CHAPTER 4

RESULTS

Cultivation of fresh limbal and oral mucosal tissues

After three weeks of cultivation, all fresh specimens including three limbal and three oral mucosal biopsies were successfully grown to form the epithelial sheets covering the entire denuded AM. From hematoxylin-eosin staining examination, the cultured epithelia showed stratification with two to five layers, no goblet cells were observed. Cultivated epithelial sheets from oral mucosal tissues showed fewer cell layers than cultured sheets from limbal tissue. However, the morphology of cultured epithelial cells from limbus and oral mucosa were similar. Cuboidal cells were seen in basal layer whereas the cells were flattened in the superficial layer (Figure 4.1A, E, I and 4.2A, E, I).

The expression of p63, the stem cell marker, showed positive nuclear staining in the cells located at basal layer and intermediate layer but not in superficial layer. The p63 expression were detected in all of cultured epithelial sheets from fresh limbal tissues(Figure 4.1B, F, J and 4.2B, F) but not in one sheet from fresh oral mucosal tissue (rabbit No. 6)(Figure 4.2J). The differentiated corneal epithelial cell marker, K3, showed positive staining in intermediate and superficial layers of all epithelial sheets while basal cells were K3 negative (Figure 4.1C, G, K and 4.2C, G, K). The expression of p63 and K3 were strongly positive in all epithelial sheets from limbal

cultures whereas epithelial sheets from oral mucosal cultures showed weakly positive. The expression of Cx43, the gap junction protein which is a marker for differentiated cells, was dispersed in all layers throughout the epithelium from all cultures (Figure 4.1D, H, L and 4.2D, H, L). The expressions of all markers in all epithelial sheets were summarized in table 4.1. Notably, the cultured sheet from rabbit No. 3 showed the most different from others with the most thickness of epithelium. However the cell population in culture still contained different population of cells include the stem cells and differentiated corneal epithelial cell with gap junction between cells.

Table 4.1 Immunohistochemical examination of marker expression in all epithelial sheets from fresh tissues culture.

Rabbit number		Markers		
		p63	K3	Cx43
Fresh limbal tissues	1	-/++	++/-	+*
	2	-/++	++/-	+*
	3	-/++	++/-	+*
Fresh oral mucosal tissues	4	-/+	+/-	+*
	5	-/+	+/-	+*
	6	-	+/-	+*

+ indicates positive; - indicates negative; ++/- indicates strongly positive in superficial and intermediate layers; +/- indicates weakly positive in superficial and intermediate layers; -/++ indicates strongly positive in intermediate and basal layers; -/+ indicates weakly positive in intermediate and basal layers; +* indicates scattered staining throughout the epithelium

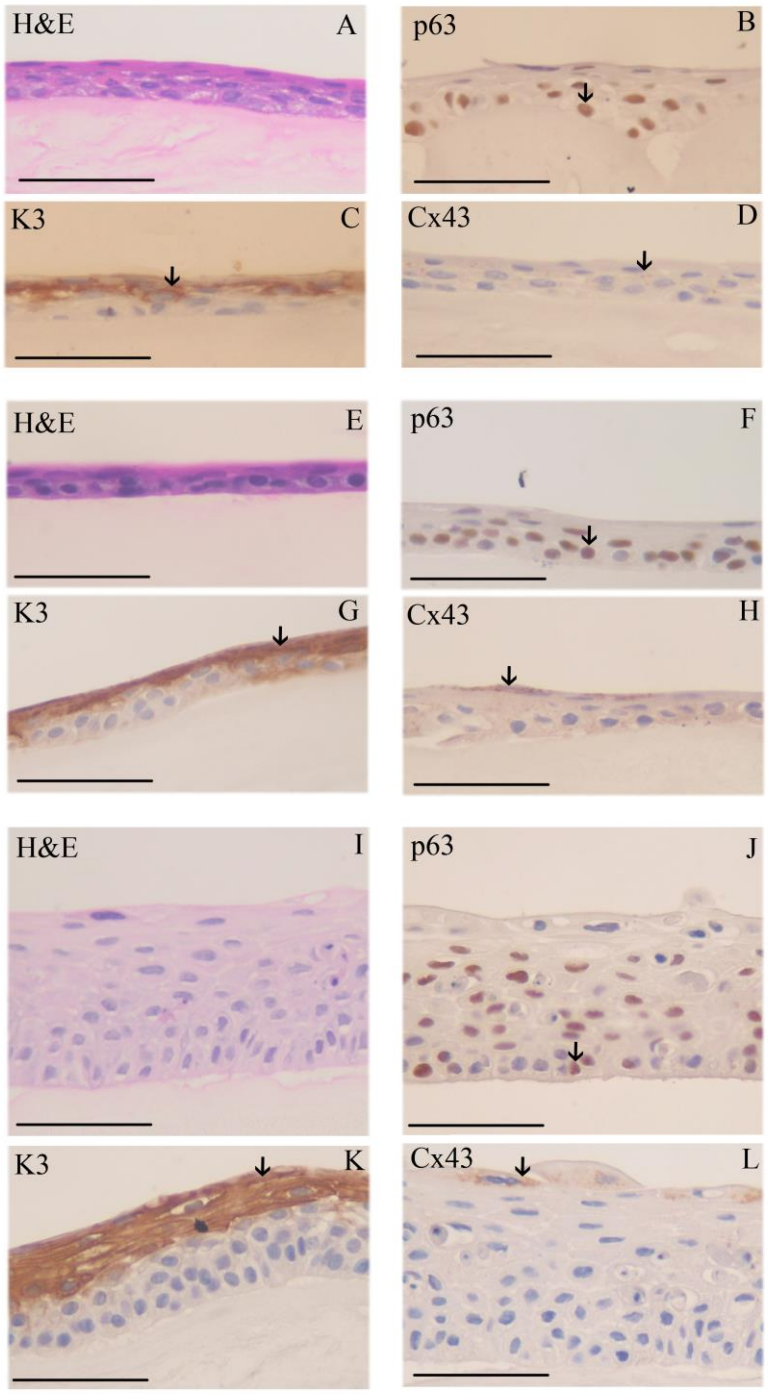

Figure 4.1 Cultured epithelial sheets generated from fresh limbal biopsies of rabbit No. 1 (A-D), rabbit No. 2 (E-H) and rabbit No. 3(I-L). Represent hematoxylin-eosin staining (A, E, I), immunohistochemical staining for p63 (B, F, J), K3 (C, G, K) and Cx43 (D, H, L). The arrows indicated positive cells. Scale bars = 50 µm.

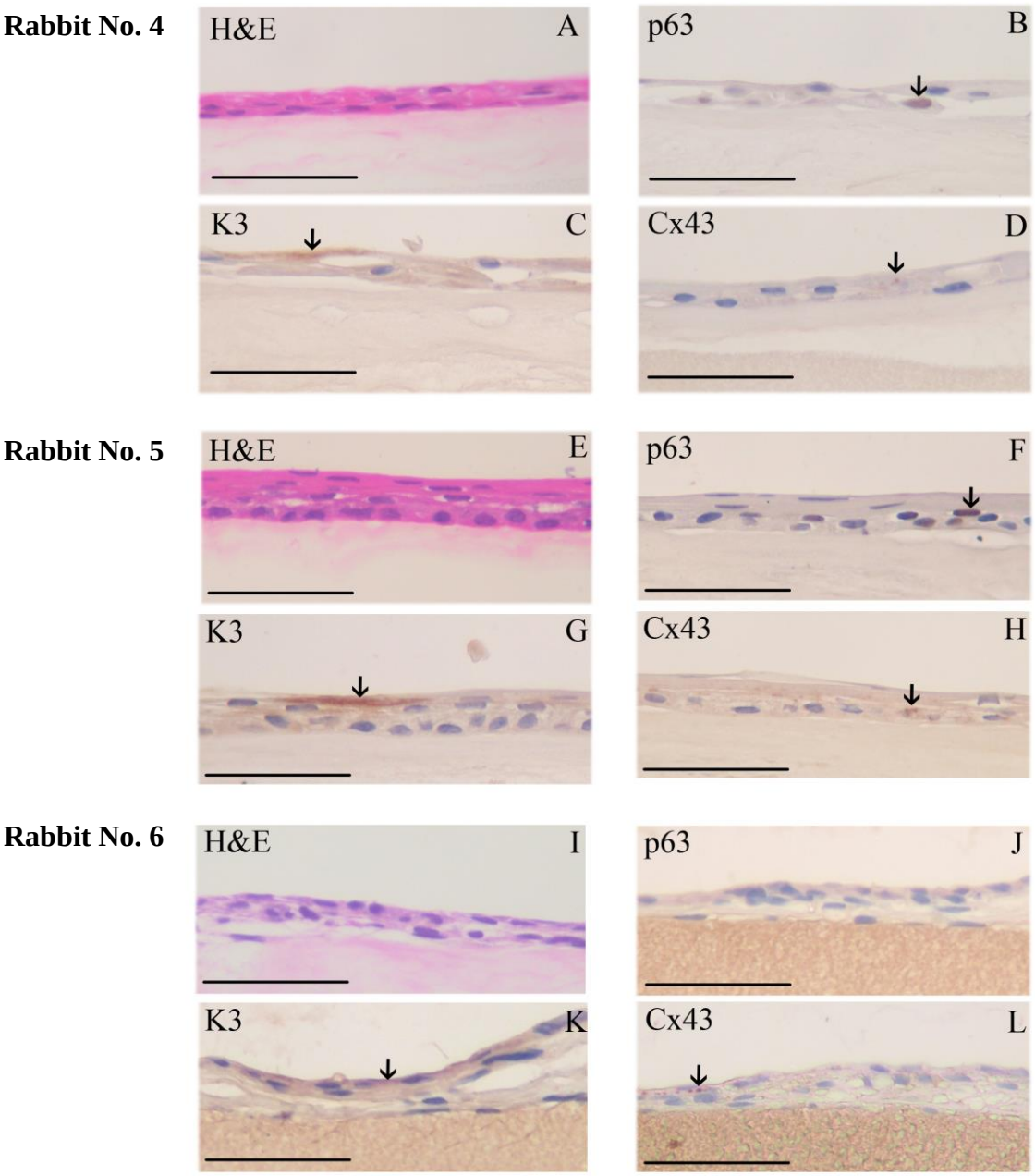

Figure 4.2 Cultured epithelial sheets generated from fresh oral mucosal biopsies of rabbit No. 4 (A-D), rabbit No. 5 (E-H) and rabbit No. 6 (I-L). Represent hematoxylin-eosin staining (A, E, I), immunohistochemical staining for p63 (B, F, J), K3 (C, G, K) and Cx43 (D, H, L). The arrows indicated positive cells. Scale bars = 50 µm.

Eye Enucleations

The right eyes of all rabbits with the induction of LSCD were transplanted with cultivated epithelial sheets by an ophthalmologist. Two months after transplantation, these eyes were enucleated. Normal eye was used as a normal control. Rabbit normal corneal epithelium showed four to five stratified layers (Figure 4.3A) whereas epithelium in limbal region was thicker and had blood vessels in the underlying stroma (Figure 4.3E). P63 was expressed mostly in basal layer and a few in suprabasal layer of limbal epithelium (Figure 4.3F) but not in corneal epithelium (Figure 4.3B). K3 was strongly positive in intermediate and superficial layer of corneal epithelium (Figure 4.3C) but was weak expression in suprabasal layer and was absent in basal layer of limbal region (Figure 4.3G). The expression of Cx43 was presented in superficial layer of corneal epithelium (Figure 4.3D) but absent in limbal region (Figure 4.3H).

Moreover, native oral mucosal tissue was examined the histology and marker expression as well. Oral mucosa showed distinct thicker epithelium than ocular epithelium underlined with lamina propria (Figure 4.4A). Immunohistochemical staining showed that p63 expressed in basal layer (Figure 4.4B) and K3 was in suprabasal layer (Figure 4.4C). Cx43-positive cells were also found in oral mucosal epithelium scattered throughout suprabasal layer (Figure 4.4D).

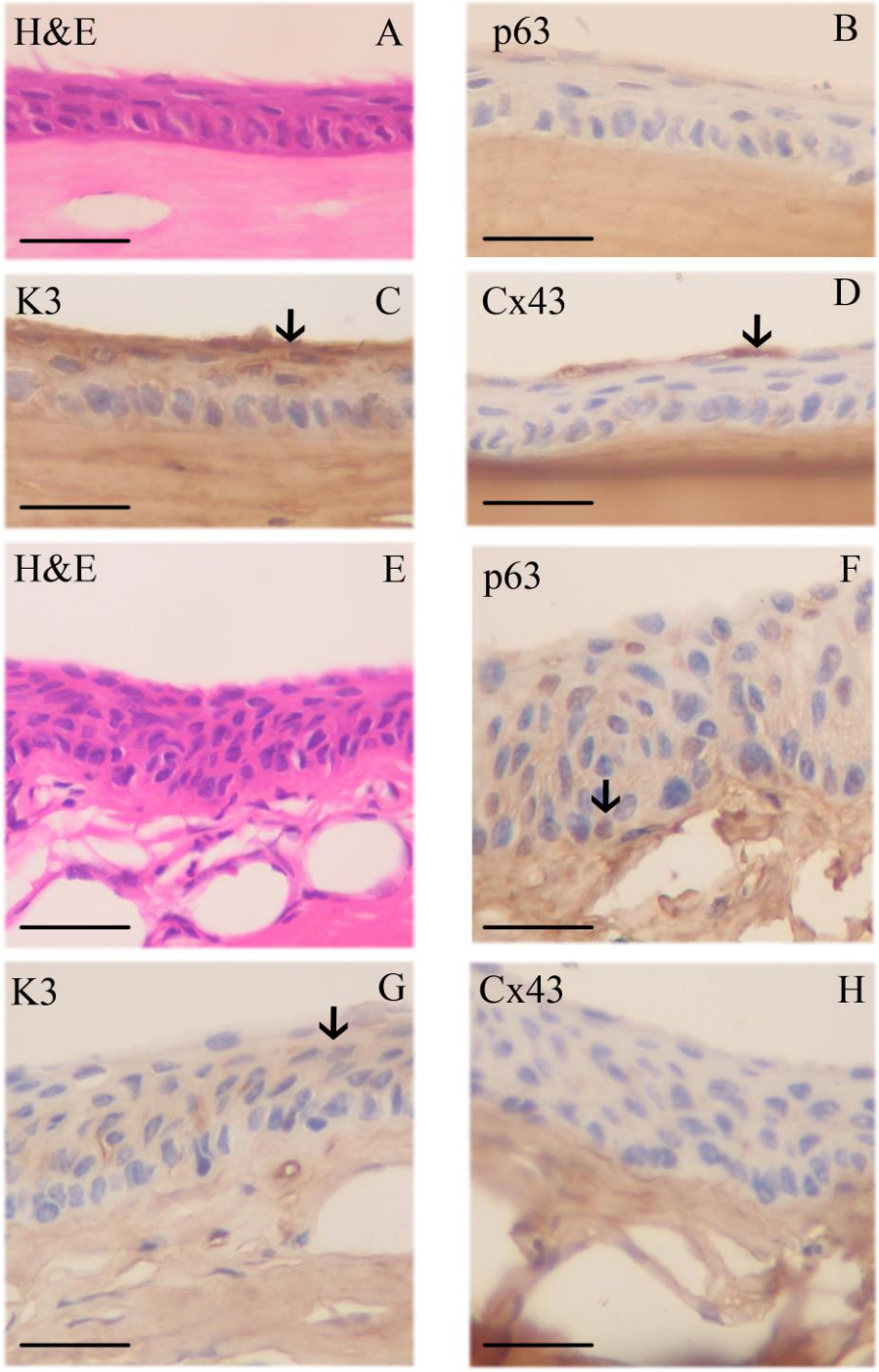

Figure 4.3 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from normal eye which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining. The arrowhead indicated blood vessels. Scale bars = 50 µm.

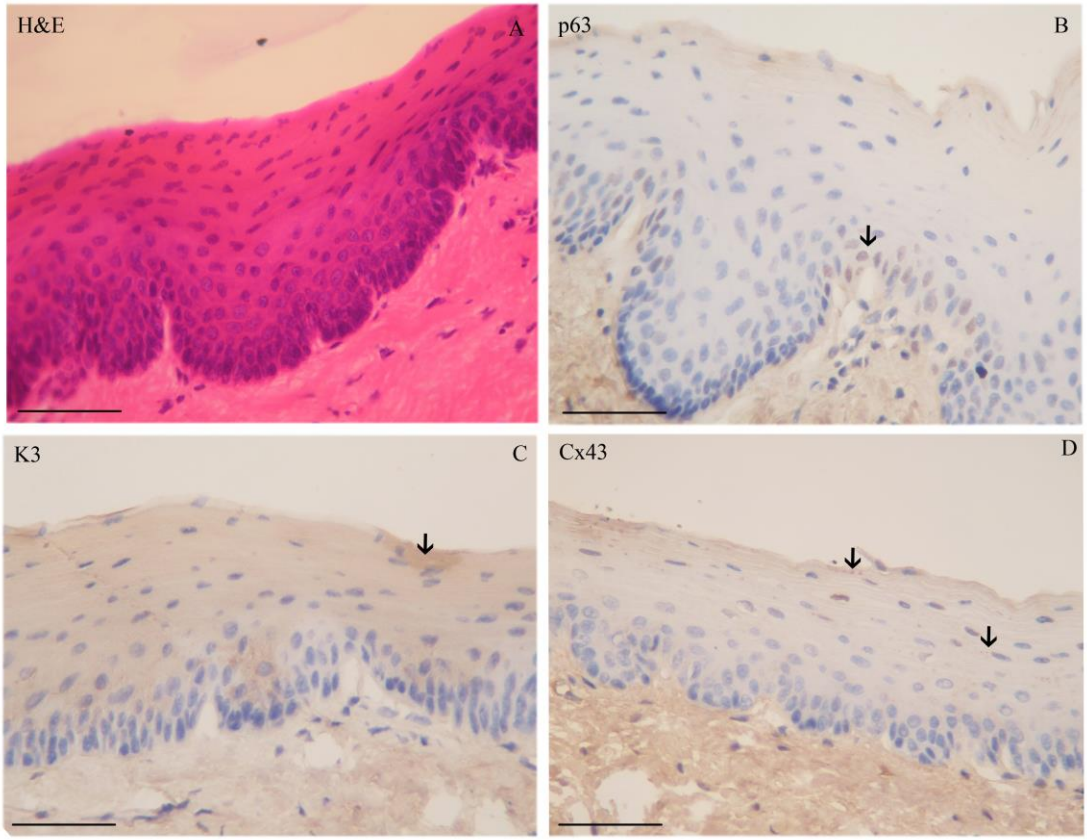

Figure 4.4 Rabbit normal oral mucosal epithelium stained with hematoxylin-eosin (A), immunohistochemistry for p63 (B), K3 (C) and Cx43 (D). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

Histology of all transplanted eyes tended to present in similar pattern with normal eye (Figure 4.5A-4.10A and 4.5E-4.10E) but some characters including goblet cells in limbal region (Figure 4.7F and 4.8F), corneal epithelial thickening (Figure 4.8B) and corneal stromal vascularization (Figure 4.11) were observed and summarized in Table 4.2.

Phenotypic characterizations of transplanted ocular surface were also examined. P63 was strongly expressed in intermediate and basal cells of limbal region of rabbit No. 1, 2, 3, 5 (Figure 4.5F, 4.6F, 4.7F and 4.9F) but absent in rabbit No. 4, 6 (Figure 4.8F, 4.10F). In corneal epithelium, p63-positive cells were found in intermediate and basal layers of all rabbits excepted rabbit No. 5 (Figure 4.5B-4.10B). Expression of K3 in limbal epithelium showed weakly stained in superficial and intermediate layers of rabbit No. 2, 5, 6 (Figure 4.6G, 4.9G and 4.10G) while the rest showed negative staining (Figure 4.5G, 4.7G and 4.8G). Strongly positive of K3 was presented in superficial and intermediate layers of corneal epithelium of all rabbits (Figure 4.5C-4.10C). In the limbus, weak staining of Cx43 was observed in superficial layer of all rabbits (Figure 4.5H-4.10H). In the cornea, Cx43-positive cells were confined to superficial layer (Figure 4.5D-4.7D, 4.9D and 4.10D) excepted rabbit No. 4 that showed scattered staining throughout the epithelium (Figure 4.8D). The markers expressions were summarized in Table 4.2.

Table 4.2 Markers expressions and histological observation of transplanted eyes.

Rabbits	p63		K3		Cx43		Goblet cells		Epithelial thickening		Corneal stromal vascularization
	Limbal epithelium	Corneal epithelium	Limbal epithelium	Corneal epithelium	Limbal epithelium	Corneal epithelium	Limbal epithelium	Corneal epithelium	Limbal epithelium	Corneal epithelium	
1	-/++	-/+	-	++/-	+#	+#	-	-	-	-	-
2	-/++	-/++	+/-	+/-	+#	+#	-	-	-	-	-
3	-/+	-/+	-	++/-	+#	+#	+	-	-	-	-
4	-	-/+	-	++/-	+#	+*	+	-	-	+	+
5	-/++	-	+/-	++/-	+#	+#	-	-	-	-	-
6	-	-/++	+/-	++/-	+#	+#	-	-	-	-	-

+ indicates present; - indicates absent; ++/- indicates strongly positive in superficial and intermediate layers; +/- indicates weakly positive in superficial and intermediate layers; -/++ indicates strongly positive in intermediate and basal layers; -/+ indicates weakly positive in intermediate and basal layers; +* indicates positive scattered staining throughout the epithelium; +# indicates positive in superficial layer

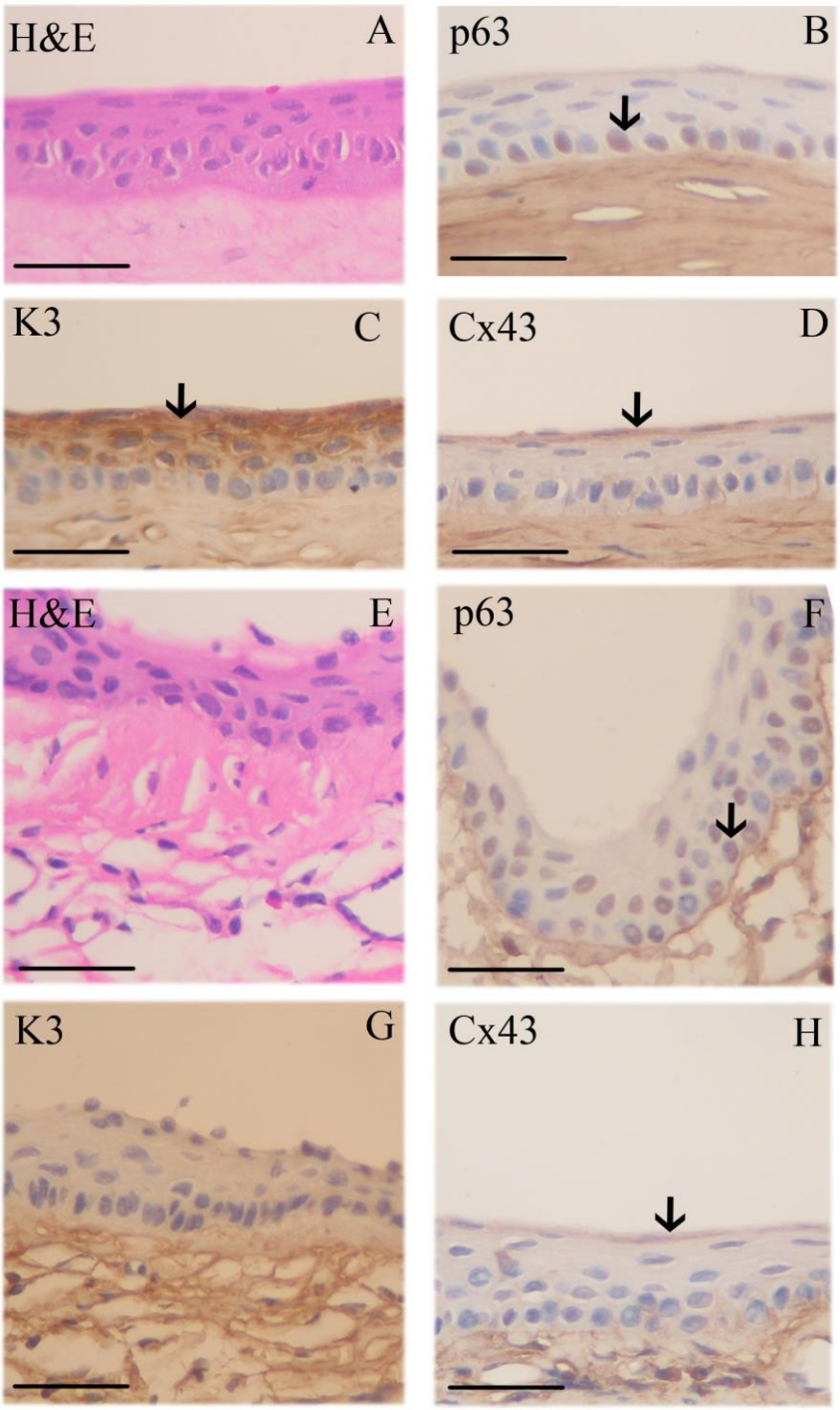

Figure 4.5 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 1 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

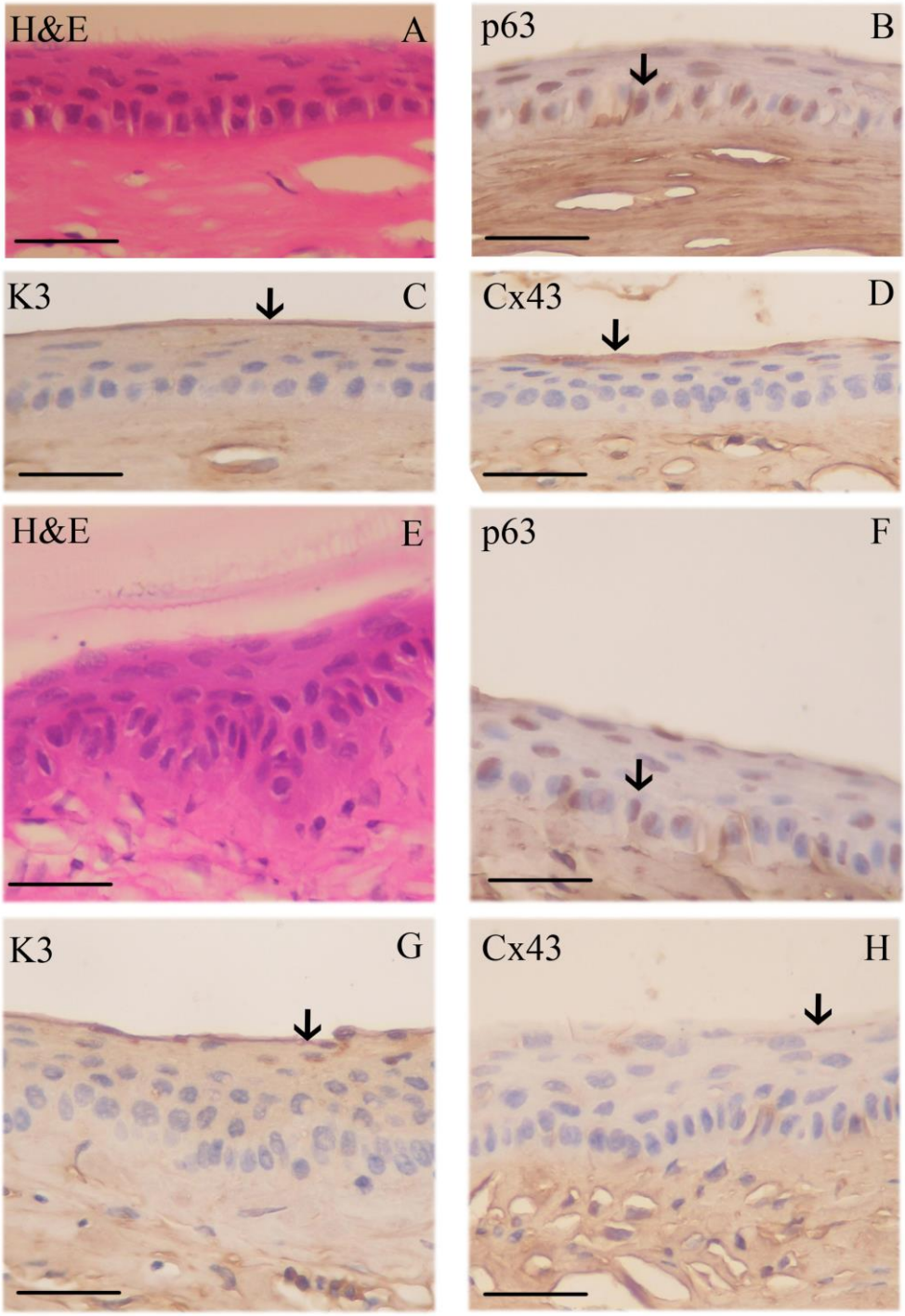

Figure 4.6 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 2 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

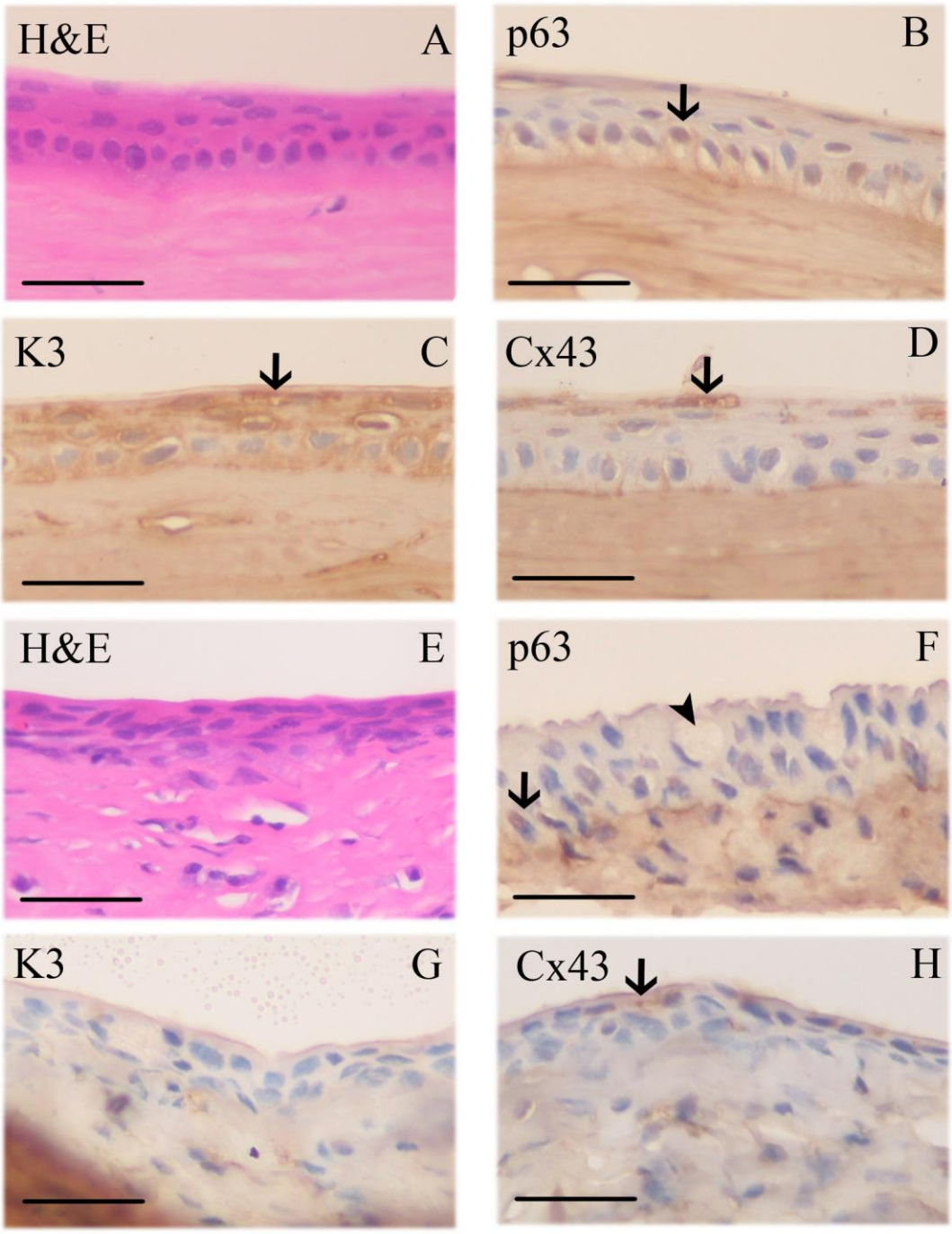

Figure 4.7 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 3 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining and arrowhead indicated goblet cell. Scale bars = 50 μm.

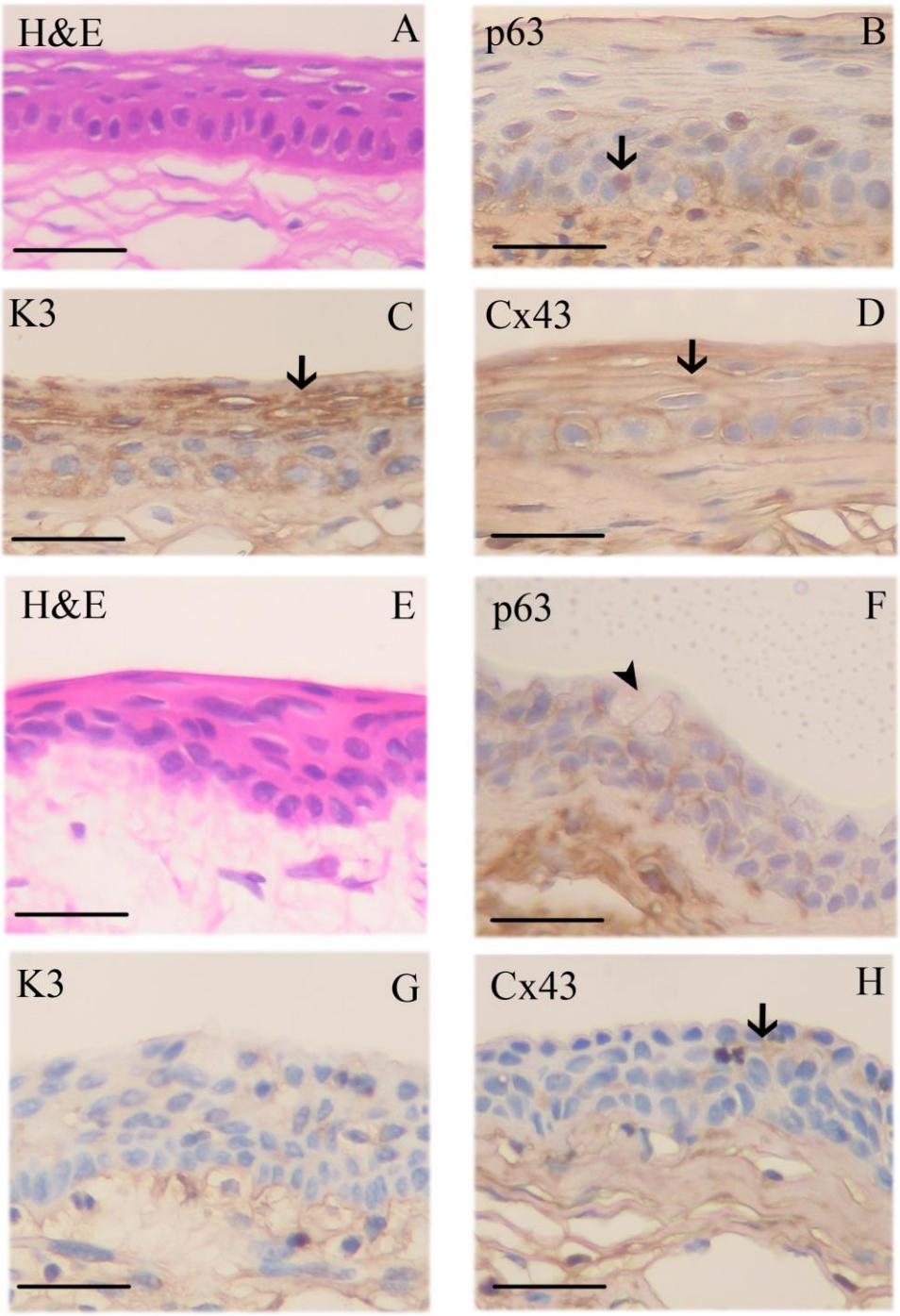

Figure 4.8 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 4 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining and arrowhead indicated goblet cell. Scale bars = 50 μm.

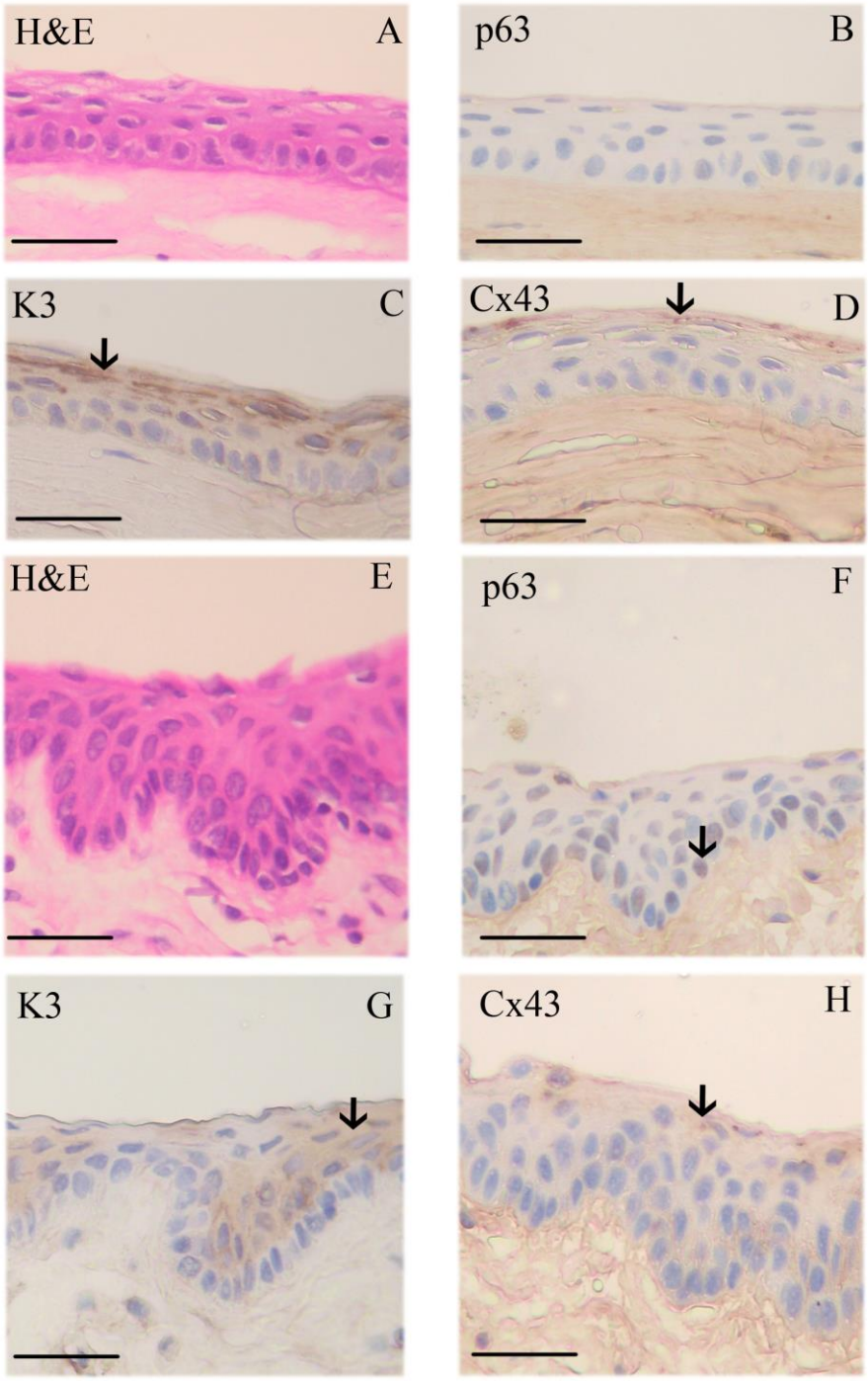

Figure 4.9 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 5 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

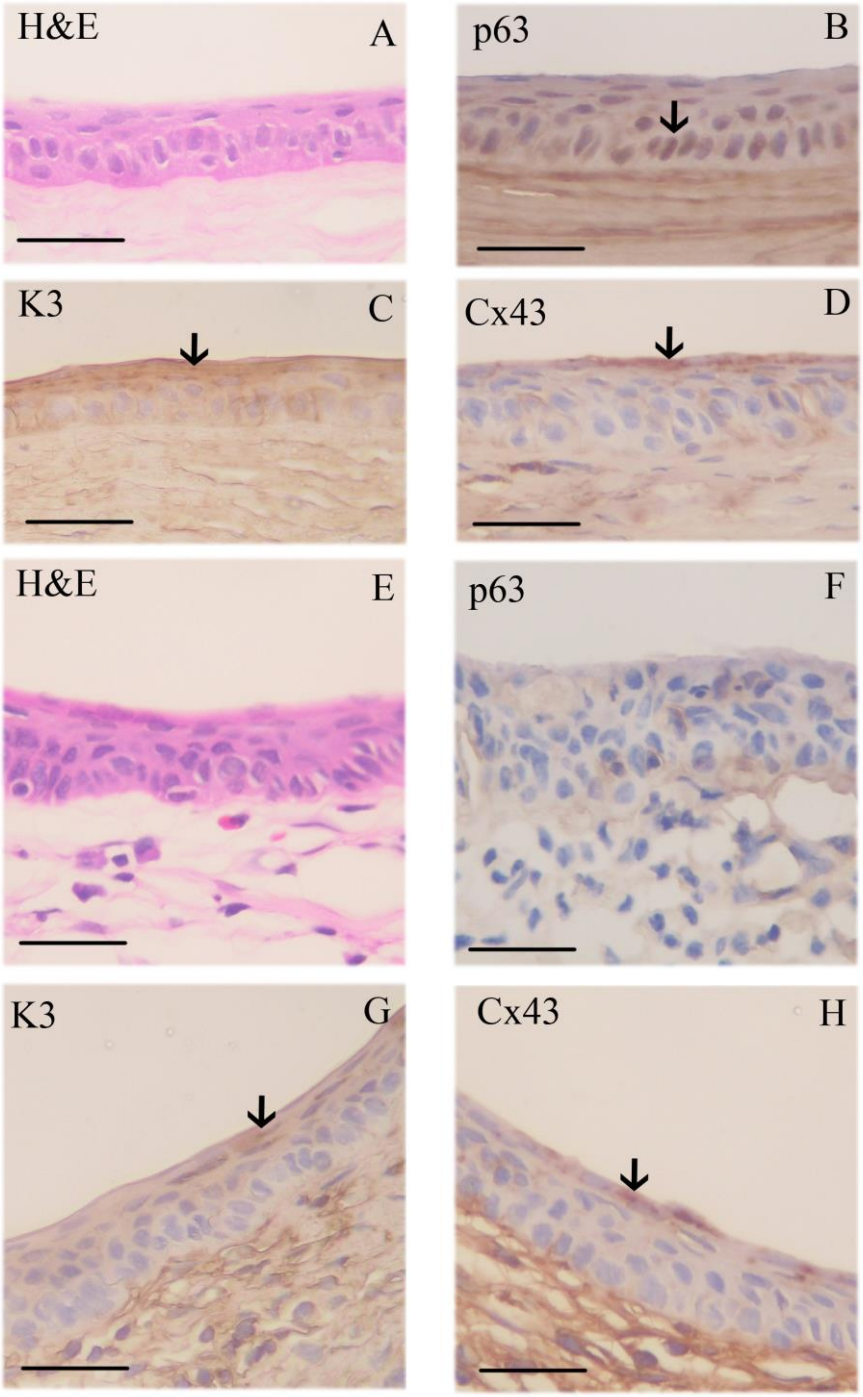

Figure 4.10 A-D represented corneal epithelial tissues and E-H represented limbal epithelial tissues from eye of rabbit No. 6 which were stained by hematoxylin-eosin staining (A, E), immunohistochemical staining of p63 (B, F), K3 (C, G) and Cx43 (D, H). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

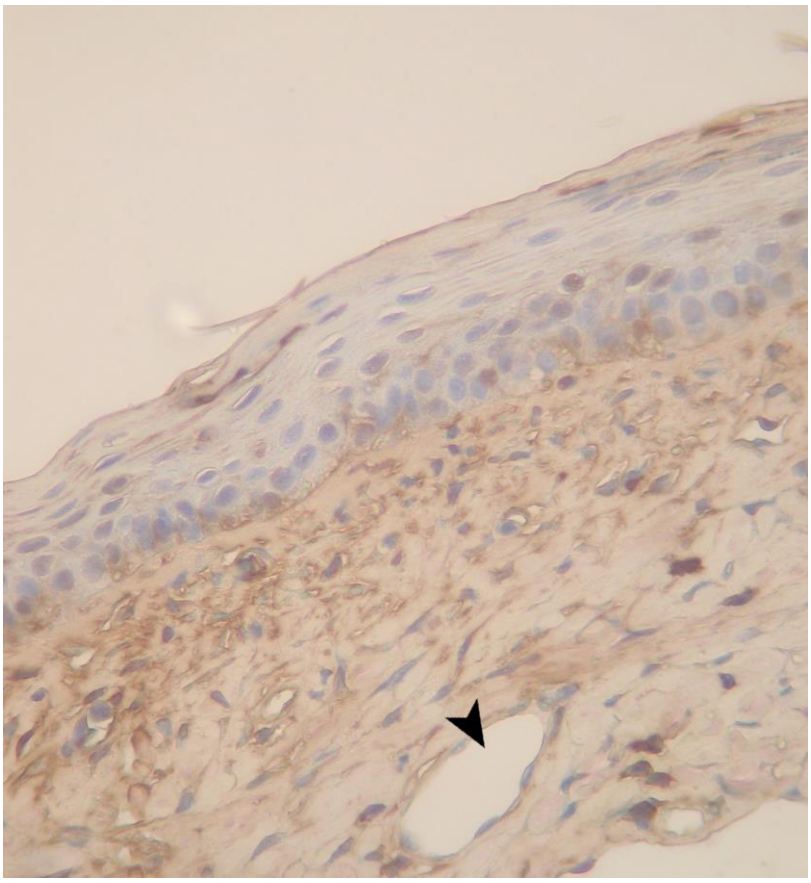

Figure 4.11 Vascularization in corneal stroma of rabbit No. 4. The arrowhead indicated blood vessel.

Selection of the vitrification formula

There were three vitrification formulas including (1) 10% DMSO, (2) 25% glycerol, 25% propylene glycol and (3) 25% DMSO, 25% propylene glycol. During freezing, the second and the third vitrification solutions were transparent suggested that there were no ice crystal formation while the first was opaque. Thawing and cultivation were performed after 1 week of cryopreservation, the best outgrowth was found in tissue that preserved by using the third formula while slower growth in the first formula and no growth in the second formula. So the third formula was used in cryopreservation in this study.

Cultivation of cryopreserved limbal and oral mucosal tissues

After two months of cryopreservation, the tissues were thawed and cultured. All cryopreserved tissue biopsies were able to form the epithelial sheets within three weeks and had similar morphology to the fresh tissue cultures. The epithelia composed of two to five layers and no goblet cells were observed. Cuboidal cells were seen in basal layer whereas in the superficial layer, the cells were flattened (Figure 4.12A, E, I and 4.13A, E, I). Consistent with fresh tissue cultures, the morphology of cultured epithelial sheets from cryopreserved limbus and cryopreserved oral mucosal tissues were similar.

Immunohistochemical examination of three markers showed positive staining in all cultures. P63 were positive in basal and intermediated cell layers (Figure 4.12B, F, J and 4.13B, F, J). K3 positive cells located in superficial and intermediate layers (Figure 4.12 C, G, K and 4.13C, G, K). However, the expression of K3 showed weaker staining in cryopreserved oral mucosal tissue cultures than cryopreserved

limbal tissue cultures. Cx43 were scattered staining throughout the epithelium (Figure 4.12D, H, L and 4.13D, H, L). The expressions of all markers in all epithelial sheets were summarized in table 4.3.

Table 4.3 Immunohistochemical examination of marker expression in all epithelial sheets from cryopreserved tissue cultures.

Rabbits number		Markers		
		p63	K3	Cx43
Cryopreserved limbal tissues	1	-/++	++/-	+*
	2	-/+	++/-	+*
	3	-/++	++/-	+*
Cryopreserved oral mucosal tissues	4	-/+	+/-	+*
	5	-/+	+/-	+*
	6	-/++	++/-	+*

+ indicates positive; - indicates negative; ++/- indicates strongly positive in superficial and intermediate layers; +/- indicates weakly positive in superficial and intermediate layers; -/++ indicates strongly positive in intermediate and basal layers; -/+ indicates weakly positive in intermediate and basal layers; +* indicates scattered staining throughout the epithelium

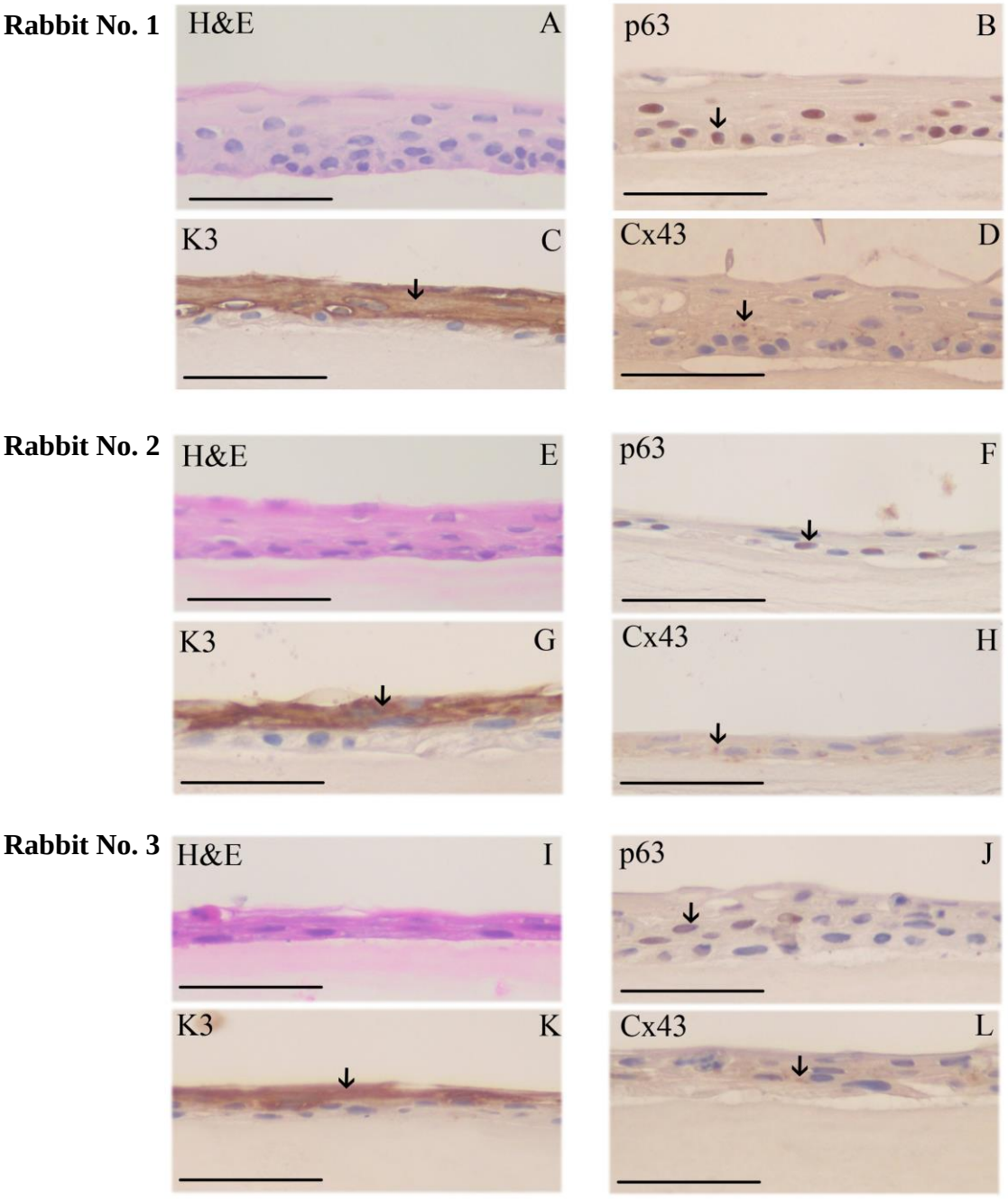

Figure 4.12 Cultured epithelial sheets generated from cryopreserved limbal biopsies of rabbit No. 1 (A-D), rabbit No. 2 (E-H) and rabbit No. 3 (I-L). Represent hematoxylin-eosin staining (A, E, I), immunohistochemical staining for p63 (B, F, J), K3 (C, G, K) and Cx43 (D, H, L). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 μm.

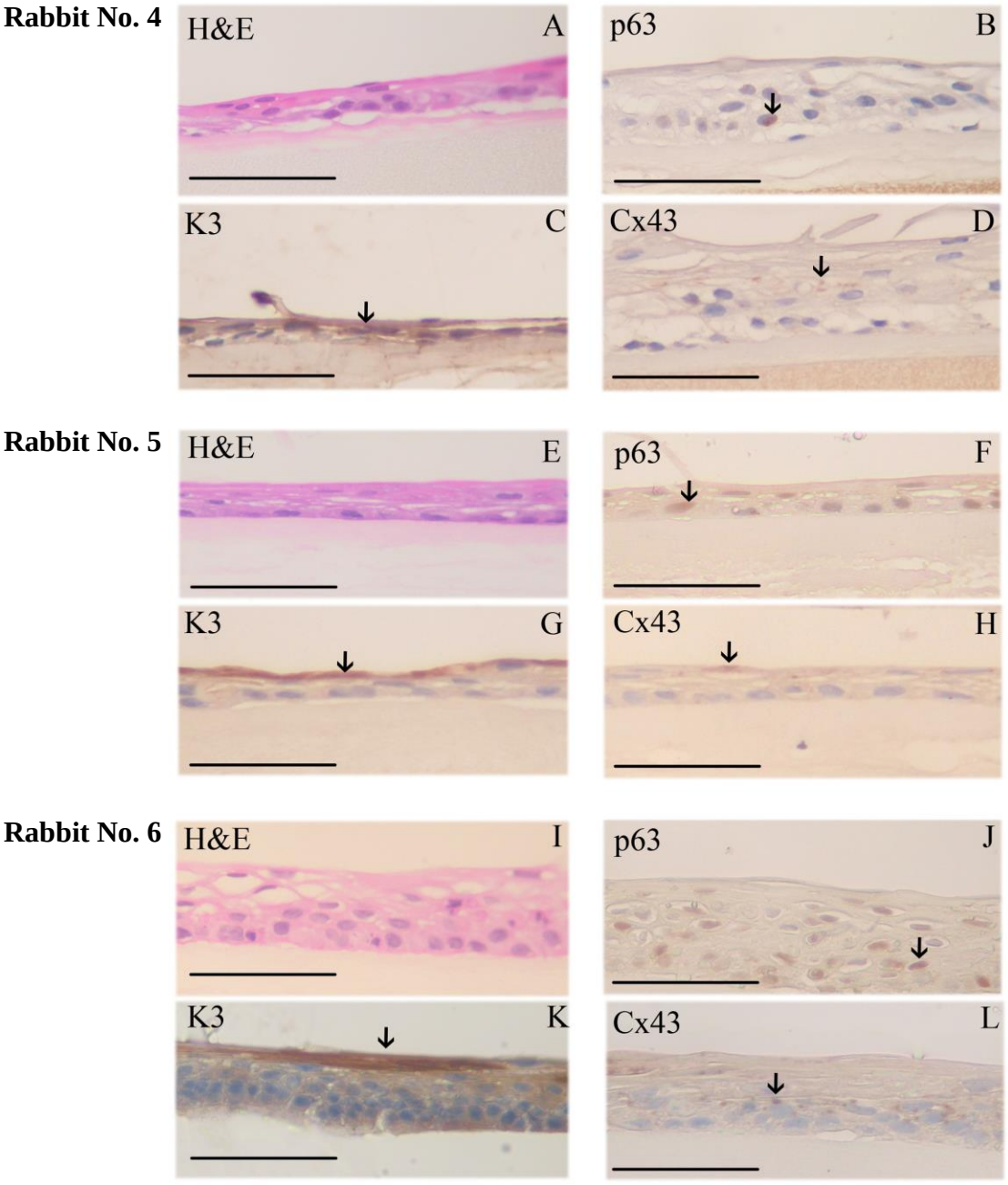

Figure 4.13 Cultured epithelial sheets generated from cryopreserved oral mucosal biopsies of rabbit No. 4 (A-D), rabbit No. 5 (E-H) and rabbit No. 6 (I-L). Represent hematoxylin-eosin staining (A, E, I), immunohistochemical staining for p63 (B, F, J), K3 (C, G, K) and Cx43 (D, H, L). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.

Cultivation of cryopreserved cultured cells from limbal and oral mucosal tissues

After two months of cryopreservation, the cells were thawed and cultured. A small amount of cell suspension was used for counting the cell viability using trypan blue exclusion test. The result showed that mean percentage of cell viability from cryopreserved cultured cells from limbal and oral mucosal tissues were 59.99 ± 5.89% and 70.57 ± 14.71% respectively. After cell culture process, only one culture from limbal specimen of rabbit No. 2 was able to generate epithelial sheet. The culture sheet composed of approximately 7 layers with cuboidal basal cells and flatted superficial cells (Figure 4.14A). Immunohistochemical study of p63 showed positive staining in cells scattered in basal and intermediated layers (Figure 4.14B). Weak staining of K3 was observed throughout the epithelium (Figure 4.14C). Cx43 positive staining were mainly in superficial cells (Figure 4.14D).

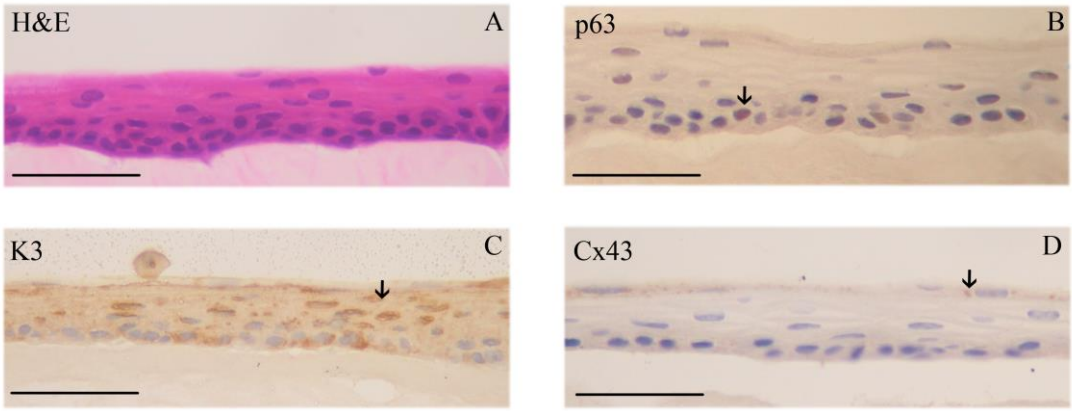

Figure 4.14 Epithelial sheet generated from cryopreserved cultured cells of rabbit No. 2. Represent hematoxylin-eosin staining (A), immunohistochemical staining for p63 (B), K3 (C) and Cx43 (D). The arrows indicated positive cells for immunohistochemical staining. Scale bars = 50 µm.