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Survival of donor-derived cells in human corneal transplants

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ABSTRACT

Purpose. To determine the fate of donor epithelial, stromal, and endothelial cells after corneal transplantation in humans.

Methods. Fifty-two transplanted corneal buttons were explanted over a two-year period from patients with donor corneas of opposite gender who required re-grafting. Fluorescent in-situ hybridization of the sex chromosomes of the epithelial, stromal, and endothelial cells was performed in histological sections prepared from each freshly-explanted graft. Fluorescence microscopy was subsequently used to determine the origin of cells in the graft (donor or recipient) and to quantify the relative proportion of donor and recipient cells of each corneal cell type.

Results. Donor epithelial cells were completely replaced by recipient epithelium in all corneal buttons examined, as early as 3 months after transplantation. Donor stromal and endothelial cells, however, were found in all fifty-two buttons, with 4 – 95% of stromal cells and 6 – 95% of endothelial cells being of donor origin. No significant correlation, however, between donor cell proportion and the age of the graft could be found. Donor-derived cells were found in significant numbers up to 32 years after transplantation. Eight corneas in this study were transparent, compensated grafts, and a similar long-term survival of donor stromal and endothelial cells was found in these cases.

Conclusions. While donor epithelial cells are promptly replaced, a high proportion of donor stromal and endothelial cells can survive within the corneal transplant in the long-term. The proportion of surviving donor cells is highly variable, however the source of this variability remains unknown.

Introduction.

In 1905 Zirm performed the first successful human corneal transplant. One hundred years later, corneal transplantation (penetrating keratoplasty) is a well-established and successful treatment modality for a range of corneal pathologies. In the United States it is estimated that around 50 000 surgeries are performed each year while in Sweden, with a population of 9 million, 500 – 600 corneal transplants are carried out annually according to the Swedish Corneal Register.¹ While the majority of corneal transplants are successful, an overall two-year rejection rate of 15% in Sweden has been reported¹ while in so-called high-risk cases the rejection rate can be much higher.² These events underscore the need for a more complete understanding of the pathogenesis of immune rejection after keratoplasty in the normally avascular, transparent, immune-privileged cornea. Of particular interest in this regard are the interactions between the recipient cornea and the new graft at the cellular level where healing, antigen activity, and the ultimate transparency of the cornea are mediated. Unfortunately, these cellular interactions are not well understood; even the basic question as to whether donor cells survive after transplantation is a fundamental biological problem that to this day remains unanswered. In fact, this important question has been the source of scientific discussion and debate ever since Zirm first showed that penetrating keratoplasty could be performed in humans. An excellent review of the literature relevant to this question was presented by Dohlman³ and later by Wollensak and Green.⁴ Depending on the technique employed for analysis, some investigators have concluded that transplanted cells are replaced by cells from the recipient cornea while others have claimed that donor cells in the transplanted tissue survive indefinitely. Without a sensitive and specific technique that could separate and unequivocally identify donor and host cells, both viewpoints could be argued. In a 1999 study by Wollensak and Green,⁴ the technique of fluorescent in situ hybridization (FISH) analysis of the X and Y chromosomes was for the first time used to distinguish

between individual recipient and donor cells in the human corneal epithelium, stroma, and endothelium with a high reliability in cases of sex mismatch between donor and recipient. In 14 failed, sex-mismatched grafts obtained retrospectively, Wollensak and Green found complete replacement of donor epithelium and endothelium by recipient cells in all grafts, while donor keratocytes were found in only three grafts, with a maximum survival time of 4.5 years. They concluded that “all cell types of corneal transplants tend to be replaced by recipient cells in the long term”, although “individual variability in the process of replacement exists.” Moreover, they proposed further studies investigating donor cell replacement in transparent, clinically-successful transplants, as their study was limited to failed grafts. In the present study, we re-examined the question of donor cell survival in the corneal transplant by applying the FISH technique to a larger sample of freshly-explanted corneal buttons at re-operation – a small subset of which were transparent, otherwise successful grafts removed for refractive reasons.

Materials and Methods.

Patients.

With approval from the Gothenburg University ethics committee and following the tenets of the Declaration of Helsinki, between February 2002 and January 2004 sixty corneal buttons were collected prospectively from patients with failed corneal transplants at the time of re-operation by members of the Swedish Society of Corneal Surgeons. All specimens were collected in sex-mismatched cases where the patient and the original donor were of different gender. Corneal button diameter varied from 7.25 to 8mm. Immediately following surgery, the explanted buttons were fixed in formalin for 24 hours, then placed in 70% ethanol and sent to a single laboratory at Gothenburg University for histochemical preparation. Eight specimens were unsuitable for analysis leaving a study sample of 52 corneal buttons.

Recipients were distributed almost equally between female (24) and male (28), with a mean

recipient age of 64.7 ± 15 y (mean \pm SD) at the time of re-operation. The time from initial penetrating keratoplasty to removal of the original donor button at re-operation ranged from 3 months to 32 years. The most common indications for the primary transplant were keratoconus (22 cases) and corneal edema (12 cases). The main indication for re-operation was decompensation of the graft (described in clinical records as edema, endothelial rejection, or graft failure), however ten non-edematous, compensated grafts were removed for other reasons, eight of which were substantially or completely transparent. Details of the patients and the explanted corneal buttons are given in Table 1.

Histological pre-treatment

In the laboratory, the formalin-fixed, ethanol-immersed corneal buttons were dehydrated and embedded in paraffin. Two full cross-sections (5 μ m thickness) were cut from the center of each button and mounted onto a glass slide. Sections were subsequently de-paraffinized in xylene and re-hydrated through a series of rinsing steps with decreasing concentrations of ethanol. Sections were then treated with 0.2 M HCl for 10 min at room temperature.

Following a PBS rinse, sections were treated with a proteinase K (Sigma) solution (50 μ g/ml) in 100 mM Tris-HCl with 50 mM EDTA, pH 8.0 at 37°C for 20 min. Enzyme digestion was stopped by immersing the slides for 5 min in 0.2 % glycine in PBS at room temperature. After rinsing with distilled water the sections were brought to 99% ethanol concentration through a series of rinsing steps with increasing concentrations of ethanol. Fluorescent in situ hybridisation (FISH) analysis of the sex chromosomes was then performed. The FISH technique has been described in detail elsewhere⁵ and is outlined briefly below.

FISH procedure

The samples were fixed in a 3:1 Et-OH/acetic acid solution at room temperature for 15 min. FISH was performed with directly labelled DNA probes specific for the X and Y

chromosomes (CEP -X/Y, Vysis Inc., USA). The DNA probe for chromosome X (DXZ1) was directly labelled with red fluorochrome (Spectrum Orange) specific for the AT rich alpha satellite DNA sequence at the centromeric region of chromosome X (Xp11.1-Xq11.1). The DNA probe for chromosome Y (DYZ1) was a collection of DNA segments (satellite III), directly labelled with green fluorochrome (Spectrum Green) and hybridised to most of Yq11.2 and all of Yq12, the telomere of the Y-chromosome. Probe hybridization was accomplished by placing 9µl of the DNA probe mixture on each slide. An 18x18 mm coverslip was attached and sealed with rubber cement. Target and probe were denatured simultaneously at 80° C for 5 min on a heat block. Hybridization was taken place at 37°C in a moist chamber for 3 hours. The slides were washed at 42° C in 50% formamid/2XSSC, pH 7.6 for 15 min. If the cover slip had not fallen off after 2 minutes it was carefully removed. The slides were further washed in 2XSSC, pH 7.0 for 10 min and transferred to 0.01% Nonident-P40 (NP-40), pH 7.0 for 5min. The samples were allowed to air-dry and counter stained with 9µl of 4',6-diamidino-2-phenylindole (DAPI II) (Vysis Inc., USA). Cover slips were attached before analysis.

Evaluation of FISH signals

Only cells with two distinctive signals per nucleus were enumerated. Overlapping interphase nuclei or cells with an indistinct nuclear membrane were excluded from the evaluation. Signals of lower intensity were interpreted as minor binding sites (cross-hybridization) and similarly ignored. Paired fluorescent spots were counted as one signal when appearing less than one signal diameter from each other. Less than 2% cells with only one signal present is a realistic standard of acceptance for the DNA labelling procedure (Vysis Inc., USA). The analysis was performed on a Nikon fluorescent microscope equipped with a digital camera for image capture. An X-chromosome centromere exhibited an orange signal, a Y q-arm exhibited a green signal and the nucleus was counter stained with DAPI-blue. A triple

bandpass DAPI/FITC/TRITC filter (360/490/570nm) was used to view all three fluorescent signals simultaneously.

All cell counts were performed by a single observer, and two transverse sections from each corneal button were used for cell counting. Cell nuclei were identified as being male- or female-derived based solely upon the presence of two orange signals (female) or a green and orange signal (male) within a single nucleus. At least 100 nuclei from the epithelium and at least 100 nuclei from the stroma were counted from each button. The endothelium, however, was missing in eight specimens and less than 30 endothelial cells were found in nine specimens, so these buttons were excluded from endothelial analysis. In the remaining 35 corneal buttons, between 30 and 100 endothelial cell nuclei from each button were counted for the analysis.

Cell counting results were tabulated in a spreadsheet and the Student *t*-test was used to compare means. Linear relationships among variables were analyzed with the Pearson correlation coefficient. In all cases a p-value of < 0.05 was considered significant. All statistics were performed with spreadsheet software (Excel 2003, Microsoft Inc., Redmond, WA).

Results

No donor-derived epithelial cells were detected in any of the corneal buttons, while all 52 corneas had some donor-derived stromal cells (keratocytes) and 26 of 35 corneas had some donor-derived endothelial cells (Table 1, Figure 1). Donor cells were distributed throughout the corneal sections in a seemingly random fashion.

Donor-derived keratocytes were found with a proportion ranging from 4 to 95%, with no significant correlation of donor keratocyte survival with graft age (Figure 2). The lack of correlation with graft age (time period within the recipient cornea) persisted after stratification of the data along gender, graft transparency (8 cases), recipient age, and indication for the

initial transplant. In 33% of cases (17 cases), donor keratocytes persisted for more than 10 years after transplantation and in one patient with a transparent graft, 65% of keratocytes counted were donor-derived 32 years postoperatively.

Analysis of endothelial cells from 35 corneal buttons revealed 9 cases in which donor endothelial cells were completely replaced by recipient endothelium, 24 cases in which donor and recipient endothelial cells co-existed, and 2 cases in which only donor-derived endothelium was present (Table 1). In cases where both donor and recipient endothelial cells were found, the proportion of donor-derived cells ranged from 6 to 95%. Similar to donor keratocytes, donor endothelial cell survival was not significantly correlated with age of the graft (Figure 3), regardless of transparency or indication for the initial transplant. When stratified by gender and recipient age, however, a significant decline in the frequency of donor endothelial cells with increasing postoperative time was found in females ($p = 0.04$, 14 corneas) and in patients older than the mean age of 64.7 years at the time of explantation ($p = 0.03$, 16 corneas). Additionally, these variables were correlated as female recipients in this study (age $70.6 \pm 10y$, mean \pm SD) were significantly older than male recipients ($59.6 \pm 17y$; $p = 0.006$).

Ten of the explanted corneal buttons in this study were removed for reasons other than edema. Of these, two were removed due to suture-related infection, five were completely transparent buttons removed due to intractable astigmatism and three were transparent except for small, localized scars on the visual axis. Eight grafts therefore exhibited general stromal transparency and endothelial compensation. As seen in Table 1 and in Figures 2 and 3, the frequency of donor-derived keratocytes and endothelial cells in these eight grafts did not generally differ from those of edematous, decompensated grafts. No clear trend in donor cell survival with graft age was noted in this small sample of transparent grafts.

Keratoconus patients in this study were significantly younger ($54.5 \pm 14y$) than those with edema as an initial indication for transplantation ($72.1 \pm 13y$; $p = 0.001$), and the survival

time of the original transplant was significantly longer for keratoconus (158.9 ± 104 mos) compared to edema (27.8 ± 16 mos; $p < 0.001$). Similarly, the seven patients with pseudophakic bullous keratopathy as an initial indication for transplantation tended to be older (75.6 ± 8 y) with a relatively short time interval until re-operation (36.7 ± 24 mos). Although the seven patients with endothelial dystrophy also tended to be older (69.1 ± 9 y), transplant survival time varied widely; however, in all seven of these cases the proportion of surviving donor keratocytes was high at the time of explantation (66 – 93%).

Discussion

We have successfully performed the largest study to date using the FISH technique to analyze human corneal donor buttons after penetrating keratoplasty. In all 52 corneal buttons analyzed, complete epithelial replacement by recipient cells was observed, as early as 3 months postoperatively in one case and after 6 months in two cases. In earlier studies using the FISH technique, complete epithelial cell turnover was similarly observed to occur within six months to one year post-transplantation.^{4,6,7} Although donor-derived epithelial cells have been shown to persist for years after limbal transplantation in patients with limbal stem cell deficiency,⁶⁻⁹ patients in this study did not have initial transplant indications consistent with limbal stem cell deficiency, and it is therefore not surprising that complete replacement of donor epithelial cells occurred in the promptly re-epithelialized graft. Confirmation of the recipient gender in all 52 sex-mismatched cases by epithelial cell analysis of the donor buttons served to validate the FISH procedure in this study in the absence of sex-matched control cases.

While donor epithelial cells were quickly and completely replaced, the hypothesis that donor keratocytes and endothelium are gradually replaced over time^{3,4} could not be supported by the results of this study. Long-term survival of donor keratocytes and endothelium was observed despite a substantial variability in patient characteristics and presumed transplant conditions

(initial operations having been performed across multiple centers over a 32-year period).

While a tendency for donor endothelial cells to be replaced over time was observed in patients over the age of 65, even within this group the long-term survival of a substantial proportion of donor endothelial cells was found in some patients (Table 1). Furthermore, the presence of eight transparent grafts in this study – exhibiting similarly variable long-term survival of donor keratocytes and endothelial cells – suggests that donor cells may survive in the long term in most corneal transplants, including successful ones.

The results of this study differ most notably from those reported by Wollensak and Green, who concluded that all cell types of the corneal transplant tended to be replaced by recipient cells in the long term.⁴ Both the small sample size that was used (14 buttons) and the retrospective nature of the study (where archived samples were used for analysis) may in part account for the discrepancy with the present results. In the present study, corneal buttons were obtained prospectively, with fresh specimens used for analysis in all cases. The time from removal of the original donor button to cellular analysis in this study did not exceed more than a few days.

Our findings support the contention that donor keratocytes and endothelial cells can survive in the transplanted cornea indefinitely, a theory supported by earlier animal experiments.¹⁰⁻¹⁵

Prior to this study, the longest period donor keratocytes have been reported to survive within a transplanted cornea was 6.5 years;¹⁶ our findings suggest that individual keratocytes (or keratocytes derived from a single source cell) may remain in the corneal stroma for life.

While damaged donor keratocytes can be replaced by cell division in primates,¹⁷ it is unclear whether the donor keratocytes observed in this study were original or have been replenished through cell division.

As endothelial cells do not generally proliferate mitotically,¹⁸ donor endothelial cells observed in this study were likely original and some of these cells may be expected to persist in the transplant for life. These findings contradict those of Espiritu and co-workers,¹⁹ who observed

complete replacement of donor endothelial cells in rabbits after 7 months using the sex chromatin method, and Dohlman³ who reported the disappearance of isotopically-labelled endothelial cells in rabbits 10 days after transplantation. Methodological limitations in these earlier studies, however, have been noted.^{10,11} The findings in this study are in agreement with experiments conducted by several investigators,^{10,11,14} who observed donor endothelial cell survival in rabbits for 12 to 21 months using both radio-labelling and sex chromatin methods. Previous studies of corneal transplantation in cats,²⁰ rabbits,^{11,13-15,21} and more recently in mice,²² have suggested that the long-term presence of donor-derived endothelial cells and keratocytes may be a necessary condition for graft transparency and long-term survival, as opaque grafts are typically characterized by invading recipient cells, vascularization, and a conspicuous paucity of donor cells. Our observations of significant proportions of surviving donor keratocytes and endothelial cells in grafts having remained transparent and avascular for long periods appear to support the observations of these earlier studies. Notably, in one study Polack and colleagues¹¹ used radio-labelling to study donor cell survival in grafted rabbit corneas, and concluded that the scar tissue in failed, opaque grafts was ‘primarily of host origin’. In grafts that remain transparent and avascular, it has been speculated that the immune privilege of the cornea may protect donor cells for long periods within the graft,²⁰ whereas in highly vascularized tissue, such as skin grafts, donor cells do not survive.^{4,11} Another possible explanation for the long-term persistence of donor cells that cannot be excluded is the potential existence of donor stem cells within the graft. Although the presence of stem cells was not investigated in this study, the future testing of explanted buttons with suitable stem cell markers could address this hypothesis.

Although in the present study donor keratocytes and endothelial cells survived in the long term, the proportion of surviving donor cells was subject to considerable individual variability. While recipient age, gender, indication for transplantation, graft survival time, and transparency of the graft did not appear to impact the proportion of donor cells that

survived, other variables such as donor characteristics (donor age, time in culture, corneal preservation method), the type and degree of surgical trauma, preoperative risk level, and postoperative complications, vascularization, and rejections may help to explain the wide variability in donor cell proportion observed, although unfortunately this detailed data was not available in the present study. Future studies controlling for these variables may help to determine why a large proportion of donor cells thrive in some transplants while their numbers are severely diminished in others.

Our finding of significant populations of both donor and recipient keratocytes and endothelial cells within the graft in the long term is positive evidence of cell migration into the graft, but instead of complete donor cell replacement, we speculate that keratocytes and endothelial cells of both the donor and recipient may reach equilibrium within the transplanted cornea in the long term, although the relative proportion of surviving donor cells may be subject to considerable individual variability. Factors such as immunological reaction, postoperative complications, and certain dystrophies present in the recipient cornea may upset this equilibrium and tip the balance in favour of the recipient cells.

Several limitations to the present study are worth noting. The use of only a few histological sections from the central region of each corneal button provided us with only a small sampling of the cell population within the transplanted cornea. That these sections are representative of the entire corneal button was implicitly assumed; non-uniformity, however, in the distribution of donor and recipient cells (with more cells of one type appearing at the graft edge, for example) would affect the relative proportion of donor cells detected. Future studies using either numerous sections taken across the entire corneal button or a flat mount technique would provide a more accurate indication of the distribution of surviving donor cells. Another limitation in this study was the presence of only a small number of non-edematous, transparent grafts. The detection of significant differences in donor cell survival between transparent (i.e., clinically successful) and failed transplants could yield insights into

the pathogenesis of graft failure; however, such an analysis was not possible in this study as this would require a larger group of clinically-successful transplants removed for refractive reasons. Finally, although a relatively large number of excised corneal buttons were examined in this study, the cross-sectional nature of the samples resulted in missing or incomplete medical information about the donor, surgical conditions, and postoperative period in many cases. Such information may be critical for determining the factors that correlate with or confound the observed degree of donor cell survival.

In summary, the results of this study indicate that while donor epithelial cells in corneal transplants are promptly replaced by recipient epithelium, both keratocytes and endothelial cells from the donor can survive in significant proportions within the transplanted human cornea in the long-term (up to 32 years), with no detectable trend towards replacement by recipient cells over time. The proportion of donor-derived keratocytes and endothelial cells surviving within the corneal transplant is subject to considerable individual variability, however, no source for this variability could be found. Examination of eight transparent grafts in this study revealed a similar variability in donor cell survival. Further studies are required to investigate the origins of the wide variability in donor cell survival following corneal transplantation and may help to elucidate the relationship between donor cell survival, immunologic activity, and the ultimate success of the corneal transplant.

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Recipient	Sex	Age*	Indication for primary transplant†	Graft age‡ (months)	Surviving donor cells (%)		Compensated grafts§
					Keratocytes	Endothelial	
1	F	81	edema	24	12	0	
2	M	88	band keratopathy	48	59	100	
3	F	85	keratoconus	264	13	0	
4	M	41	edema	6	71		
5	F	80	endothelial dystrophy	55	77	24	
6	M	73	edema	18	5		
7	F	78	edema	3	91	100	Infection
8	F	62	endothelial dystrophy	52	68	57	
9	F	81	endothelial dystrophy	134	66	61	
10	M	62	keratoconus	90	68	79	
11	F	53	keratoconus	72	53		
12	M	61	keratoconus	28	21	0	
13	F	59	endothelial dystrophy	156	68		
14	M	58	keratoconus	17	83	0	
15	M	46	keratoconus	45	15	0	Astigmatism
16	F	77	keratitis	6	84	75	
17	M	29	keratoconus	13	38	0	
18	M	75	PBK	26	95		
19	F	77	lattice dystrophy	193	78	24	
20	F	72	PBK	21	45		
21	M	70	edema	37	58		
22	F	59	endothelial dystrophy	36	93	41	
23	F	58	keratoconus	156	86	0	Astigmatism
24	M	87	PBK	84	58	17	
25	M	50	keratoconus	216	21	6	Astigmatism
26	M	42	keratoconus	192	89	77	Astigmatism
27	M	44	herpetic macula	111	15	0	Scar
28	M	39	keratoconus	120	74	52	
29	M	60	keratoconus	166	66	17	
30	M	85	edema	60	16		
31	M	55	keratoconus	196	8	95	
32	F	70	endothelial dystrophy	24	81		
33	M	83	edema	27	34	18	
34	M	77	edema	47	23	33	Scar
35	F	80	PBK	28	13		
36	F	71	edema	28	7		
37	F	71	edema	27	79		
38	F	82	edema	36	33	69	
39	M	81	keratoconus	210	52	18	
40	F	77	PBK	18	95	84	Infection
41	F	77	PBK	27	6		
42	F	58	keratoconus	72	93	70	
43	M	31	keratoconus	103	34	67	
44	F	73	endothelial dystrophy	216	84	42	
45	M	45	keratoconus	240	4		
46	M	45	keratoconus	216	58	31	
47	F	61	PBK	53	72		
48	M	62	keratoconus	240	13	8	Scar
49	M	56	keratoconus	384	65	62	Astigmatism
50	F	53	keratoconus	96	24		
51	M	53	edema	20	64		
52	M	71	keratoconus	360	9	0	

Table 1. Patient (recipient) details and results of FISH analysis of cells in explanted corneal buttons.

*age at time of explantation

†PBK = pseudophakic bullous keratopathy

‡ time interval between initial transplantation and explantation

§ reason for explantation of well-compensated, non-edematous grafts. Infections were suture-related, astigmatism was intractable in totally transparent grafts, scars were small and localized to the visual axis in otherwise transparent grafts.

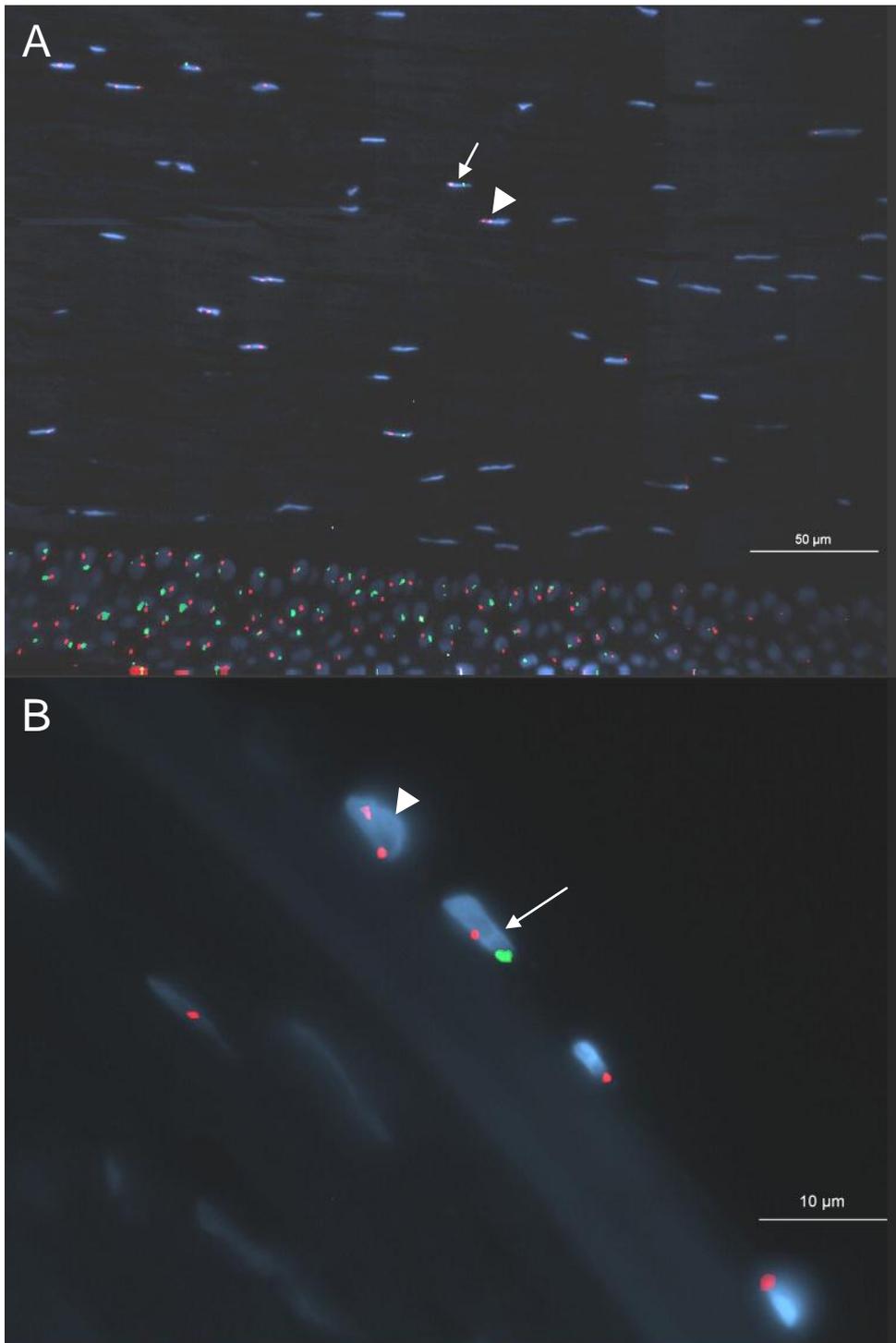


Figure 1. Typical fluorescence microscope images used for FISH analysis of corneal sections. (A) epithelial (bottom) and stromal (top) cells in a female donor corneal button removed from a male recipient. All epithelial cells with two distinct signals had one red and one green signal per cell. Keratocytes had either one red and one green signal (arrow) or two red signals (arrowhead) per cell. Scale bar = 50 μ m, 20 \times objective. (B) endothelial cells at the posterior surface of a male donor corneal button removed from a female recipient. Endothelial cells with one red and one green signal (arrow) or two red signals (arrowhead) per cell were observed. Scale bar = 10 μ m, 100 \times objective..

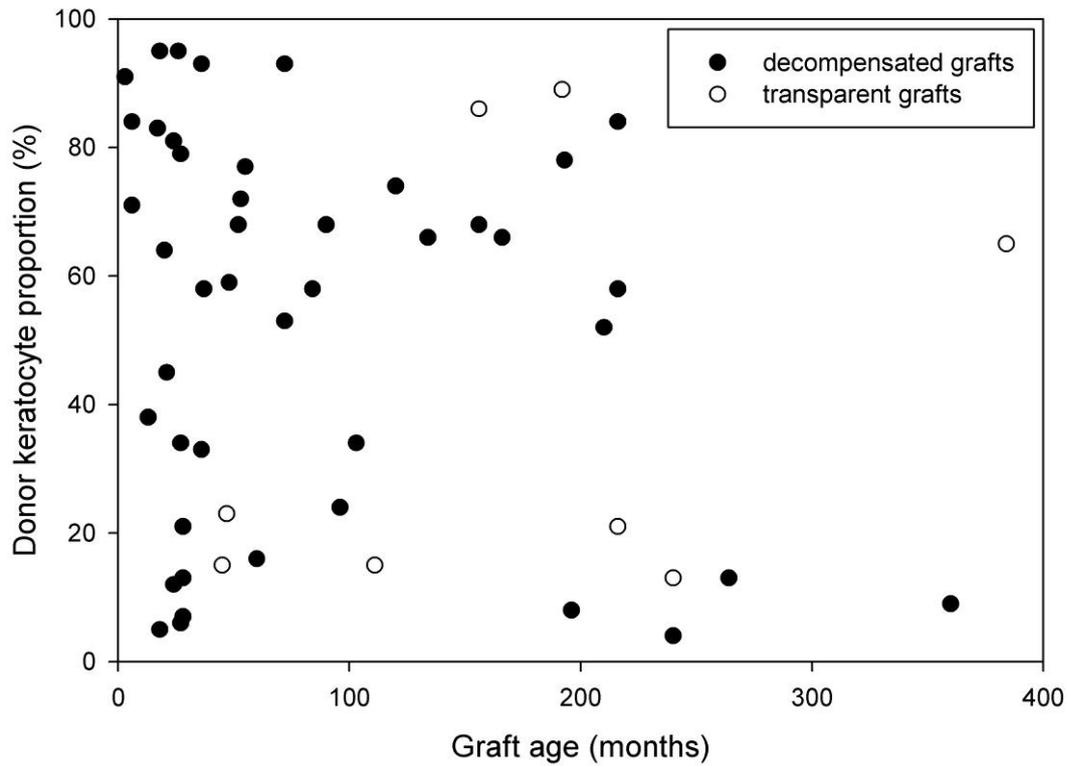


Figure 2. Donor keratocytes as a proportion of total keratocytes counted in each of 52 corneal grafts, plotted against graft age (time period the graft remained within the recipient). The subset of eight transparent grafts removed due to reasons other than endothelial decompensation is indicated by open circles. Donor keratocyte survival did not correlate with graft age.

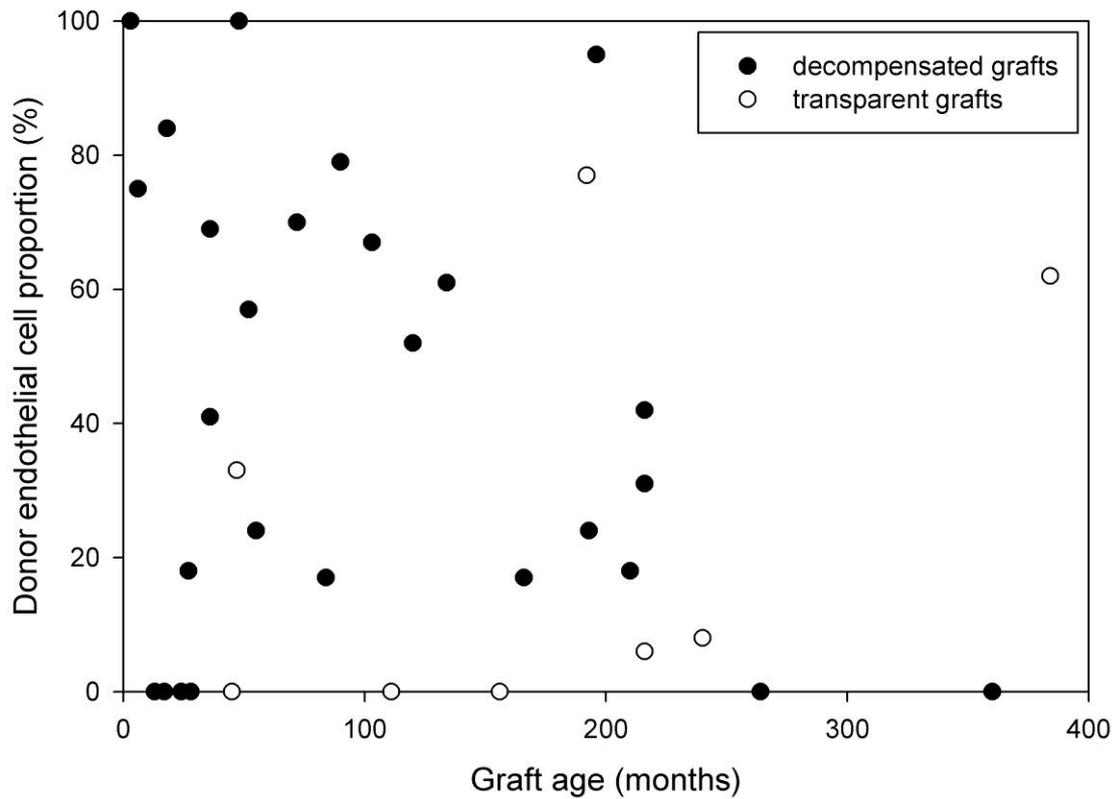


Figure 3. Donor endothelial cells as a proportion of total endothelial cells counted in each of 35 corneal grafts, plotted against graft age. The subset of eight transparent grafts removed due to reasons other than endothelial decompensation is indicated by open circles. No donor endothelial cells were found in 9 grafts, and no recipient endothelial cells were found in 2 grafts. Donor endothelial cell survival did not correlate with graft age.