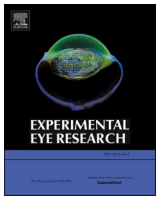
Experimental Eye Research 218 (2022) 108985

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer



Short communication

# Impact of the transcription factor IRF8 on limbal epithelial progenitor cells in a mouse model

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ARTICLE INFO

*Keywords:*
Cornea
Corneal epithelium
Limbus
Stem cells
Stem cell marker
IRF8

ABSTRACT

The limbus of the eye is the location of the corneal epithelial stem cell niche. These cells are necessary for continuous renewal of the corneal epithelium. In the case of limbal stem cell deficiency, these cells are damaged, and the whole cornea becomes opaque. It is important to be able to identify stem cells that could be applied for new therapeutic strategies. There are various known markers to characterize these cells, including p63, Nanog, oct4 and FGFR2. However, none of these markers are exclusively expressed in these stem cells (they are also expressed in transient amplified cells). It seems likely that a combination of stem cell markers will be necessary for corneal stem cell identification. The aim of this study was to detect IRF8 in limbal epithelial stem cells and to determine its function. In a mouse model, IRF8 could be detected in limbal and basal epithelial cells of the cornea by histological and immunohistological staining of wild-type mouse eyes. Furthermore, the limbus of the eye was significantly smaller in IRF8-knockout mice than in wild-type mice, and the expression of Nanog was lower in IRF8-knockout mice. This suggests that IRF8 has an influence on the maintenance of stem cell properties in the limbus, possibly by affecting the expression of Nanog. Furthermore, IRF8 has an impact on E-cadherin and N-cadherin expression in the mouse eye.

The transcription factor interferon-regulatory factor 8 (IRF8), also known as interferon consensus sequence-binding protein (ICSBP), was first described in 1990 and belongs to the IRF family. The expression of the 50-kDa IRF8 protein is upregulated by interferon-γ (IFN-γ). With its N-terminal DNA-binding domain, IRF8 binds to a specific DNA sequence called the interferon-stimulated response element (ISRE), which is located at the promoter region of the MHC I gene. IRF8 can interact with other transcription factors and influence the translation of genes. IRF8 is mainly found in the cell nucleus and is present at lower concentrations in the cytoplasm (Driggers et al., 1990; Nelson et al., 1993; Tamura et al., 2008).

The expression of IRF8 is mainly described in various haematopoietic cells, where its functions vary. In recent years, IRF8 expression has also been demonstrated outside of the haematopoietic system for example in the retina and the lens (Kim et al., 2015; Li et al., 1999). To date, nothing is known about its expression in the cornea or limbus.

The limbus, the location of the stem cell niche, is defined as the transition zone of the corneal stroma into the sclera and the corneal epithelium into the conjunctiva. This transition zone has a length of 1.0–1.5 mm in humans and can be divided into anterior (bluish) and posterior (white) parts. The limbus is the origin of epithelial stem cells and protects the stem cells from various harmful internal and external influences. The niche also protects stem cells from differentiating into corneal epithelial cells (Tseng, 1996; Watt and Hogan, 2000). Damage to limbal epithelial stem cells leads to limbal stem cell deficiency syndrome, a severe disease that frequently leads to severe visual impairment of the affected eye.

It is therefore important to determine the location of stem cells to develop better therapeutic options. Different markers for stem cell identification have already been established. These include cytokeratin 14 (Kasper et al., 1988), p63 (Pellegrini et al., 2001), FGFR2 (De Iongh et al., 1997), N-cadherin (Hayashi et al., 2006; Higa et al., 2009), Oct4 and Nanog (Luo et al., 2013; Pauklin et al., 2011; Zhou et al., 2010).

However, it is not yet possible to completely differentiate between limbal epithelial stem cells and progenitor cells. To date, a combination of multiple stem cell markers is considered the best way to identify limbal epithelial stem cells (Schlötzer-Schrehardt and Kruse, 2005).

The aim of this study was to detect IRF8 in the cornea and limbus and

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https://doi.org/10.1016/j.exer.2022.108985
Received 25 October 2021; Received in revised form 7 February 2022; Accepted 10 February 2022
Available online 25 February 2022

**Abbreviations**

| | |
|---|---|
| IRF8 | interferon-regulatory factor 8 |
| FGFR2 | Fibroblast growth factor receptor 2 |
| ICSBP | interferon consensus sequence-binding protein |
| IFN-γ | interferon-γ |
| DNA | deoxyribonucleic acid |
| ISRE | interferon-stimulated response element |
| mm | milimeter |
| CK14 | Cytokeratin 14 |
| IRF8$^{-/-}$ | interferon-regulatory factor 8 knock out |
| μm | micrometer |
| PAS | Periodic acid–Schiff staining |
| WT | wild type |
| Fig | figure |
| IHC | immunohistochemistry |
| sd | standard deviation |
| av | average |
| TGF-β | Transforming growth factor beta |
| EMT | epithelial-mesenchymal transition |

to examine the impact of IRF8 knockout in mouse eyes.

The experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The mouse eyes were provided by the Department for Haematology and Oncology of the University Hospital Halle (Salle). The mice, which were 8–12 weeks old, were killed as part of another experimental project that is not related to this project. Studies on mouse eyes were carried out on wild-type (C57BL/6) and IRF8 knockout (IRF8$^{-/-}$) mice (C57BL/6) (Holtschke et al., 1996). They were only killed for organ removal (killing application for these experiments number: AZK6IVM1). The eyes were fixed in 4% paraformaldehyde overnight at 4 °C and then embedded in paraffin according to standard protocols. Then, a microtome was used to make 4 μm sections of the eyes. After dewaxing, Periodic acid–Schiff (PAS) staining or immunohistochemical staining was performed according to standard protocols. During immunohistochemical staining, heat-induced antigen retrieval was performed with EDTA buffer. The primary antibodies used were against FGFR2 (abcam ab10648), p63 (abcam ab124762), Nanog (abcam ab80892), N-cadherin (abcam ab76011), E-cadherin (abcam ab76055), Oct4 (abcam ab19857), IRF8 (thermo fisher PA5-20088), ICSBP (santa cruz sc-365042) and CK14 (abcam ab181595). After staining, different images were taken with a Nikon Eclipse 80i fluorescence microscope and digitally recorded with the associated Nikon DS-Fi3 colour camera system. Subsequent image processing was carried out with the ImageJ program. The statistical evaluation was carried out using Microsoft Excel version 2010 and using the *t*-test.

Regarding the expression of IRF8, a total of 37 stains on seven different WT mouse eyes were evaluated. As a negative control, immunohistochemical staining of IRF8$^{-/-}$ mouse eyes was performed. The retinal photoreceptors and the microglial cells of the ganglion cell layer served as a positive control for the antibody (Kim et al., 2015).

In the area of the central cornea, IRF8 was expressed primarily in basal epithelial cell nuclei. Cells that are located more superficially expressed IRF8 (Fig. 1: IHC on a WT mouse eye A-C). Expression increased in the direction of the limbus, and superficial cells were also increasingly involved. The cytoplasm also showed weak expression of IRF8. Stroma fibroblasts and corneal endothelial cells expressed the transcription factor, whereas basal cells in the limbal region of the cornea showed the strongest expression, and superficial cells show weaker expression (Fig. 1: IHC on a WT mouse eye D-F). In the ciliary body, a few cell nuclei were positive for IRF8. In the iris, no IRF8 could be detected. IRF8 was also expressed in the cell nuclei of equatorial lens fibres. Retinal ganglion cells in the ganglion cell layer and the outer segments of the photoreceptors of the retina expressed IRF8. The cells with the strongest expression were photoreceptors. The retinal pigment epithelium was also positive for IRF8 (Fig. 1G–I). In IRF8$^{-/-}$ mice, no expression of IRF8 could be detected.

No differences could be detected in the expression of the stem cell markers p63, Oct4, CK14 and FGFR2 in WT and IRF8$^{-/-}$ mice. Nanog expression was significantly weaker in the basal epithelial cells of the limbus and the cornea in IRF8$^{-/-}$ mice than in those of WT mice.

In the cornea of WT mouse eyes, particularly in the central corneal epithelium, there was strong expression of E-cadherin in the cell membrane (Fig. 2A–C). In comparison, staining intensity was weaker in IRF8$^{-/-}$ mouse eyes than in the WT eyes and was stronger in superficial cells than in basal cells (Fig. 2D–F). In WT eyes, the loss of E-cadherin expression in the peripheral cornea was detectable (Fig. 2M–O). In this area, expression was only observed in the most superficial cells of the corneal epithelium. In the area of the limbus, the expression of E-cadherin was confirmed in all layers of WT and IRF8$^{-/-}$ mouse eyes (Fig. 2).

N-cadherin was only expressed in the apical pole of the basal cells of the peripheral cornea (Fig. 2P–R). In IRF8$^{-/-}$ mouse eyes, N-cadherin expression was detected throughout the entire cornea (Fig. 2D–F); in WT eyes, it was mainly expressed in the peripheral cornea, which corresponds to the area where E-cadherin can only be detected in superficial cells. The central cornea showed only low expression of N-cadherin in WT mouse eyes (Fig. 2A–C). In the area of the limbus, there was no N-cadherin expression in the epithelium in either the WT or IRF8$^{-/-}$ mouse eyes (Fig. 2G–L).

The images of the PAS stains were used to determine the size of the limbus region. In these images, the distance between the end of the Bowman layer and the end of the Schwalbe line (anterior limbus) and the distance between the Schwalbe line and the iris root (posterior limbus) were calculated to determine the size of the limbus region. This procedure was carried out on four WT and three IRF8$^{-/-}$ mouse eyes. A total of 97 PAS-stained WT mouse eye samples and 57 PAS-stained IRF8$^{-/-}$ mouse eye samples were evaluated. The significance was determined by means of a *t*-test.

When measuring the limbus size of the WT mouse eyes, an average (av) limbus size of 163.78 μm (standard deviation (sd) ±21.29) was obtained. The anterior limbus (av 101.25 μm, SD ± 17.51) was larger than the posterior limbus (av = 62.94 μm, SD ± 38.19). The limbus region of the IRF8$^{-/-}$ mouse eyes was significantly smaller than that of WT mouse eyes ($p = 2.78 \times 10–34$). The total limbus size of the IRF8$^{-/-}$ mouse eye was on average only 116.13 μm (SD ± 7.21). In these eyes, the anterior limbus (av 77.94 μm, SD ± 7.5) was also larger than the posterior limbus (av 38.19 μm, SD ± 5.1).

Cytokeratin 14 was examined as another size marker of the limbus region. The images of the limbal region of the immunohistochemical staining with the antibody against CK14 were evaluated. The length measured from the iris root to the end of CK14 expression was measured. These results were compared with the results from PAS staining to determine whether CK14 is suitable as a size marker for the limbal region of the mouse eye. A total of 23 IHC preparations with CK14 staining were measured on the WT and 20 IHC preparations with CK14 staining on the IRF8$^{-/-}$ mouse eyes. This results in an average size of 158.43 μm (SD ± 9.80) for the CK14-positive region in the WT mouse eyes. The difference between the averages of the limbus size for the PAS and CK14 staining procedures was only 5.35 μm and was not significant ($p = 0.12$). In IRF8$^{-/-}$ mouse eyes, the CK14-positive region averaged 111.24 μm (SD ± 11.88), which was not significantly different from the PAS measurement ($p = 0.47$). The difference between the averages of the CK-14-positive region of WT versus IRF8$^{-/-}$ mouse eyes was highly significant according to PAS staining ($p = 8.46 \times 10–18$).

Notably, there are no studies on the expression of IRF8 or its influence on limbal cells or corneal cells. This is the reason why other organ systems, especially the haematopoietic system, have to be used as a model. However, it is unclear whether the same signalling pathways that

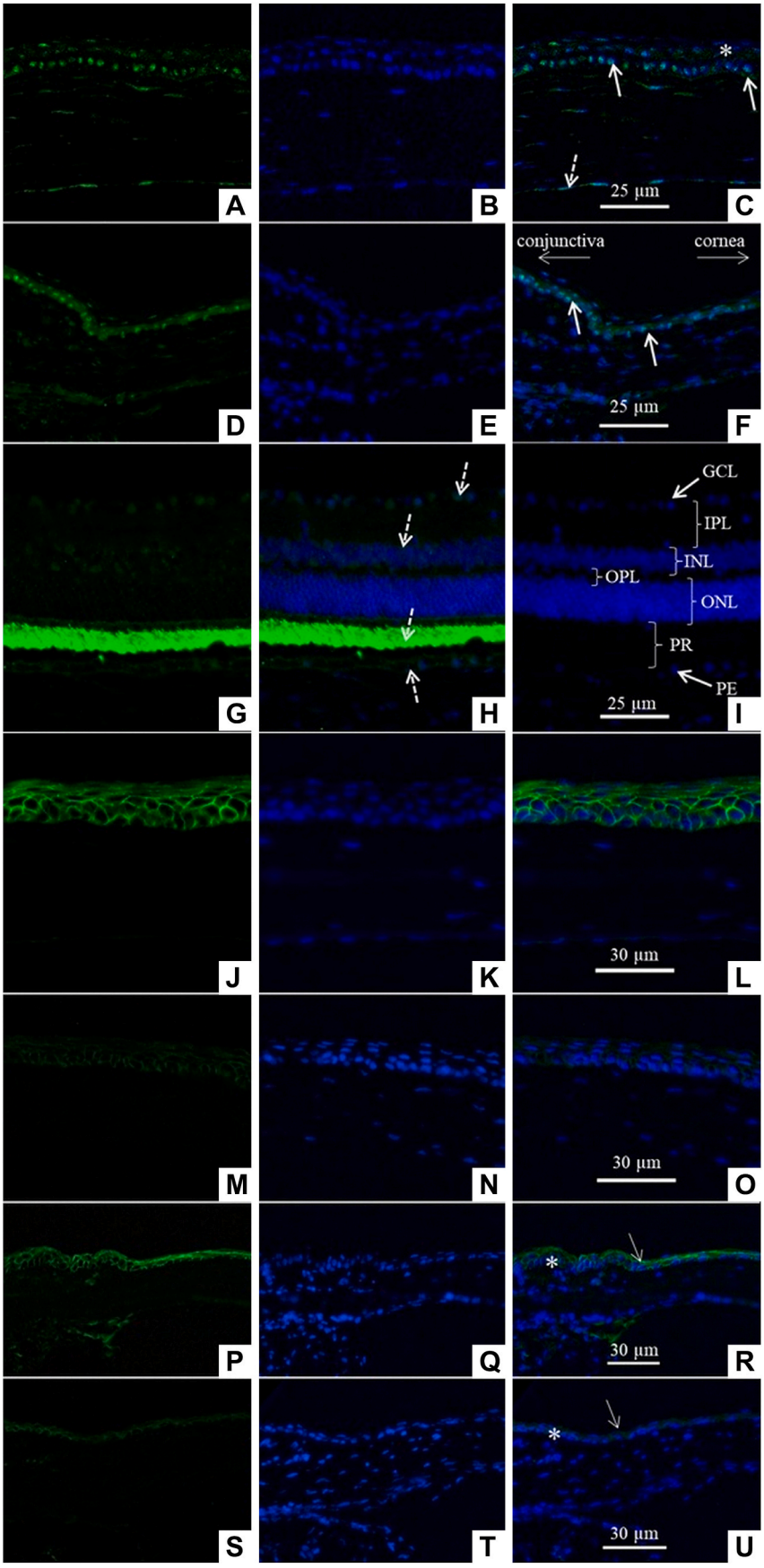


**Fig. 1.** A–C: Representative IRF8 expression in the central cornea, which is mainly in the basal cells of the epithelium; D–F: representative IRF8 expression in the limbus, where the expression becomes focused in the cytoplasm; G–I: representative IRF8 expression in the retina, especially in the GCL, INL, PE and PR.
J–
L: Central cornea of a WT mouse shows consequent E-cadherin expression in all layers; M–
O: central cornea of an IRF8$^{-/-}$ mouse shows weaker expression of E-cadherin in comparison to the WT; P–R: limbus of a WT mouse shows consequent E-cadherin expression; S–U: the limbus of an IRF8$^{-/-}$ mouse shows weaker expression of E-cadherin; green in A–I: IRF8; green in J–U: E-cadherin expression; blue: nucleus; arrow in J–U: limbus; star in R + U: conjunctiva.
Arrow in A–
I: IRF8-positive cells; dashed arrow: IRF8-positive cells in the retina; star in C: epithelium; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; PR: photoreceptor; PE: pigment epithelium.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

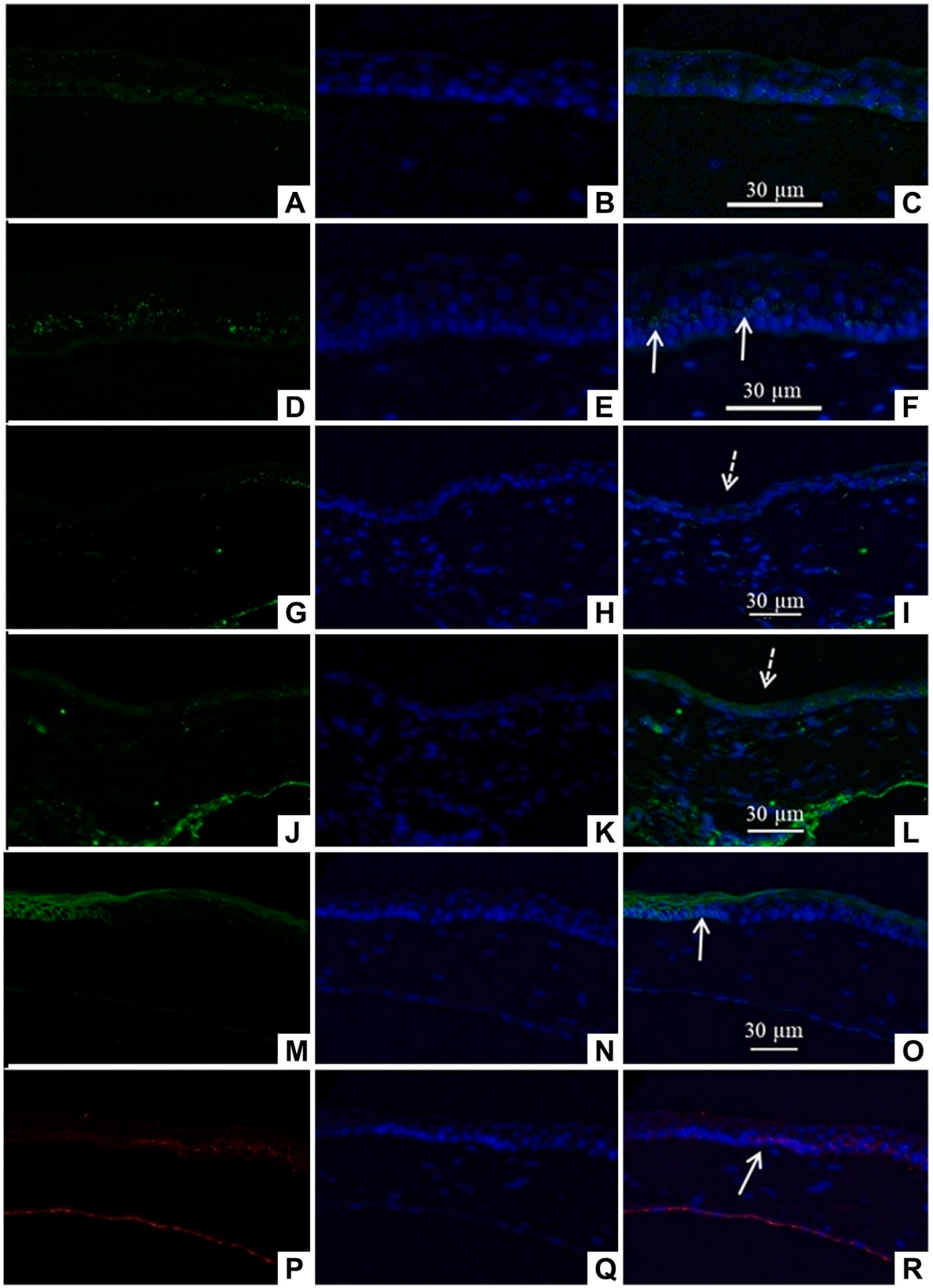


**Fig. 2.** A–C: The central cornea of a WT mouse shows only very weak N-cadherin expression at the top of the basal cells; D–F: the central cornea of an IRF8$^{-/-}$ mouse shows strong expression of N-cadherin in the apical pole of the basal cells; G–I: the limbus of a WT mouse shows no expression of N-cadherin, and the expression is lost before reaching the limbal region; J–L: the limbus of an IRF8$^{-/-}$ mouse shows no expression of N-cadherin; M–O: there is a gap in E-cadherin expression in the peripheral cornea of a WT mouse; P–R: in this gap, N-cadherin expression begins; green in A–L: N-cadherin; green in M–R: E-cadherin; red: N-cadherin; blue: nucleus; arrow in F: N-cadherin positive cells; dashed arrow: limbus; arrow in O + R: beginning of the E-cadherin-negative and N-cadherin region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

occur in model systems (for example, haematopoietic cells) also take place in limbal cells.

In 2015, IRF8-positive cells were first detected in the photoreceptors, ganglion cell layer and inner granular cell layer of the retina (Kim et al., 2015). In the context of this work, the transcription factor IRF8 was detected for the first time in limbal and basal corneal cells of the murine eye using IHC. The retina of the mouse eye, in which the expression of IRF8 in the photoreceptors and the microglial cells of the ganglion cell layer of the retina is known, served as a positive control for the antibody (Kim et al., 2015). The expression of IRF8 in these cells was proven by IHC performed on the mouse eye.

In mice that had received a transplantation of labelled haematopoietic stem cells, these cells could be detected in the stroma and epithelium of the cornea, which suggests migration of the haematopoietic stem cells. Since these cells have a high differentiation capacity, they can presumably transdifferentiate into corneal keratinocytes (Nakamura et al., 2005). It is possible that IRF8-expressing cells can identify these cells as originating from the bone marrow, since progenitor cells of monocytes, granulocytes and dendritic cells express IRF8 (Kurotaki et al., 2014; Wang et al., 2014). However, it is questionable whether this phenomenon only occurs in the state after transplantation of haematopoietic stem cells or is also present without transplantation. However, the results of this work may support the hypothesis that haematopoietic stem cells or progenitor cells migrate into the cornea, as these cells are known to be IRF8-positive.

The results of the immunohistochemical staining of the mouse eye support the hypothesis of the coexpression of IRF8 with Oct4, Nanog and FGFR2. The cells of the mouse eye that express IRF8 also showed Oct4, Nanog, CK14 and FGFR2 expression in the same region. These related cells were mainly limbal and basal corneal cells. The presence of IRF8 in stem cells and various progenitor cells has been described in the haematological system (Kurotaki et al., 2014; Wang et al., 2014). The fact that IRF8 is also expressed in the haematological system by progenitor cells indicates that this possibility also exists in limbal cells and that IRF8 is also expressed by progenitor cells of the cornea. This hypothesis was confirmed by immunohistochemical staining of IRF8 in the mouse eye. The expression of IRF8 is significantly stronger in the basal cells at the limbus of the mouse eye than in the basal cells of the central cornea. The basal cells of the cornea exhibit weaker expression, while the superficial cells of the cornea and limbus are negative for IRF8. This indicates that IRF8 is not a unique stem cell marker because it is also expressed by progenitor cells in the basal cells of the central cornea.

In the haematopoietic system, IRF8 is involved in determining the fate of immune cells and differentiation (Scharton-Kersten et al., 1997). Transferred to the results of this work, this can mean that IRF8 may also be important in determining the fate and differentiation of limbal epithelial cells. Through its function as a transcription factor, it can, for example, influence the regulation of genes that are important for differentiation and thus maintain the characteristics of stem cells. Investigations on the measurement of the limbal region provide further evidence of this. The limbal region of IRF8$^{-/-}$ mouse eyes is significantly smaller than the limbus region of WT mouse eyes (p value = 2.78 × 10–34). This means that the absence of IRF8 has a negative effect on the size of the limbus. Thus, it can be deduced that fewer stem and progenitor cells are present in IRF8$^{-/-}$ eyes. Another interesting point is that CK14 seems to be a good marker for the size of the limbus in a mouse model because there were no significant differences between the sizes of the limbus according to PAS staining versus CK14 staining.

The results from the immunohistochemical staining showed that there were no differences in the expression distribution of the stem cell markers Oct4, p63 and FGFR2 in the IRF8$^{-/-}$ mouse eye compared to the WT mouse eye. This means that IRF8 probably has no influence on the expression of these markers. However, the studies show differences with regard to Nanog expression, which is weaker in IRF8$^{-/-}$ eyes than in WT mouse eyes. This can mean that a lack of IRF8 leads to reduced expression of the pluripotency factor Nanog. This can have a negative impact on the proliferation and maintenance of the stem cell properties of limbal stem cells, since low levels of Nanog are associated with an increased tendency to differentiate (Kalmar et al., 2009; Luo et al., 2013). It is possible that this leads to a reduced number of limbal epithelial cells, which may also explain the smaller limbal region of the IRF8$^{-/-}$ mouse eyes.

The results for the expression of the stem cell markers CK14 and p63 were in accordance with the results of Guo et al., who described the expression differences in stem cell markers in the development of mice (Guo et al., 2020). It would be interesting to examine the expression of IRF8 in the cornea during mouse development and to determine whether there are similarities.

In addition, it is possible that IRF8 influences other signalling pathways that maintain stem cell properties in the limbus and counteract differentiation. An example of this is the TGF-β signalling pathway. In a leukaemia cell line, IRF8 was found to influence the TGF-β signalling pathway, which is also important for limbal stem cells (Joyce and Zieske, 1997; Nishida et al., 1995). Increased expression of IRF8 upregulates the expression of members of the TGF-β signalling pathway. This results in increased cell proliferation and cell migration (Sung et al., 2011, 2014). Since the TGF-β signalling pathway is also important in limbal cells (Watabe and Miyazono, 2008), the loss of IRF8 can lead to the loss of cells in the limbal region. The reduced proliferation and migration, as well as the reduced potential for maintaining stem cell properties, can result in a smaller limbus region in IRF8$^{-/-}$ mouse eyes, as proven in this work.

In the IHC experiments of cadherin expression in the IRF8$^{-/-}$ mouse eye, it is striking that N-cadherin expression was found throughout the entire epithelium of the cornea, whereas in WT mouse eye, it was only detected in the peripheral cornea. In addition, E-cadherin expression in the corneal epithelium was significantly weaker than that in WT mouse eyes. This indicates that a lack of IRF8 may lead to epithelial-mesenchymal transition (EMT). During EMT, there is a switch from E-cadherin to N-cadherin as the dominant marker (Gheldof and Berx, 2013; Nieman et al., 1999). This also seems to happen in IRF8$^{-/-}$ mouse eyes. β-Catenin is important for the induction of EMT and other processes (Cohen et al., 2015; Sánchez-Tilló et al., 2011). A relationship has already been confirmed between β-catenin and IRF8. On the one hand, β-catenin acts as an inducer of IRF8 during the pathogenesis of pterygium (Cohen et al., 2015), and on the other hand, IRF8 lowers β-catenin activity in myeloid cells (Huang et al., 2010). If IRF8 is no longer available due to knockout, the inhibition of β-catenin secretion can be eliminated, since IRF8 acts as a repressor. This can lead to increased β-catenin levels, which can result in EMT and may explain the cadherin switch. It is also possible that more β-catenin is secreted in an effort to increase the IRF8 level, but this is not possible due to the knockout. This can also lead to an increased β-catenin level and thus to EMT. This may indicate that IRF8 plays a role in EMT. Conversely, since the absence of IRF8 favours EMT or the change from E-cadherin to N-cadherin as the dominant marker, IRF8 counteracts EMT and maintains the epithelial characteristics of cells.

We were able to prove that IRF8 is expressed in the corneal epithelium, especially in the limbus. Unfortunately, IRF8 was not determined to be a unique stem cell marker, as it is also expressed in progenitor cells. Furthermore, we found that IRF8 probably influences the maintenance of stem cell properties and that knockout of the IRF8 gene decreased the size of the limbal region in a mouse model. Additionally, IRF8 influences the expression of E-cadherin and N-cadherin. Nevertheless, further investigations are necessary to prove our assumptions and to assess whether IRF8 is also expressed in the human limbus.

## Funding

This research was not supported by any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Author contributions

Christiane Kesper, Arne Viestenz, Cornelia Wiese-Rischke, Marina Scheller and Thomas Hammer designed the study; Christiane Kesper, Cornelia Wiese-Rischke and Marina Scheller performed the experiments; Christiane Kesper analysed the data and wrote the manuscript.

## Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

## Declarations of competing interest

None.

## Acknowledgements

The authors gratefully acknowledge Dr. Marina Scheller, who provided the mouse eyes for our investigations. Furthermore, we thankfully acknowledge the Department for ENT of the University Hospital Halle (Saale) as the owner of the Nikon Eclipse 80i fluorescence microscope and the associated Nikon DS-Fi3 colour camera system, which was used for documentation.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.108985.

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