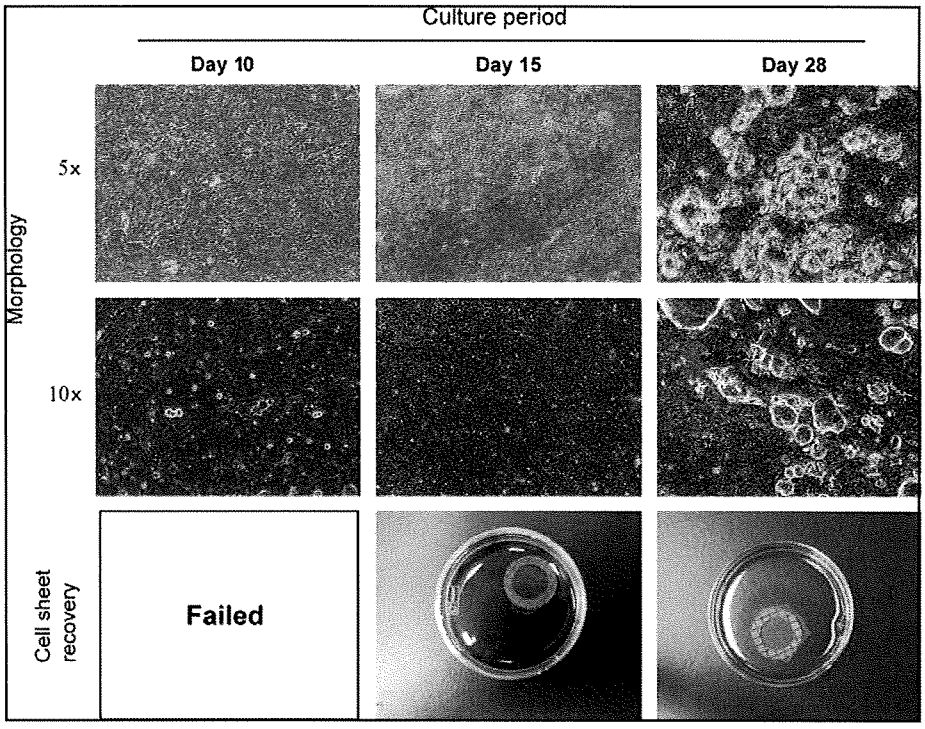

FIG. 2. Cell morphology and cell sheet harvest. Cell morphology was examined by phase contrast microscopy. Harvested cell sheets with supporter membranes were transferred into culture medium in 60-mm dishes. Cells cultured for 10 days were not successfully harvested from temperature-responsive inserts (denoted as "Failed"). Color images available online at www.liebertonline.com/ten.

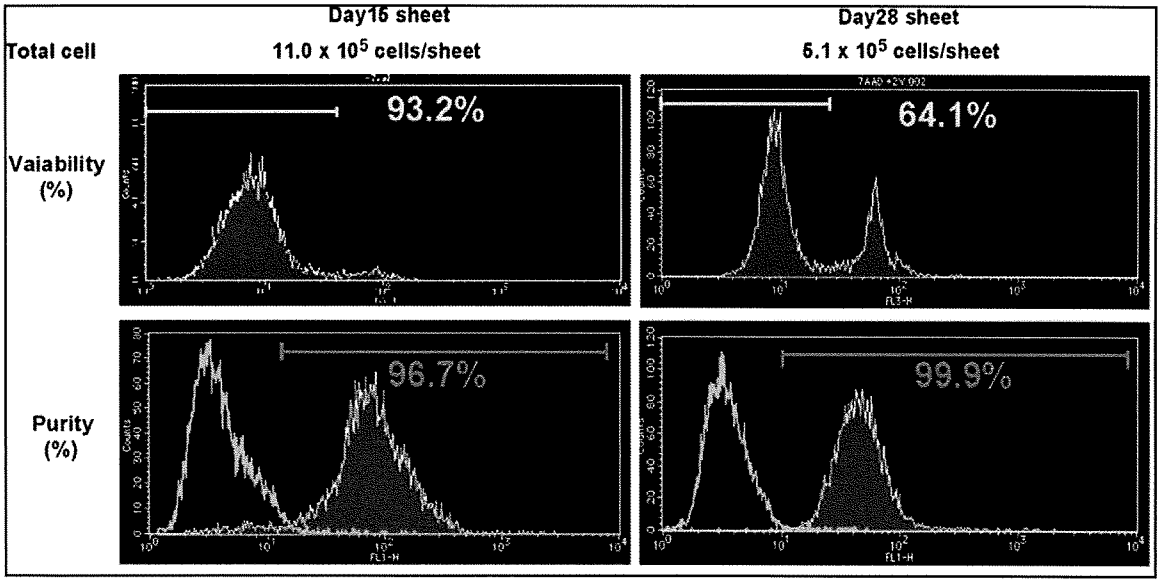

FIG. 3. Flow cytometric analyses. Harvested cell sheets were incubated with trypsin–ethylenediaminetetraacetic acid to obtain single-cell suspension. Resuspended cells were analyzed in cell viability and epithelial cell purity by staining with 7-aminoactinomycin D (7′AAD) and antipancytokeratin antibody, respectively. Color images available online at www .liebertonline.com/ten.

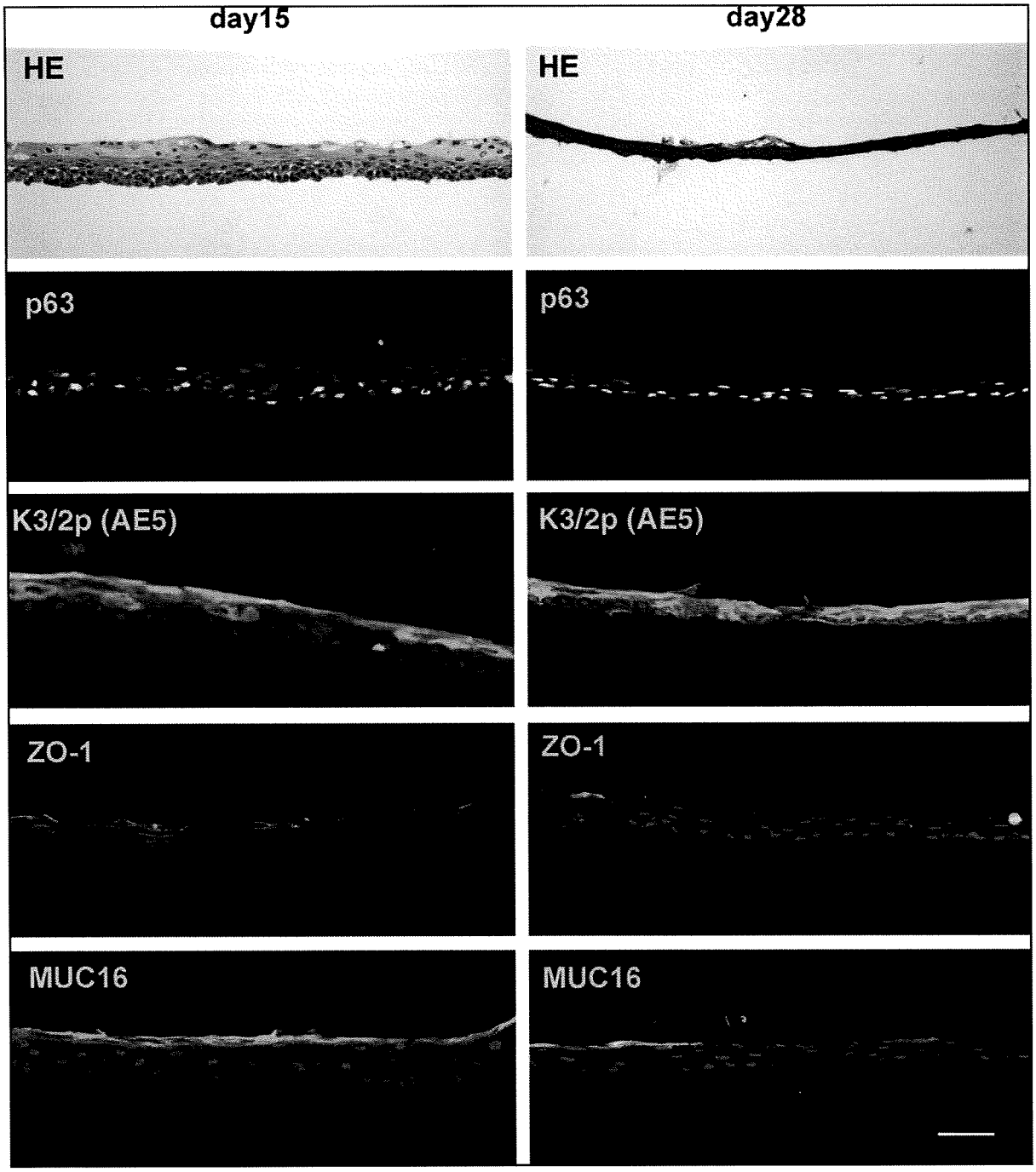

FIG. 4. Histological analyses. Frozen sections of harvested cell sheets were subjected to HE staining and immunofluorescence with anti-p63, anti-K3/2p, anti-ZO-1, or anti-MUC16 antibodies.

TABLE 1. SUMMARY OF VALIDATION OF HUMAN CORNEAL EPITHELIAL CELLS CULTURED FOR DIFFERENT PERIODS

	Phase contrast	*Detachment test*	*Cell ($\times 10^5$)*	*Viability (%)*	*Purity (%)*	*Stratification (HE)*	*p63*	*K3/2p*	*Muc16 ZO-1*
Day 10	Low density	Impossible	—	—	—	—	—	—	—
Day 15	Normal	Possible	11.0	93.2	96.8	Normal 4–8 layers	Posi	Posi	Posi
Day 28	Defects	Partially broken	5.1	64.1	99.9	Thin 2–3 layers	Posi	Posi	Faint

HE, hematoxylin and eosin; Posi, positive.

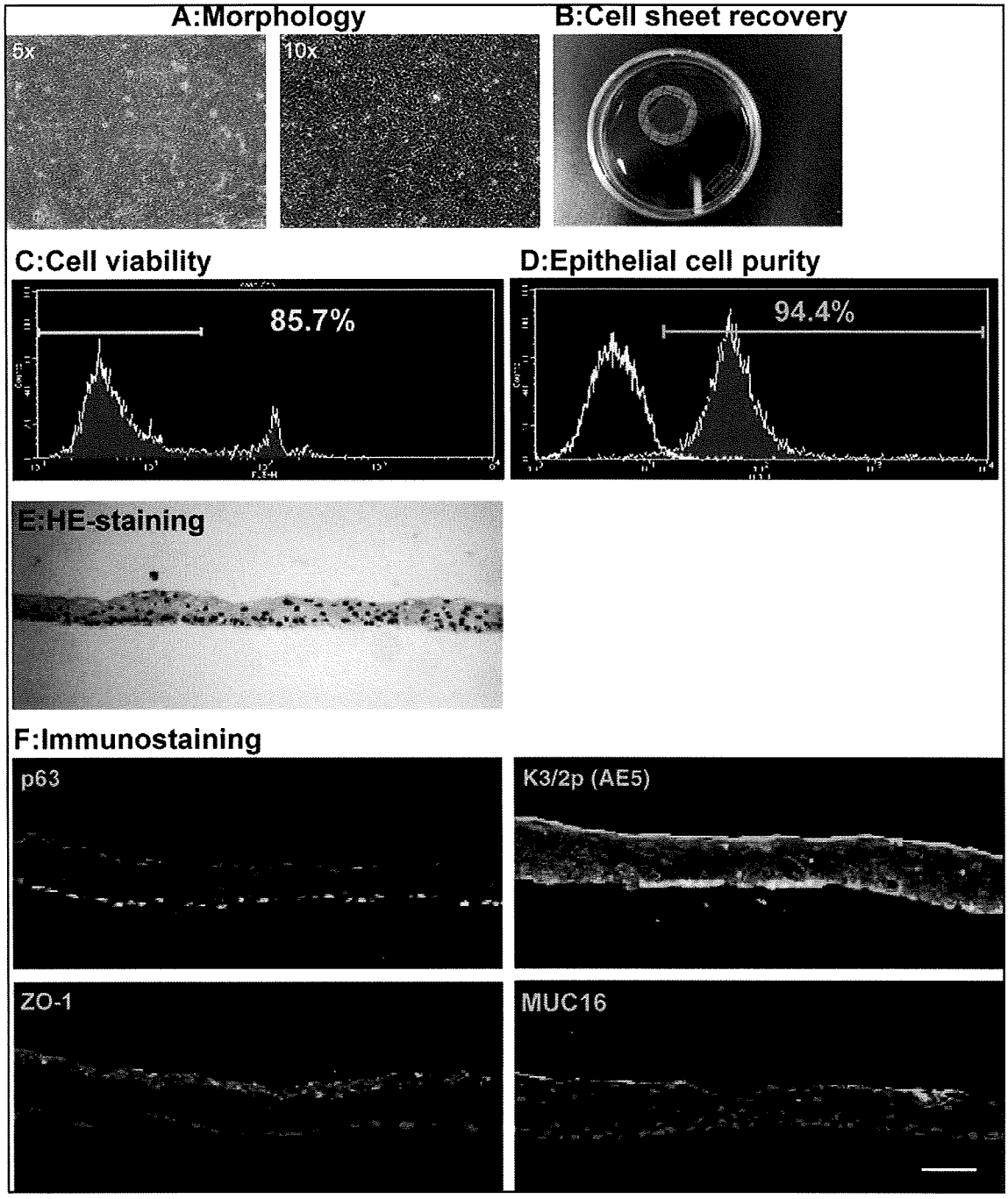

FIG. 5. Validation of human oral mucosal epithelial cell sheets. Human oral mucosal epithelial cells were cultured on temperature-responsive cell inserts for 15 days. Cell morphological examination was performed by phase contrast microscopy (**A**), and then cultured epithelial cells were harvested by reducing temperature to 20°C (**B**). The harvested cell sheet was used for flow cytometric analyses (**C, D**), HE staining (**E**), and immunostaining for p63, K3/2p, ZO-1, and MUC16 (**F**), to validate the quality of cell sheets.

determination of appropriate culture periods before harvest is crucial to fabrication of transplantable cell sheets. In the total cell number determination test, day 28 sheet showed fewer total cell number than day 15 sheet. This indicated that excessive culture period promoted the epithelial cell turnover as phase contrast observation showed, and finally resulted in decrease of total cell numbers. This result corresponded with the result of HE staining, which showed that day 28 sheet had fewer cell layers than day 15 sheet. In addition, the decrease in cell viability in day 28 sheet was also

TABLE 2. SUMMARY OF VALIDATION OF HUMAN CORNEAL AND ORAL MUCOSAL EPITHELIAL CELLS

	Phase contrast	*Detachment test*	*Cell ($\times 10^5$)*	*Viability (%)*	*Purity (%)*	*Stratification (HE)*	*p63*	*K3/2p*	*Muc16 ZO-1*
CO 1	Normal	Possible	10	92.8	97.9	Normal	Posi	Posi	Posi
CO 2	Normal	Possible	11	93.1	95.3	Normal	Posi	Posi	Posi
CO 3	Normal	Possible	8.0	87.1	93.0	Normal	Posi	Posi	Posi
OR 1	Normal	Possible	11	85.7	94.4	Normal	Posi	Posi	Posi
OR 2	Normal	Possible	16	83.3	98.7	Normal	Posi	Posi	Posi
OR 3	Normal	Possible	9.5	89.8	98.1	Normal	Posi	Posi	Posi

CO, human corneal epithelial cell; OR, human oral mucosal epithelial cell; Posi, positive.

caused by excessive cell turnover. These results of day 28 sheet indicated that most of the 3T3 cells did not remain beyond 2 weeks on the culture dish due to mitomycin C treatment, and could no longer support proliferation or maintenance of the epithelial stem/progenitor cells after 2 weeks. In actuality, when the feeder layer was replaced by a new one at the point of day 15, epithelial cells can be maintained without any aberration after additional 14 days culture (data not shown). Interestingly, in the epithelial cell purity analysis and p63 immunostaining, there were no remarkable differences between day 15 and 28 sheets. This result suggested that some defects in day 28 sheets were possibly caused by the loss of the appropriate regulations for proliferation and differentiation of stem/progenitor cells in the additional 2 weeks of culture, rather than the maintenance of stem/progenitor cells by 3T3 cells. In addition, the long culture periods did not promote nonepithelial cell proliferation such as fibroblasts.

We performed the validation not only for the corneal but also for the oral mucosal epithelial cell sheet, because for bilateral corneal disease, we perform transplantation with the cultured epithelial cell sheet fabricated from the patient's own oral mucosal epithelium. The results of each of the three cell sheets showed that there were no remarkable differences between the two epithelial cell sheets. It should be noticed that the ocular surface–specific mucin MUC16, which is not expressed in oral mucosa *in vivo*, was expressed in all three oral mucosal epithelial cell sheets. This corresponded with our previous report,[21] suggesting that this culture condition promoted oral mucosal epithelial cells to express MUC16.

We previously showed that the tissue-engineered epithelial cell sheets that had cultured for 2 weeks were successfully transplanted to patient' eyes and restored their ocular surface.[11] These previous and present results strongly suggested that culturing epithelial cells for around 15 days is the most appropriate for clinical application. Further, our results suggested that among the nine items in the validation system, especially (1) cell morphology, (2) cell recovery, (3) total cell number, and (4) cell viability, in which results were notably different between day 15 sheet and the others, were thought to be the most important factors, and (7) existence of stem/progenitor cells is also an important factor in general for regenerative medicine.

Conclusion

In the present study, our validation system worked well with both corneal and oral mucosal epithelial cell sheets. Using this system, we could standardize the quality of cell sheets for clinical use even in different facilities. This validation system would contribute to the establishment of safe and effective regenerative therapy with cell sheet techniques as a standard therapy.

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Disclosure Statement

No competing financial interests exist.

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