle (Omori et al., 2020). The expression of p16 triggers an irreversible cell-cycle arrest, equivalent to cellular aging (LaPak and Burd, 2014). Elimination of p16-positive senescent cells has been reported to bring longer life expectancy and delay the progression of age-related disease (Baker et al., 2011). The expression of p16 is increased not only in senescent cells but also in various conditions such as injury. p16-positive senescent cells play a critical role in

Abbreviations. DGF, delayed graft function; CNI, calcineurin inhibitor; IF/TA, interstitial fibrosis and tubular atrophy; eGFR, estimated glomerular filtration rate; HE, hematoxylin-eosin; IHS, immuno-histological score; IQR, interquartile range; LAST, The Lower Anogenital Squamous Terminology; mAb, monoclonal antibody; SA, same age; SASP, senescence-associated secretory phenotype; SD, standard deviation; O-Y, old-to-young; WHO, the World Health Organization.

tissue repair and the removal of these cells resulted in delayed wound closure (Demaria et al., 2014). In the kidney, age-dependent p16 upregulation was normally observed in tubules and interstitial cells. Rejected grafts also showed higher p16 expression in the glomeruli, tubules, and interstitial cells (Safwan-Zaiter et al., 2022). Also, p16 expression in glomeruli and interstitial cells was significantly prominent in glomerular disease, especially presenting proteinuria, fibrosis, or interstitial inflammation, when compared with normal-aged kidneys (Sis et al., 2007; Safwan-Zaiter et al., 2022). The mechanism of the strengthening of p16 in rejection and glomerular diseases, whether it reflects senescence or reduced potential of cell repair, remains unclear. In this context, we excluded those kidney transplant cases with episodes of rejection, calcineurin inhibitor (CNI) nephropathy, infection, recurrence of the primary disease, or high interstitial fibrosis and tubular atrophy (IF/TA) rate from this study to evaluate "true aging" in the transplanted kidney and to remove p16-related factors other than aging.

Materials and methods

Study design

Sixty-eight cases of ABO-compatible living-donor kidney transplantations (68 donors, 68 recipients) were conducted from 2013 to 2020 at the University of Tsukuba Hospital (Fig. 1). Among them, 34 transplants were excluded because of ABO-incompatibility or minor mismatch transplantation; two transplants lacked specimens. A final total of 32 transplants were thus analyzed in this study. We collected the donor and recipient patients' age, gender, estimated glomerular filtration rate (eGFR), and clinical history post-transplantation as baseline data. The donor eligibility was determined according to the guidelines and the marginal donor criteria of the Japanese Society for Transplantation and the Japanese Renal Transplant Society (in Japanese: http://www.asas.or.jp/jst/pdf/manual/008.pdf). The guideline included a donor of (1) 20 ≤age ≤70 years, (2) without active infection, anti-HIV

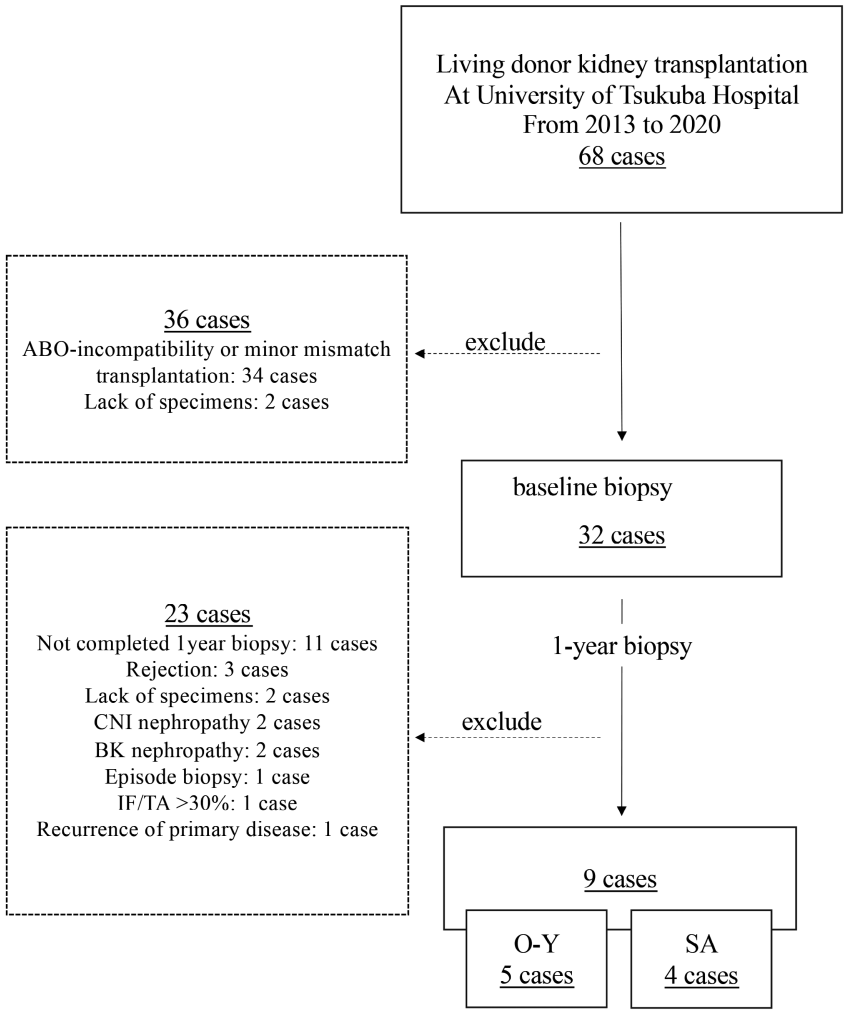

Fig. 1. Inclusion and exclusion criteria of this study.

antibody positive, and Creutzfeldt-Jakob disease, (3) blood pressure (BP) <140/90 mmHg, (4) Body mass index (BMI) ≤30 kg/m^2, (5) creatinine clearance ≥80 ml/min/ 1.73 m^2, (6) urinary protein <150 mg/day or <150 mg/gCr or albuminuria 30 mg/gCr, (7) fasting blood glucose ≤126 and hemoglobin A1c (HbA1c) ≤6.2, and (7) without structural disease including malignancy and urinary infection. The marginal donor included (1) age ≤80 years, (2) blood pressure (BP) ≤130/80 mmHg with antihypertensive medications, (3) BMI ≤32 kg/m^2, (4) creatinine clearance ≥70 ml/min/ 1.73 m^2, and (5) hemoglobin A1c (HbA1c) ≤6.5 without oral medication for diabetes or ≤6.5 with oral medication for diabetes.

A baseline kidney biopsy was conducted during the transplant surgery one hr after revascularization (n=32), and another kidney biopsy was performed one year post-transplantation (n=9). All these biopsy specimens were evaluated pathologically, including the immuno-histological score (IHS) of p16. The pathological features are described as the global/segmental sclerosis rate, the IF/TA rate, and the degree of atherosclerosis. The global/segmental sclerosis rate was calculated by the number of global/segmental sclerosis glomeruli divided by the total glomeruli number. The IF/TA rate is described as a percentage in steps of 5%. The degree of arteriolosclerosis was scored as normal (0 points), mild (1 point), moderate (2 points), and severe (3 points). The IHS for p16 was determined in each biopsy, following the method described below.

Of the total of 32 cases, we excluded 23 cases from the one-year biopsy analysis for reasons including not completed one-year biopsy (11 cases), rejection (three cases), lack of specimens (two cases), CNI nephropathy (two cases), BK nephropathy (two cases), episode biopsy (one case), IF/TA >30% (one case), and recurrence of primary disease (one case). We divided the remaining nine cases into two groups according to the age difference between the donor and recipient (subtracting the age of the recipient from the age of the donor): the O-Y group (age difference: -31 to -21 years) and the same-age (SA) group (age difference: -15 to +8 years).

This research protocol was approved by the Ethics Committee of the University of Tsukuba Hospital (H30-200). Each patient's informed consent to participate in the study was required by the Institutional Review Board. An announcement of the study was simultaneously posted at the outpatient clinic of our institute. The study complied with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research involving Human Subjects in Japan.

p16 immunohistochemistry

We selected the immunohistological score based on the positivity of p16 as the representative marker of renal senescence in the present retrospective analyses. The selection of human anti-p16 antibodies was carried out with reference to the positive distribution of mouse p16 tracer experiments (Omori et al., 2020). We selected the clone E6H4 (Roche, Mannheim, Germany), which is approved by the U.S. FDA and used for cervical cancer diagnoses, as a human anti-p16 antibody instead of the clone JC8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which has been used in many studies of renal senescence. In the field of gynecology, p16 is known to be overexpressed in response to the HPV viral genome, and p16 positivity is seen in squamous cell carcinoma, precancerous-state cervical atypia, and cervical adenocarcinoma. The Lower Anogenital Squamous Terminology (LAST) standardization and the World Health Organization (WHO) recommend the adjunctive use of p16 to diagnose cervical cancer. (Kurman, 2014; Stoler et al., 2018) The p16 antibody clone E6H4 is the only validated tool to diagnose cervical cancer and it has been observed to be highly accurate.

Regarding p16 staining with clone E6H4 in the kidneys, no specific staining was observed, according to the package insert (https://www.unilabs.sk/media/2022/02/1/5/p16C-E6H4.pdf). The above-summarized information led us to use clone E6H4 instead of JC8 to more accurately evaluate senescence in the kidney.

An immunohistological analysis of p16 using the clone JC8 (Santa Cruz Biotechnology) was performed as follows. Tissue sections were deparaffinized and hydrated. Autoclaving of deparaffinized sections was used for antigen retrieval, and the sections were then immersed in 3% hydrogen peroxide in methanol and blocked with 10% normal goat serum. The sections were incubated with a primary antibody overnight at 4°C and with a secondary antibody for 1h at room temperature. The primary antibody was a monoclonal (mAb) anti-p16 antibody (lot #K1813, mouse IgG: 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was a peroxidase-conjugated anti-rabbit IgG polyclonal (pAb) antibody (#H2004A, Nichirei Bioscience, Tokyo). DAB detection kits (Dako, Glostrup, Denmark) were used. The sections were subjected to nuclear staining using hematoxylin-eosin (HE).

For the clone E6H4 (CINtec® p16 Histology, 109912, Roche, Mannheim, Germany), the protocol provided by the manufacturer was performed using ULTRA Cell Conditioning Solution (ULTRA CC1) (950-224, Roche) for 64 min and an ultraView Universal DAB detection kit (109431, Roche).

Scoring method

P16-positive and -negative nuclei were counted by QuPath software (ver. 0.4.3) (https://qupath.github.io/) (Bankhead et al., 2017). The p16-positive rate was obtained by dividing the number of all nuclei by the number of p16-positive nuclei.

Statistical analysis

The quantitative data are presented as the mean

value ± standard deviation (SD) or the median and interquartile range (IQR) in each figure. The quantitative variables were compared between two groups with the Student's t-test and the Mann-Whitney U-test, and categorical variables were compared with Fisher's exact test. Statistical significance was established at $p<0.05$. Bell Curve for Excel ver. 3.00 software (Social Survey Research Information Co., Tokyo) was used for all the statistical analyses.

Results

Patient characteristics

The median age of the 32 donors was 62.5 (52.5-68) years. The O-Y kidney transplantation group included five cases and the SA group four. The O-Y group had a median age of 68 (67-72) years for the donor and 41 (39-44) for the recipient, whereas the SA group had 53 (49.75-57.25) years for the donors and 51.5 (45.75-56.25) for the recipients (Table 1). The age difference (subtracting the recipient age from the donor age) was -27 years in the O-Y group and -3.5 in the SA group. All donors met the guideline and marginal donor criteria of the Japanese Society for Transplantation. All recipients received basiliximab, methylprednisolone, mycophenolate mofetil, and tacrolimus according to the ABO-compatible protocol in our hospital. There were no DGF cases.

p16 immunohistological scores

The expression of p16 was observed mainly in the loop of Henle or glomerular parietal epithelial cells (PECs) but also partially in distal tubules or podocytes when stained with clone E6H4, although an earlier report indicated that tubules would be the most appropriate structures for our evaluation of renal senescence (Melk et al., 2004). In addition, the positive rate and localization were far more limited when the p16 antibody of clone E6H4 was used compared to clone JC8. The p16-positive rate in the 32 baseline biopsies ranged from 0 to 0.2. Figure 2 provides representative examples of p16 staining. As shown in Figure 3, the donor age and the baseline p16-positive rate were weakly correlated.

The p16-positive rate of the baseline biopsies was 0.08 (0.04-0.12) in the O-Y group and 0.03 (0.01-0.07) in the SA group, and at one-year post-transplantation, the corresponding values were 0.12 (0.09-0.24) and 0.03 (0.02-0.04). The p16-positive rate of the one-year biopsies of the O-Y group was thus not significantly different than that of the SA group (p=0.19).

The p16-positive rate, eGFR, and pathological diagnoses

Patients' renal function was evaluated based on their eGFR values. The donor pre-transplant eGFR was 63.4 (61.1-95.5) in the O-Y group and 87.6 (82.3-99.9) in the

Table 1. Clinical characteristics and the p16-positive rate of the old-to-young and same-age groups of kidney transplant patients.

			Old-to-young group (n=5)	Same-age group (n=4)	*p*-value
Age	Donor	(Median and IQR 25%-75% years)	68 (67-72)	53 (49.75-57.25)	
	Recipient	(Median and IQR 25%-75% years)	41 (39-44)	51.5 (45.75-56.25)	
	Difference	(Median and IQR 25%-75% years)	-27 {(-28)-(-24)}	-3.5 {(-11.25)-(4.25)}	
Gender	Donor	(Male / Female)	1/4	1/3	
	Recipient	(Male / Female)	5/0	3/1	
P16-positive rate	Baseline	(%)	0.08	0.03	0.73
	1 year	(%)	0.12	0.03	0.19
eGFR	Donor (pre-transplantation)	(Median and IQR 25%-75% mL/min/1.73 m^2)	63.4	87.6	0.41
	Recipient (one year post-transplantation)	(Median and IQR 25%-75% mL/min/1.73 m^2)	48.8	55.0	0.06

The p16-positive rate of the old-to-young group was not decreased at one year post-transplantation. eGFR, estimated glomerular filtration rate; IQR, interquartile range.

Table 2. Pathological characteristics of the old-to-young and same-age groups.

		Baseline			1 year		
		Old-to-young group (n=5)	Same-age group (n=4)	*p*-value	Old-to-young group (n=5)	Same-age group (n=4)	*p*-value
Global/segmental sclerosis	(Median and IQR 25%-75%; %)	7.1 (3.8-14.3)	6.2 (4.4-10.3)	0.98	14.3 (3.8-14.3)	22.6 (16.7-26.0)	0.45
IF/TA	(Median and IQR 25%-75%; %)	5 (5-10)	5 (3.8-6.3)	0.76	10 (5-20)	5 (3.8-8.8)	0.37
Arteriolo-sclerosis	(Median and IQR 25%-75%; points)	1 (0-1)	1 (0.75-1.25)	0.98	1 (1-1)	1 (0.75-1)	0.76

The baseline and one-year pathological features were not significantly different between the old-to-young and same-age groups. IF/TA: interstitial fibrosis and tubular atrophy.

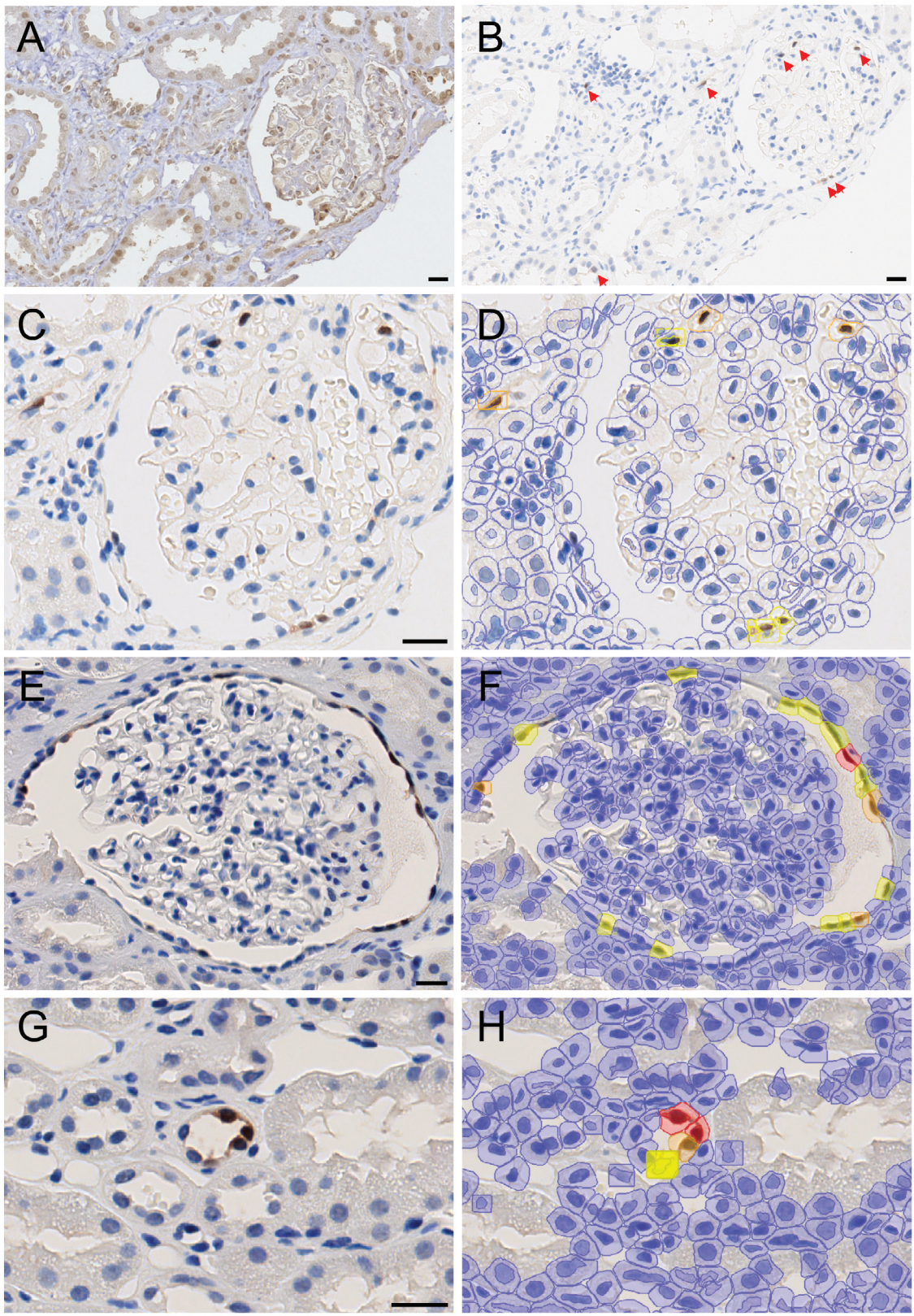

Fig. 2. Representative images of p16-positive cells. Scale bars represent 20 µm. **A.** Immunohistochemistry of p16 with the anti-p16 antibody of clones JC8, and E6H4 **(B-H)**. All nuclei are marked in blue, and positive cells in yellow, orange, and red according to the positive intensity **(D, F, H)**. **C, D.** Glomeruli. **E, F.** Parietal epithelial cells. **G, H.** Tubules. A, B, x 100; C-F, x 200; G, H, x 400.

SA group, and for recipients at one year post-transplantation, the eGFR was 47.1 (42.7-55.8) in the O-Y group and 49.4 (44.5-54.2) in the SA group.

The pathological findings were evaluated based on the global/segmental sclerosis rate, the IF/TA rate, and the degree of arteriolosclerosis (Table 2). The baseline and one-year pathological features were not significantly different between the O-Y and SA groups. The results for the global/segmental sclerosis rate, IF/TA rate, and degree of arteriolosclerosis each showed a tendency to have worsened in the one-year biopsies compared with the baseline biopsies, however, none of the changes were significant.

Discussion

Renal senescence results in poor graft function in renal transplants. Due to the worldwide increase in aged populations and the lack of donor organs, it is desirable to prevent the aging of organs and/or to rejuvenate organs. Investigation of the aging process and rejuvenation trials have been conducted for many years, for example, in parabiosis murine models in multiple organs (Rebo et al., 2016; Palovics et al., 2022) and in young bone marrow transplantation for aged kidney (Yang et al., 2011), nevertheless, the details of the underlying mechanisms remain to be elucidated.

It has been demonstrated that cellular senescence is induced by multiple factors such as oxidative stress, oncogenic stimuli, irradiation, mitochondrial dysfunction, and chemical toxicants (Zhou et al., 2020). Multiple factors including TP^{53} and $P21^{cip1}$ were reported to influence these processes. Many factors in the human kidney have been investigated, including p16INK4a, p14/p19ARF, p21CIP1/WAF1, heat shock proteins, TGF-β, PAI-1, metallothioneins, and GADD45. Among them, p16, also known as the *CDKN2A* gene product, is the marker that is most strikingly correlated with kidney senescence (Melk, 2003); it is stimulated by DNA damage, resulting in an inhibition of CDK complexes and retinoblastoma protein phosphorylation, inducing senescent progression (Sturmlechner et al., 2017).

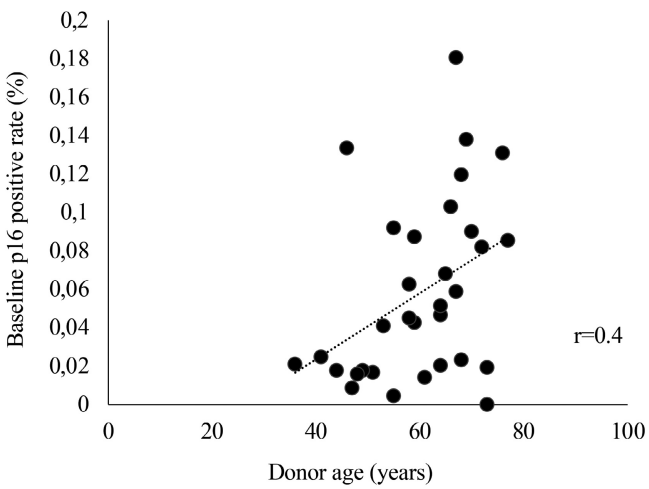

Fig. 3. The donor age was weakly related to the baseline p16-positive rate (r=0.4).

The expression of p16 Ink4a in the healthy human kidney was increased with age in a variety of renal cell types but most prominently in tubules (Melk et al., 2004; Sofue et al., 2018). The p16-positive rate differed from 0% to > 80% in the investigations reported by Melk et al. (2004) and Sofue et al. (2018), respectively. In 2004, Melk et al. reported that the p16-positive rate was 7.8% in young kidneys and 30.8% in adult kidneys. In the mouse kidney too, Melk et al. reported p16-positive rates of almost 20%-70% (Melk et al., 2009). In contrast, Omori et al. reported that the percentage of $p16^{high}$ Tom+ cells in the kidney was almost 0.5% in two-month-old mice and 1.5% in 12-month-old mice (Omori et al., 2020). The positive rate was different in previous p16 staining reports and actual reporter mice. In addition, manual staining depends on the temperature, technique, and operator's experience level.

To solve these problems, we used the Roche clone E6H4 to improve the accuracy of the positive rate evaluation in the present study. E6H4 is a universally applied diagnostic tool for cervical cancer. Comparative studies of antibody clones have also been performed not only in cervical cancer but also in mesopharyngeal cancer. Shelton et al. compared the p16 antibody clones E6H4, JC8, and G175-405, finding that the positive predictive values for high-risk HPV status by RNA *in situ* hybridization ranged from 98% to 100% in these three clones, however, negative predictive values were variable: clone E6H4, 86%; JC8, 69%; and G175-405, 56% (Shelton et al., 2017). With Kaplan-Meier survival plots in that study, the E6H4 clone had the largest differential in disease-specific and overall survival between p16-positive and -negative results. On the whole, the E6H4 clone was the best in all aspects including the strongest staining intensity, the greatest differential in outcomes between positive and negative results, lowest interobserver variability, and lowest background nonspecific staining (Shelton et al., 2017).

Based on this highly accurate staining, our present findings demonstrated a weak correlation between the donor's age and the p16-positive rate, as in these earlier studies. The weak correlation between donor age and the p16-positive rate revealed by our analysis suggests that p16 could be a marker for estimating kidney aging, at least in healthy populations. The correlation also indicates the possibility that the p16-positive rate declined when a kidney was transplanted into a younger donor. To the best of our knowledge, there are no published reports of investigations of age disparity in transplantations, indicating the novelty and importance of our present results.

Kidney aging might be caused by a loss of the capacity for the repair and replication of cells and tissues (Melk et al., 2009). Each type of tissue can repair and replicate itself for survival, however, aging reduces this capacity as a result of oxidative and cellular stress. In

addition, it has been demonstrated that senescent cells not only cause cell-cycle arrest but also secrete many inflammatory proteins. This phenomenon is called the senescence-associated secretory phenotype (SASP). We speculated that an old graft can regain its capacity to repair and replicate when it is transplanted into a young recipient, since the existence of some circulating factors may contribute to its rejuvenation. However, our present results revealed that the rejuvenation of the kidney did not occur when old donor kidneys were transplanted into young recipients. Instead, senescent cells tended to increase when old donor kidneys were transplanted into younger recipients. Also, the p16 positive rate of the SA group at one year was the same as at baseline, suggesting that SA transplantation did not bring drastic environmental differences to the transplanted kidneys. These findings also suggest that the one-year follow-up period may have been too short to evaluate the rejuvenation, and/or multiple factors other than age influenced the rejuvenation.

Several study limitations should be considered. The number of cases was small (n=32). We included only cases that did not present any severe clinical course, and we excluded ABO minor mismatch or incompatible cases. The follow-up period (one year) was relatively short. More parameters must be examined over a longer follow-up period. Since the positive rate of p16 is very small, this would scarcely make a difference. In addition, all specimens were obtained from a needle biopsy, not an excisional biopsy, and the specimens may not necessarily have fully reflected the patient's condition.

Conclusions

This is apparently the first study to evaluate the age difference in renal transplantations by using a marker of senescence. Even though our present results revealed that old kidneys did not rejuvenate when transplanted into young recipients, further investigations with larger numbers of cases and longer follow-ups will clarify the impact of donor-recipient age differences through transplantation.

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Disclosure. The authors declare that there are no conflicts of interest related to this study.

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