Graefes Arch Clin Exp Ophthalmol (2015) 253:2187–2201
DOI 10.1007/s00417-015-3128-6

BASIC SCIENCE

Interplay of proliferation and differentiation factors is revealed in the early human eye development

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Received: 1 March 2015 / Revised: 20 July 2015 / Accepted: 28 July 2015 / Published online: 11 August 2015

Abstract
Background Eye development is a consequence of numerous epithelial-to-mesenchymal interactions between the prospective lens ectoderm, outpocketings of the forebrain forming optic vesicles, and surrounding mesenchyme. How different cell types forming eye structures differentiate from their precursors, and which factors coordinate complex human eye development remains largely unknown. Proper differentiation of photoreceptors is of special interest because of their involvement in the appearance of degenerative retinal diseases.
Methods Here we analyze the spatiotemporal expression of neuronal markers nestin, protein gene product 9.5 (PGP9.5), and calcium binding protein (S100), proliferation marker (Ki-67), markers for cilia (alpha-tubulin), and cell stemness marker octamer-binding transcription factor 4 (Oct-4) in histological sections of 5-12 -week human eyes using immunohistochemical and immunofluorescence methods.
Results While during the investigated developmental period nestin shows strong expression in all mesenchymal derivatives, lens, optic stalk and inner neuroblastic layer, PGP9.5 and S100 expression characterizes only neural derivatives (optic nerve and neural retina). PGP9.5 is co-localized with nestin and S100 in the differentiating cells of the inner neuroblastic layer. Initially strong proliferation in all parts of the developing eye gradually ceases, especially in the outer neuroblastic layer. Proliferating Ki-67 positive cells co-localize with nestin in the retina, lens, and choroid. Strong Oct-4 and alpha-tubulin immunoreactivity in the retina and optic nerve gradually decreases, while they co-localize in outer neuroblastic and nerve fiber layers.
Conclusions The described expression of investigated markers indicates their importance in eye growth and morphogenesis, while their spatially and temporally restricted pattern coincides with differentiation of initially immature cells into specific retinal cell lineages. Alterations in their spatiotemporal interplay might lead to disturbances of visual function.

Keywords Eye development · Nestin · PGP9.5 · S100 · Oct-4 · Alpha-tubulin

Abbreviations

nestin	intermediate filament protein
PGP9.5	protein gene product 9.5, ubiquitin-C-terminal hydrolase (UCHL-1), neuron specific gene protein
S100	calcium binding protein
Ki67	nuclear protein, proliferation marker
Alpha tubulin	protein that make up microtubules in eukaryotic cells, marker for primary cilia
Oct-4	octamer-binding transcription factor 4, POU domain, class 5, transcription factor 1(POU5F1), marker for stemness

Introduction

Human eye development starts with the formation of the optic vesicles, which fold inward to form the optic cups, consisting of an inner layer that gives rise to the retina, and an outer layer that forms the retinal pigment epithelium. The lens develops

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from thickened surface ectoderm by a similar invagination process, followed by formation of cornea, which is invaded by the neural crest cells [1].

Eye cellular inventory and development are generally maintained across many vertebrate species, although histological and functional differences between them exist. As investigations on early human eye development are very scarce, it is still obscure how different cell types forming eye structures that differentiate from their precursors and which factors coordinate the complex human eye development.

At the earliest stages of eye development, one of the first markers, which appear in immature cells, is nestin, an intermediate filament protein, which is classified as a type IV neurofilament [2]. Its expression is found primarily in neuroepithelial stem cells [2] and proliferating neural progenitor cells [3]. Nestin is widely expressed in developing zebrafish brain and eyes, having a key role in protecting progenitor cells from apoptosis, thus, enabling their differentiation. On the other hand, its deficiency leads to enhanced progenitor cell apoptosis and formation of defective brains, eyes, and cranial nerves [4]. During differentiation, nestin is downregulated and replaced by specific intermediate filaments, such as neurofilaments and glial fibrillary acidic protein [5]. While in the developing mouse eye, nestin is expressed in extraocular muscles, Müller cells, and lens epithelium [6, 7], in the adult mouse, it is restricted only to the astrocytes of the optic nerve [6]. Nestin is in the postnatal period present in rat retina, and in the human eye within the trabecular meshwork and the corneal endothelial transition zone [8]. Nestin has also been detected in human melanomas, neuroblastomas, angiosarcomas, and glioblastomas [9–12], and; therefore, it is suggested as a diagnostic and prognostic marker for cancer progression and malignancy [13]. The only two studies investigating expression of nestin during human eye development detected nestin in corneal stem cells [14], neural retina, optic nerve, choroid, sclera, and lens [15]. Among other intermediate filaments, neurofilaments appeared in differentiating neurons during human retinal development. One of the neuron-specific protein gene products is protein gene product 9.5 (PGP9.5), which is included in the ubiquitin C-terminal hydrolase subclass [16] and is highly expressed in vertebrate neurons and neuroendocrine cells [17]. In the rat retina, PGP9.5 first appears in a cell population of the neuroblast layer [18], while studies on adult retina disclose its presence in various species including humans [19]. The only study investigating both PGP9.5 and calcium-binding protein (S100 protein) on the human developing eye was performed at later developmental stages than those analyzed in this study [20]. The S100 proteins are characterized by two calcium binding sites [21] and are involved in regulation of enzyme activities and in different intracellular and extracellular functions. In the cell, S100 is normally present in the neural crest derivatives, epithelial and mesodermal cells, while in the extracellular matrix, it has a regulatory influence on inflammatory cells, neurons, astrocytes, and microglia [22]. So far, S100 protein expression has been detected in human retina during later fetal development [23].

One of the factors that seem to play an essential role in the maintenance of stemness and undifferentiated cell phenotype is octamer-binding transcription factor 4 (Oct-4) , which is also found in progenitor cells during development [24–26]. Ectopic expression of Oct3/4 in mice leads to formation of dysplastic skin and intestinal lesions due to an increase in the number of progenitor cells [27], while it is also expressed by stem cells in different types of cancer [28]. It has been investigated in mammalian embryos [27] and in human kidneys during development [29]. However, by now it has not been investigated in the developing human eye, but only on human induced pluripotent stem cells [30].

During development, the process of proliferation precedes differentiation of different cell types and is important for growth and normal eye morphogenesis. One of the most commonly used marker for detection of proliferation is Ki-67 nuclear protein, expressed in all active phases of the cell cycle [31] and, therefore, generally used in numerous histopathological studies [32]. By now, Ki-67 has been detected in the human late embryonal [33] and early fetal [34] eye development, but also in fetal and postnatal hippocampal tissue [35] and the cerebellar cortex [36]. Besides proliferation, other developmental processes such as differentiation and cell death seem to be controlled by the primary cilia, a peculiar organelle, which participates in different signaling pathways crucial for tissue homeostasis. It may serve as a cellular sensory structure, as described for the vertebrate olfactory organs and the outer segments of photoreceptors [37–39]. Acetylated alpha-tubulin as a marker for primary cilia is present in microtubules, where it stabilizes their structures [40, 41]. By now, primary cilia has been mostly associated with the maintenance of stem cell proliferation and differentiation during early embryonic development [42], but was also described in postnatal mouse cornea [43]. Possible roles of primary cilia have been studied in various cell types, from human kidney tubules [29] and neurons [44] to the modified cilium of the retinal photoreceptors [45]. Cilia dysfunction in the eye has been accompanied with defective patterning of corneal endothelium during postnatal development [43]. However, up to now investigations dealing with the role of the primary cilia in human early eye development are missing.

Information regarding development and structure of human retina has become increasingly important. Proper differentiation and synaptogenesis of photoreceptors or retinal ganglion cells is of special interest because of their involvement in many hereditary degenerative retinal diseases, age-related macular degeneration, or glaucoma [30]. Some of the keys to future clinical management lie in understanding the molecular mechanisms of eye development. Potential strategies for

treatment could include gene therapy and transplantation of stem cells in order to regenerate photoreceptors or replace them. Understanding of the early human eye development will be essential in order to pursue these strategies.

In the present study of early human eye development, we followed a shift from initially strongly proliferating cells characterized by the Ki-67 marker, over the phase of undifferentiated postmitotic stem cells showing strong expression of intermediate filament nestin, transcriptional Oct-4 marker and primary cilia marker acetylated alpha-tubulin, to the phase of gradual differentiation of main cell types in the eye characterized either by neuronal marker PGP9.5 or S100 marker of glial, or supporting cells. Analysis of their co-expression in the eye cells enabled us to determine their spatiotemporal interplay during the normal course of development, which, if altered, might lead to disturbed visual function.

Materials and methods

Tissue procurement and processing

All embryonic and foetal tissues was obtained and processed with approval of the Ethical and Drug Committee of the University Hospital of Split in accordance with the 1964 Helsinki Declaration (Williams, 2008). After exterior examination, any macerated or poorly maintained material was discarded. Haematoxylin and eosin staining of every tenth section of each tissue block confirmed appropriate material preservation. The age of conceptuses was estimated according to crown–rump length and the Carnegie staging system based on morphology [46]. A total of 14 normal human conceptuses between the fifth and 12^{th} developmental weeks (Table 1) were collected (after spontaneous abortions and after tubal pregnancies) from the Department of Pathology of the University Hospital of Split. Conceptuses were integrated into the age groups fifth/sixth (3 samples), seventh/eighth (4 samples), ninth/tenth (4 samples), and $11^{th}/12^{th}$ (3 samples) of the developmental week. Conceptuses were dissected and cranial parts were used for analyzes. Material was fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in graded ethanol, and embedded in paraffin. Serial 7 micrometers thick sections were cut in a transversal or a longitudinal plane, mounted on glass slides, and analyzed with an Olympus BX51 light microscope (Olympus, Tokyo, Japan).

Triple immunofluorescence staining

After deparaffinization and rehydration, the sections were treated as we previously described [44]. Briefly, sections were cooked in a microwave oven at 95 °C for 15 min in sodium citrate buffer (pH 6.0). After cooling, sections were washed in PBS and incubated with goat serum (Normal Goat Serum, X0907; DAKO, Glostrup, Denmark) for 1 hour. Sections were then incubated with the appropriate combination of primary antibodies for 1 hour or overnight at room temperature. The following primary antibodies were used: rabbit polyclonal to nestin (1:500; ab93157; Chemicon, Temecula, CA, USA), rabbit anti-PGP9.5 (1:500, ab5925; Chemicon, Temecula, CA, USA), mouse monoclonal to PGP9.5 (1:500, ab8189; Chemicon, Temecula, CA, USA), mouse monoclonal to S100 (1:300, ab4066; Chemicon, Temecula, CA, USA), mouse anti-Ki-67(1:100, M7240; DAKO, Glostrup, Denmark), mouse monoclonal [6-11B-1] to acetylated alpha-tubulin (1:800, ab24610; Abcam, Cambridge, UK), rabbit anti-Ki-67antigen (1:100, ab9260; Chemicon, Temecula, CA, USA), and rabbit polyclonal to Oct-4 (1:500, ab18976; Chemicon, Temecula, CA, USA). After multiple washes in PBS, sections were incubated for 1 hour with a combination of secondary antibodies: Rodamin (1:300, AP124R, Jackson Immuno Research Lab., PA, USA) and Alexa fluor 488 donkey anti-rabbit (1:300, Life Technologies A-11058). After secondary antibody incubation, the sections were washed in PBS and counterstained with DAPI nuclear stain. After final rinsing in PBS, sections were mounted, briefly dried, and coverslipped (Immuno-Mount, Shandon, Pittsburgh, PA, USA). Controls for antigen specificity included omitting of primary antibody from the usual staining protocol. Additionally, control experiments were carried out with nestin, PGP9.5, S100, Ki-67, Oct-4, and alpha tubulin antibodies preabsorbed with corresponding peptides. No staining was observed when the sections were incubated with antibodies solutions preabsorbed with appropriate peptides indicating that the antibodies detected the appropriate antigen sequence.

Sections were examined with a fluorescence microscope (Olympus BX61, Tokyo, Japan) and images were captured using a digital camera (DP71) and Olympus CellA software while images were assembled using Adobe Photoshop. Image J software (National Institutes of Health, Bethesda, MD, USA) was used for measuring threshold area percent, which was determined by the intensity of fluorescence of PGP9.5, S100, and nestin positive cells, respectively. In each picture

Table 1 Age and number of the human conceptuses analyzed in this study

Age (weeks)	CRL (mm)	Carnegie stage	No.
5	8	15	1
6	14	17	2
7	21	20	2
8	26	21	2
9	28	-	2
10	36	-	2
11	42	-	2
12	60	-	1

taken at×40 magnification, the surface of positive cells was marked, and then was measured their share in the squares (20×20 μm). The percentage of the surface covered with positive cells (threshold area percent) were analyzed in six randomly selected fields from each picture and compared between the regions of interest (the neural retina and optic stalk/nerve). Quantitative analyses were performed for the following developmental periods (fifth-sixth, seventh-eighth, ninth,-tenth, and 11^{th}-12^{th} weeks). In each region of interest (20×20 μm), threshold area percent was calculated and expressed as a mean±SD. For statistical analysis, the Mann Whitney test was used to examine differences between two regions of interest (the neural retina and optic stalk for the fifth-sixth developmental week), while differences between three regions of interest (optic nerve, inner, and outer neuroblastic layer for remaining periods) were analyzed with the Kruskal–Wallis test, followed by Dunn's post-hoc test (GraphPad Software, La Jolla, CA, USA) after testing distribution of the data. Statistical significance was set at $p<0.05$.

Semi-quantification

The intensity of staining of developing eye structures was semi-quantitatively described by four categories regarding the staining intensity, with (−) indicating the absence of any reactivity; (+), a mild reactivity; (++), moderate reactivity; (+++), and strong reactivity (Table 2, 3). Three researchers independently analyzed the staining intensity .

Results

The spatiotemporal expression pattern and semi-quantitative analysis of nestin, PGP9.5, S100, Ki-67, alpha-tubulin, and Oct-4 markers during human eye development were analyzed in 5 to 12-week-old human conceptuses using hematoxylin and eosin and triple immunofluorescence staining.

Embryonic period (fifth to eighth developmental week)

In the fifth to sixth week of human eye development, the lens vesicle and two walls of optic cup have formed (Fig. 1a). The inner (neural retina) and outer (pigmented layer of retina) wall of the optic cup are initially separated by the intraretinal space, which gradually disappears (Fig. 1a and b). Cells contain oval and elongated nuclei in the neural retina, and rounded nuclei in the pigmented layer, which in the cytoplasm display small brown pigment granules already during the fifth developmental week (Fig. 1a). The marginal zone beneath the neural retina contains the neural fibers (Fig. 1a) that join toward the optic stalk, while the choroid fissure enables the hyaloid artery to enter into the optic cup (Fig. 2a). In the fifth week of development, the wall of the optic vesicle is fully surrounded by the loose head mesenchymal sheath, which differentiates into an inner layer, that forms the highly vascularized and pigmented choroid, and the outer layer that develops into sclera (Fig. 1a). The reestablished surface ectoderm constitutes the anterior epithelium of developing cornea (Fig. 1a).

During the seventh developmental week, the choroid fissure closes and the mouth of the optic cup becomes a rounded opening, the future pupil (Fig. 1b). In the seventh to eighth week of development, outer and inner neuroblastic layers are clearly discerned and temporary separated by a part of the marginal zone known as the transient fiber layer of Chievitz (Fig. 1b) present only in primate mammals, that is composed of intertwined neuronal and Müller cells (Fig. 3b). This layer disappears at the end of embryonic development, but remains in the area of the future macula (Figs. 2b and 3b) throughout all investigated periods.

Early fetal period (ninth to 12^{th} developmental week)

In the ninth week of fetal eye development, the space between the lens and cornea forms the anterior chamber, while the posterior chamber is located posterior to the developing iris and anterior to the lens. The development of the neural retina is most advanced in the posterior half of the optic cup. First signs of differentiation in the neural retina occur at the border of the intraretinal space, where the ependymal cells differentiate into the rods and cones, photoreceptive cells (Fig. 1c). Neurons and supporting cells start to differentiate adjacent to the photoreceptive layer, while the ganglion cell layer beneath becomes more apparent (Fig. 1d, inset).

Nestin and PGP9.5 expression pattern

In the fifth to sixth developmental week, nestin was strongly expressed in the loose head mesenchyme (especially in external muscles of the eye) optic stalk, sclera, choroidea, lens vesicle, and some cells within the future inner neuroblastic layer of neural retina, adjacent to the marginal zone. The proportion of the nestin positive surface in this developmental period was higher in the optic stalk than in the neural retina ($p<0.0001$, Mann–Whitney test), In the early fetal period, nestin expression was localized in numerous elongated radial processes spanning along the thickness of the retina, while later on nestin was strongly expressed in the inner neuroblastic and nerve fiber layer. The proportion of the nestin positive surface in the seventh and eighth week as well as in the early fetal period was higher in the optic nerve than in the outer and inner neuroblastic layer ($p<0.0001$, Kruskal-Wallis test), while the inner neuroblastic layer had a higher proportion in comparison to the outer neuroblastic layer ($p<0.001$, Kruskal-Wallis test). Other eye structures showed decreased nestin expression toward the 12^{th} week of development. In the corneal and palpebral stroma, nestin was positive only in the

Table 2 Immunoreactivity to specific antibodies in the human eye during the fifth to 12th weeks of development

structure	antibodies											
	PGP9.5				S100				nestin			
	5/6w	7/8w	9/10w	11/12w	5/6w	7/8w	9/10w	11/12w	5/6w	7/8w	9/10w	11/12w
m	-*	-*	-*	-*	++	-*	-*	-*	+++	++	-*	-*
os/on	+	+++	+++	+++	+	+++	+++	+++	+++	+++	++	+
sc	-*	-*	-*	-*	-*	-*	-*	-*	+++	+	+	+++
Chr	-	-	-	-	-	-	-	-	+++	++	+	+
pr/RPE	+	+	-	-	+	+	-	-	-	-	-	-
nr/oNbl	++	+	+	+	+	+	+	+	+++	++	+	+
nr/iNbl		+++	+++	+++		+	+	+++		++	++	+++
mz/nf	+++	+++	+++	+++	+	+	+	+++	+++	++	+	+++
Lv/Le	+	+	+	+	+	+	+	+	+++	-	-	-
C	-*	-*	-*	-*	-*	-*	-*	-*	-*	++	+	+
Ace	++	++	+	+	-	-	-	-	-	-	-	-
ps	-*	-*	-*	-*	-*	-*	-*	-*	-*	+	+	+
pe	++	++	+	+	+	+	+	+	-	-	-	-

m the loose head mesenchyme, *os* optic stalk, *on* optic nerve, *sc* sclera, *Chr* choroidea, *pr* pigmented layer of retina, *RPE* retinal pigment epithelium, *nr* neural retina, *oNbl* outer neuroblastic layer, *iNbl* inner neuroblastic layer, *mz* marginal zone, *nf* nerve fiber layer, *Lv* lens vesicle, *Le* lens, *C* corneal stroma, *Ace* anterior corneal epithelium, *ps* palpebral stroma, *pe* palpebral epithelium, *w* weeks of development

+++ strong reactivity

++ moderate reactivity

+ mild reactivity

− no reactivity

/ structure absent in the tissue section

/* structure absent during corresponding developmental week

-* positive only in the nerve fibers or perineurium

nerve fibers during the fifth and sixth weeks, while later on its expression varied from moderate to mild within whole stroma, especially in the future limbus area (Fig. 5a). Nestin positivity was absent in the pigmented layer of retina and the anterior corneal and palpebral epithelium during the whole investigated period (Fig. 5a). The intensity of nestin expression and threshold area percent in the developing human eye structures during the described developmental period (5–12th week) are shown in Figs. 2, 4 and 6 and Table 2, respectively.

During all investigated periods, PGP9.5 was strongly expressed in the optic stalk and optic nerve, neural retina (especially in inner neuroblastic layer), and marginal zone, or nerve fiber layer. While there were no statistically significant differences in the proportion of the PGP9.5 positive surface during the fifth and sixth developmental week, later, from the seventh developmental week, the proportion of the PGP9.5 positive surface was higher in the inner neuroblastic layer than in the outer neuroblastic layer and optic nerve ($p<0.0001$, $p<0.001$, $p<0.01$, respectively, Kruskal-Wallis test), The loose head mesenchyme in scleral, corneal, and palpebral stroma was PGP9.5 positive only in areas of the nerve fibers penetration. PGP9.5 in the retinal pigment epithelium during the late embryonic period displayed no or very mild staining, while in the lens fibers of a developing lens, PGP9.5 was detected during all investigated periods. In the anterior corneal and palpebral epithelium, PGP9.5 was moderately to mildly positive from the fifth week toward the 12th week of development (Fig. 5a). PGP9.5 positivity was absent in the choroidea. The intensity of PGP9.5 expression and threshold area percent in the developing human eye structures during the described developmental period (5–12th week) are shown in Figs. 2, 3 and 6 and Table 2, respectively.

We sought to examine whether same cells would co-express nestin and PGP9.5, and these two markers co-localized in the marginal zone and inner neuroblastic layer during whole investigated period, especially in the 11th to 12th developmental week (Fig. 2a-d).

PGP9.5 and S100 expression pattern

In the fifth to sixth week of the developing human eye, S100 was moderately positive in the loose head mesenchyme, while in the later stages it was only in the perineurium of the optic nerve and myelinated nerve fibers. Similarly, in sclera, cornea, and palpebral stroma, S100 was positive only in the

Table 3 Immunoreactivity to specific antibodies in the human eye during the fifth to 12th weeks of development

structure	antibodies											
	Ki-67				Oct-4				alpha-tubulin			
	5/6w	7/8w	9/10w	11/12w	5/6w	7/8w	9/10w	11/12w	5/6w	7/8w	9/10w	11/12w
m	+++	+++	++	+	+	++	+	+	+	+	+	+
os/on	+++	+++	++	+	+	+++	++	+	+	+++	+++	+++
sc	+++	+++	++	+	+	++	+	-	+	+	+	+
Chr	+++	+++	++	+	+	++	+	+	-	+	+	+
pr/RPE	+++	+	+	+	+	++	++	+	-	+	+	+
nr/oNbl	+++	+++	+	+	+++	+	+	+	+++	+	++	+++
nr/iNbl		+++	+	+		+	+	+		++	++	+++
mz/nf	-	-	-	-	++	-	-	-	+	+++	+++	+++
Lv/Le	+++	+	+	+	/	/	+	+	/	/	+	+
C	+	+	+	+	-	-	-	-	+	+	+	+
Ace	+	+	+	+	+	+	+	+	++	++	++	++
ps	+	+	+	+	+	+	+	-	+	+	+	+
pe	+	+	+	+	++	++	+	+	++	++	++	++

m the loose head mesenchyme, *os* optic stalk, *on* optic nerve, *sc* sclera, *Chr* choroidea, *pr* pigmented layer of retina, *RPE* retinal pigment epithelium, *nr* neural retina, *oNbl* outer neuroblastic layer, *iNbl* inner neuroblastic layer, *mz* marginal zone, *nf* nerve fiber layer, *Lv* lens vesicle, *Le* lens, *C* corneal stroma, *Ace* anterior corneal epithelium, *ps* palpebral stroma, *pe* palpebral epithelium, *w* weeks of development, **elm* positive only in the external limiting membrane

+++ strong reactivity

++ moderate reactivity

+ mild reactivity

− no reactivity

/ structure absent in the tissue section

/* structure absent during corresponding developmental week

-* positive only in the nerve fibers or perineurium

perineurium during all stages (Fig. 5b). Strong S100 expression was found in the optic nerve, while choroidea and anterior corneal epithelium had been S100 negative during all developmental stages. The neural retina and the nerve fiber layer were mildly S100 positive except in the 11^{th}-12^{th} week, when the inner neuroblastic layer and the nerve fiber layer became strongly S100 positive. The proportion of the S100 positive surface during all stages was higher in the optic stalk than in the neural retina ($p<0.0001$), while the inner neuroblastic layer had a higher proportion of the S100 positive surface during seventh/eighth and $11^{th}/12^{th}$ week ($p<0.0001$, Kruskal-Wallis test). Pigmented layer and the retinal pigment epithelium were mildly S100 positive during late embryonic stages, while in the early fetal stages the retinal pigment epithelium was S100 negative. Lens and palpebral epithelia have been mildly S100 positive during the whole investigated period (Fig. 5b). The intensity of S100 expression and threshold area percent in the developing human eye structures during the described developmental period (5–12^{th} week) are shown in Figs. 3 and 6 and Table 2, respectively. We sought to examine whether the same cells would co-express PGP9.5 and S100, and these two markers co-localized in the inner neuroblastic layer during the 11^{th} to 12^{th} developmental week (Fig. 3a-d).

Ki-67 and nestin expression pattern

Ki-67 was strongly expressed during the late embryonic period in the loose head mesenchyme, optic stalk and nerve, in the sclera, choroid, pigment retina, neural retina, and lens vesicle (Fig. 5c). Ki-67 was mildly positive in all other investigated structures, except the marginal zone and nerve fiber layer where Ki-67 was negative. During the early fetal period, proliferation of the cells in the neural retina ceased especially in the posterior half of the outer neuroblastic layer. Co-localization of nestin and Ki-67 positive cells was observed in the neural retina, inner neuroblastic layer, lens, and choroidea (Fig. 4a-b, Table 3).

Oct-4 and alpha-tubulin expression pattern

During the late embryonic period, Oct-4 was strongly positive in the neural retina and optic nerve while the cornea was Oct-4

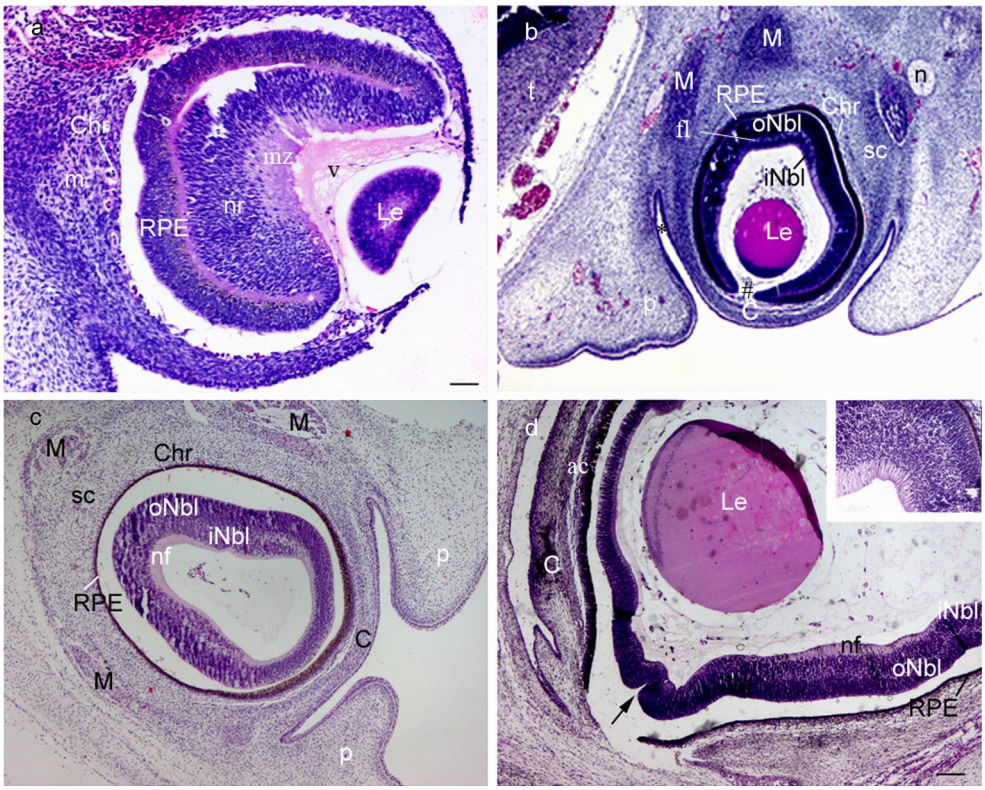

Fig. 1 Section through the eye of human embryo: (**a**) in the fifth week of development (**b**) in the seventh week of development **c** in the ninth week of development (**d**) in the 12th week of development, inset (neural retina): m – the loose head mesenchyme, sc – sclera, Chr – choroidea, pr – pigmented layer of retina, RPE – retinal pigment epithelium, nr – neural retina, oNbl – outer neuroblastic layer, fl – fiber layer of Chievitz, iNbl – inner neuroblastic layer, mz – marginal zone, nf- nerve fiber layer, v – future vitreous body, Le – lens vesicle/lens, ac – anterior chamber, C – corneal stroma, p – eyelid (palpebra), * - conjunctival sac, M – external eye muscle, t – telencephalon, n – nerve, arrow – ciliary body formation, # - future pupila. Haematoxylin and Eosin staining, (**a**) scale bar 100 μm, (**b-d**) scale bar 50 μm

negative (Fig. 5d). During the fifth to sixth developmental week, Oct-4 was mildly expressed in the loose head mesenchyme, optic stalk, sclera, choroid, pigmented layer of retina, anterior corneal epithelium, and palpebral stroma, while moderately positive in the marginal zone and palpebral epithelium. During the seventh to eighth developmental week, Oct-4 expression increased to moderate in all those structures except the nerve fiber zone, where Oct-4 was absent. During the early fetal period, Oct-4 expression in the neural retina ceased especially in the posterior half of both the inner and outer neuroblastic layer.

During the late embryonic period, alpha-tubulin was strongly positive in the neural retina, optic nerve, and nerve fiber zone (Fig. 5d), while the choroid and pigmented layer of retina were alpha-tubulin negative in the fifth to sixth developmental week. In the opposing pigmented and outer layers of the neural retina, alpha-tubulin positive cilia from the neuroblasts extended towards the surface of the pigmented cells (Fig. 4c). During the fifth to sixth developmental week, alpha-tubulin was also mildly expressed in the loose head mesenchyme, optic stalk, sclera, marginal zone, cornea, and palpebral stroma, while moderately positive in the anterior corneal and palpebral epithelium. During the seventh to eighth developmental week, alpha-tubulin expression increased to moderate in most of the described eye structures except in the head mesenchyme and palpebral stroma. During the early fetal period, alpha-tubulin expression in the neural retina remained the same as for the late embryonic period except for an increase of intensity in the inner and outer neuroblastic layer and nerve fiber layer. Co-localization of Oct-4 and alpha-tubulin positive cells was observed in the outer neuroblastic layer and in the nerve fiber layer. The intensity of Ki-67, alpha-tubulin, and Oct-4 expression in the developing human eye structures during the described developmental period (5–12th week) is shown in Fig. 4 and Table 3, respectively.

Discussion

Our study on the developing human eye dealt with the most intense period of human eye morphogenesis and differentiation: starting from period when the human eye structures were still intensively shaping (wide intraretinal space and opened optic fissure) and contained highly undifferentiated cells to the fetal period, showing advanced morphogenesis of all eye structures and clear signs of retinal cell differentiation. During the described period, analyzed markers appeared in eye structures in an overlapping spatiotemporal manner, indicating gradual differentiation of immature precursor cells into specific cell lineages. Thus, nestin was used to identify neural progenitor cells, which displayed strong expression in all mesenchymal eye derivatives, lens, optic stalk, and inner neuroblastic layer, throughout the late embryonic and early fetal period, reflecting the immaturity of these cells. However, the increase of nestin expression observed in the sixth week in the marginal and inner neuroblastic layer and optic stalk

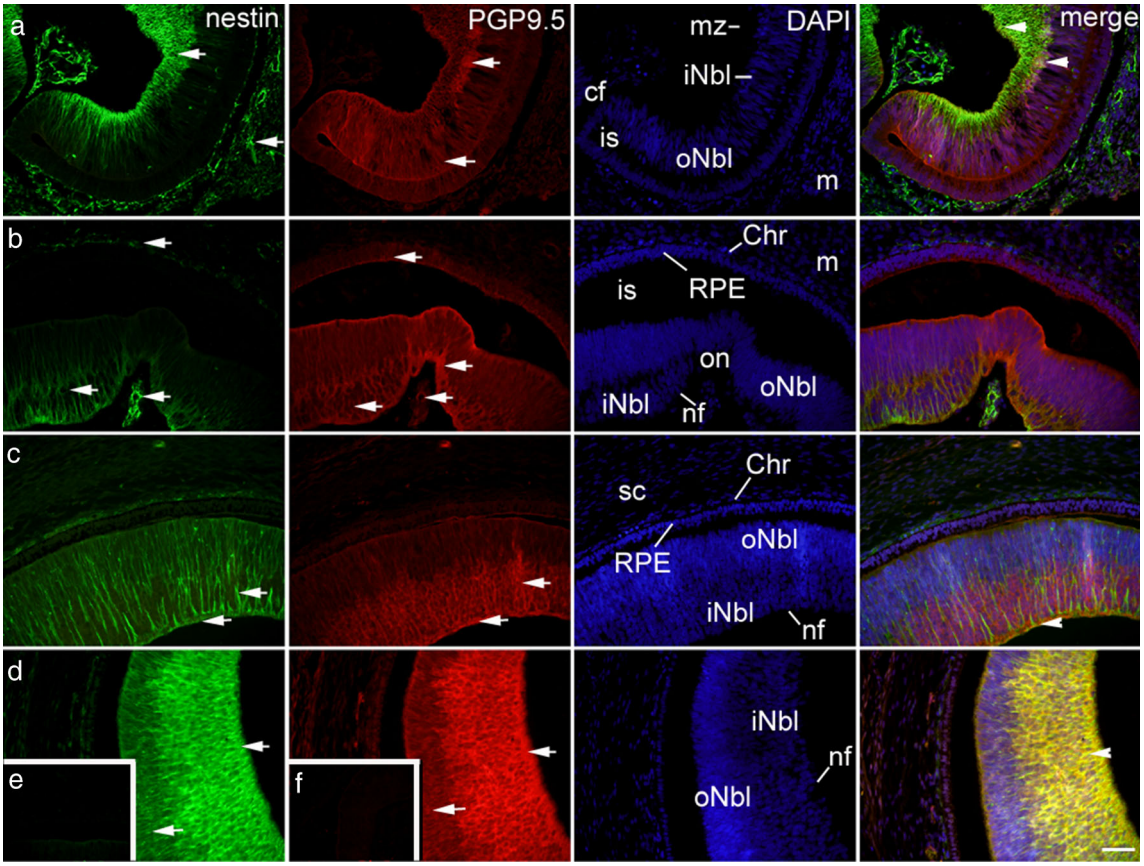

Fig. 2 (**a**) Section through the eye of a 6-week human embryo: nestin (*green*) positive cells (*arrows*) can be seen in the loose head mesenchyme (m), and marginal zone (mz), while PGP9.5 (*red*) is positive (*arrows*) in the outer neuroblastic layer (oNbl), is – intraretinal space, cf – choroid fissure. Co-localization of nestin and PGP9.5 positive cells (arrowheads) in the marginal zone (mz) and inner neuroblastic layer (iNbl). (**b**) Section through the eye of a 7-week human embryo: nestin positive cells (*arrows*) can be seen in all eye structures except in the retinal pigment epithelium (RPE). PGP9.5 positive cells (*arrows*) can be seen in the optic nerve (on), the retinal pigment epithelium (RPE), inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf), is – intraretinal space. Co-localization of nestin and PGP9.5 positive cells (*arrowheads*) in the marginal zone (mz) and inner neuroblastic layer (iNbl). (**c**) Section through the eye of a 9-week human embryo: nestin positive cells (*arrows*) can be seen in the inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). PGP9.5 positive cells (*arrows*) can also be seen in the inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). Co-localization of nestin and PGP9.5 positive cells (*arrowhead*) in the nerve fiber layer (nf). (**d**) Section through the eye of an 11-week human embryo: nestin positive cells (*arrows*) can be seen in the outer and inner neuroblastic layer (oNbl, iNbl) and in the nerve fiber layer (nf). PGP9.5 positive cells (*arrows*) can also be seen in the inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). Co-localization of nestin and PGP9.5 positive cells (*arrowhead*) can be seen in the nerve fiber layer (nf). (**e-f**) The negative control showed no nestin or PGP9.5 signal. Triple immunofluorescence staining to nestin (*green*), PGP9.5 (*red*) and DAPI (*blue*), scale bar 25 μm

indicated the possibility that those nestin positive cells migrated into the retina from the adjacent mesenchyme, as previously suggested by Edqvist and Hallbook [47]. Additionally, higher proportions of the nestin positive surface in the optic stalk in comparison to the neural retina might be explained by advanced differentiation toward mature neuronal and/or supporting cells within the neural retina. During further embryonic development, retinal separation between the inner and outer neuroblastic layer was fully accomplished, as also shown by Nag et al. [48]. During the early fetal period, the maturation of neural retina was most advanced in its posterior half, this was reflected in thinning of its wall, and outer neuroblastic layer accompanied by advanced differentiation of different cell subtypes. In the later fetal period, nestin was strongly expressed in both outer and inner neuroblastic layers, probably representing the extensions of immature Müller cells or radial glia. Also, higher proportions of the nestin positive surface in the inner neuroblastic layer in comparison to the outer layer might be explained by the cytoplasmic extensions of immature Müller cells contributing from the outer neuroblastic layer. In contrast to our study, investigations on pig eye development showed that the strongest nestin expression was localized in fibers of the ganglion cell layer, and a similar staining pattern was also described in the fifth postnatal day mouse retina and the eighth week old rat retina [26].

The role of nestin positive cells as neural progenitors was verified by co-localization with the Ki-67 proliferating marker, which revealed their co-localization throughout the

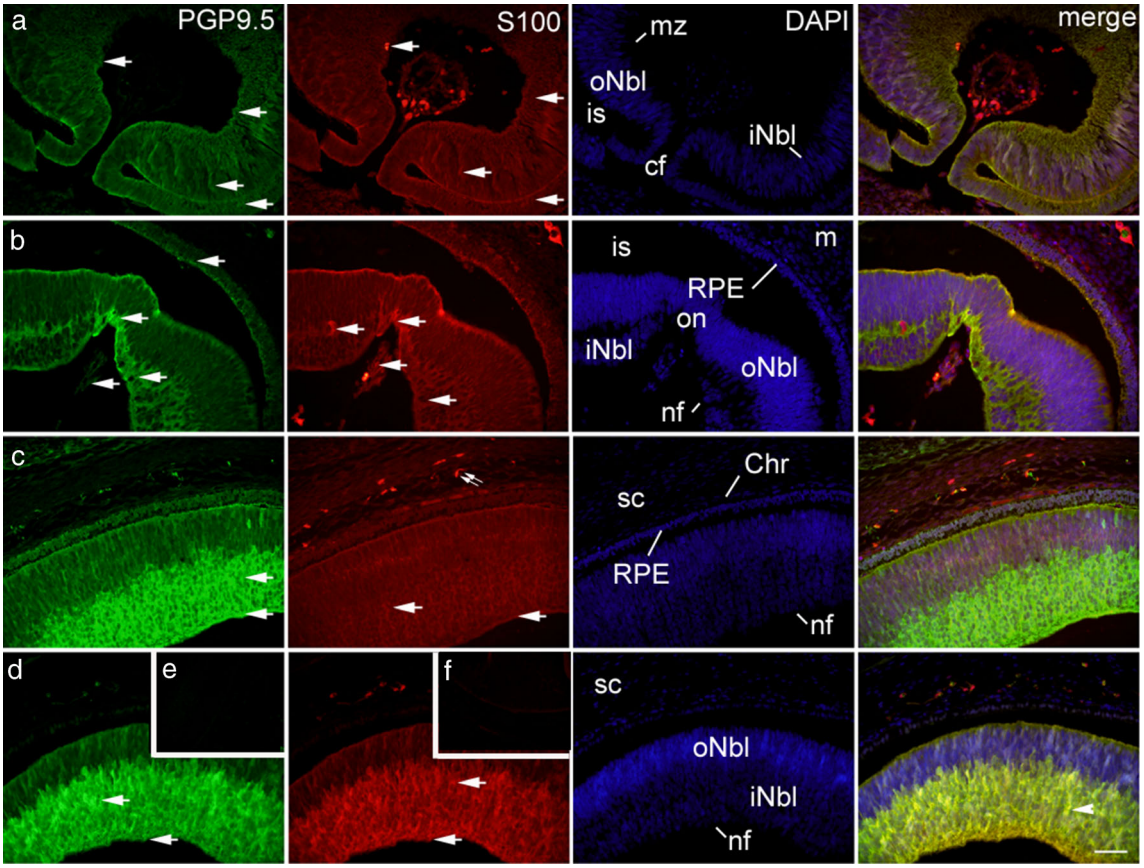

Fig. 3 (**a**) Section through the eye of a 6-week human embryo: PGP9.5 (*green*) positive cells (arrows) and S100 (*red*) positive cells (*arrows*) can be seen in the inner and outer neuroblastic layer (iNbl, oNbl) and marginal zone (mz); is – intraretinal space, cf – choroid fissure. (**b**) Section through the eye of a 7-week human embryo: PGP9.5 positive cells (*arrows*) can be seen in can be seen in the optic nerve (on), the retinal pigment epithelium (RPE), inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf); is – intraretinal space, m - mesenchyme. S100 positive cells (*arrows*) can be seen in the optic nerve (on), inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). (**c**) Section through the eye of a 9-week human embryo: PGP9.5 positive cells (*arrows*) can be seen in the inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). S100 positive cells (*arrows*) can be seen in the optic nerve (on) and in the nerve fiber layer (nf) and also in the scleral nerve fibers (*double arrows*). (**d**) Section through the eye of an 11-week human embryo: PGP9.5 positive cells (*arrows*) can be seen in the optic nerve (on), inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). S100 positive cells (*arrows*) can be seen in the inner neuroblastic layer (iNbl) and in the nerve fiber layer (nf). Co-localization of PGP9.5 and S100 positive cells (*arrowhead*) in the inner neuroblastic layer (iNbl). (**e-f**) The negative control showed no PGP9.5 and S100 signal. Triple immunofluorescence staining to PGP9.5 (*green*), S100 (*red*) and DAPI (*blue*), scale bar 25 μm

neuroblastic layers, including its innermost part, which is a source of amacrine cells. This finding is in contrast to the results of Guduric-Fuchs et al., who found no co-localization of nestin and Ki-67 in the future amacrine cells during pig retinal development [49].

Some studies indicate that Müller cells can be regarded as neural progenitor cells based on their nestin expression, but the successor generation of neurons has not been demonstrated unambiguously. Namely, nestin is not expressed only by neural progenitor cells, but rather, it can be induced in adult Müller cells after retinal injuries [50].

In our study, nestin and PGP9.5 co-localization revealed a relatively small population of retinal progenitor cells during the late embryonic and early fetal period. Contrary to our findings, the studies on retinal ganglionic cell cultures treated with or without staurosporine, trichostatin A, or succinylconcanavalin revealed high levels of nestin expression, and co-localization of nestin with PGP9.5, as well as with other mature neuronal markers such as tau, βIII-tubulin, microtubule-associated protein (MAP)-1b, and MAP2 [51]. This finding indicates that other factors might be involved in in vivo models rather than direct restriction of this co-expression, but at the same time opens the way for new treatment possibilities. However, further studies are necessary to reveal the role of nestin in the developing retina and Müller cells in order to understand retinogenesis and neuronal cell fate restrictions.

In our study, besides nestin positivity of nerves penetrating the cornea, nestin was also expressed within the cells of corneal stroma and at the border between the corneal epithelium

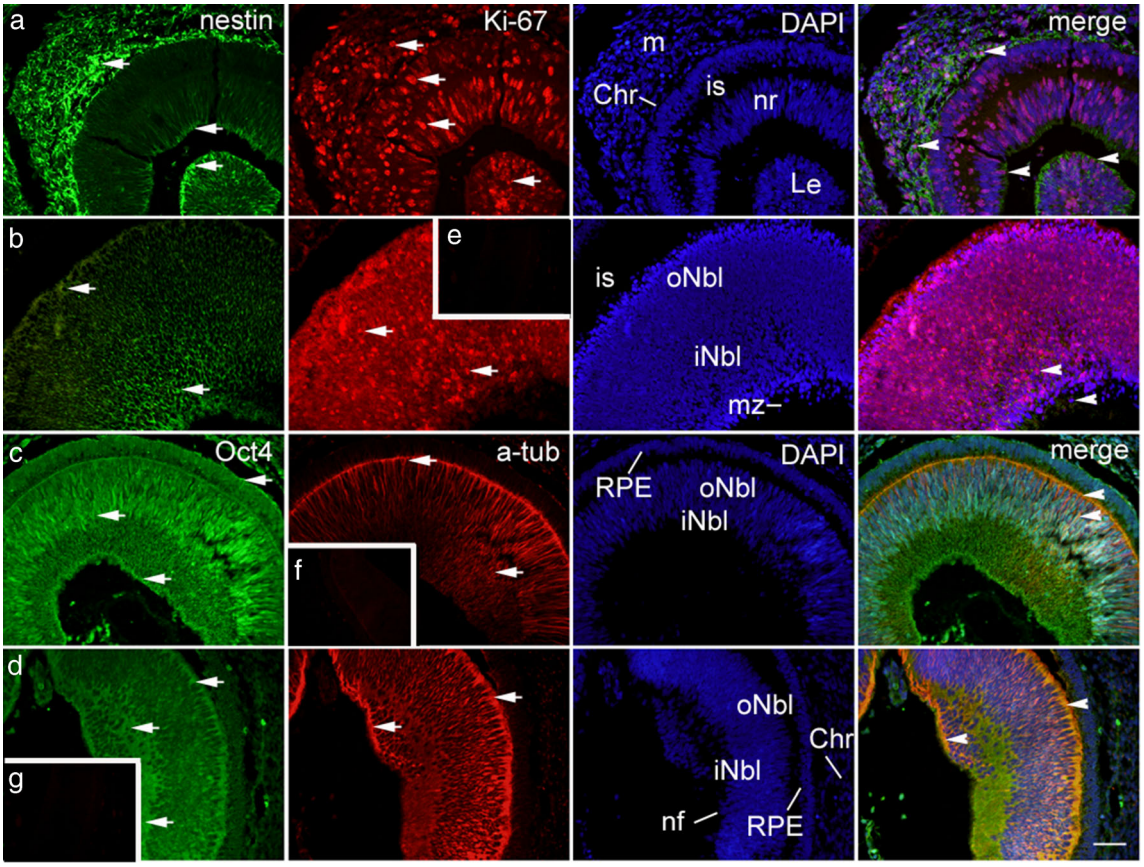

Fig. 4 (**a**) Section through the eye of a 5-week human embryo: nestin (*green*) positive cells (*arrows*) and Ki-67 (*red*) positive cells (*arrows*) can be seen in the loose head mesenchyme (m), in the neural retina (nr) and lens (Le). Blood vessels start to form choroidea (Chr); is – intraretinal space. Co-localization of nestin and Ki-67 positive cells (*arrowheads*) in the lens (Le), neural retina (nr) and future choroidea (Chr). (**b**) Section through the eye of a 8-week human embryo: nestin and Ki-67 positive cells (*arrows*) can be seen in the inner and outer neuroblastic layer (iNbl, oNbl); mz – nerve fiber layer, is – intraretinal space. Co-localization of nestin and Ki-67 positive cells (*arrowheads*) in the inner neuroblastic layer (iNbl). Triple immunofluorescence staining to nestin (green), Ki-67 (red) and DAPI (blue). (**c**) Section through the eye of a 6-week human embryo: Oct-4 (*green*) positive cells (*arrows*) can be seen in the inner and outer neuroblastic layer (iNbl, oNbl) and the retinal pigment epithelium (RPE), while alpha-tubulin (*red*) positive cells (*arrows*) can be seen only in the inner and outer neuroblastic layer (iNbl, oNbl). Co-localization of Oct-4 and alpha-tubulin positive cells (*arrowheads*) in the outer neuroblastic layer (oNbl). (**d**) Section through the eye of a 8-week human embryo: Oct-4 positive cells (*arrows*) and alpha-tubulin positive cells (*arrows*) can be seen in the in the inner and outer neuroblastic layer (iNbl, oNbl) and in the nerve fiber layer (nf). Co-localization of Oct-4 and alpha-tubulin positive cells (*arrowheads*) in the outer neuroblastic layer (oNbl) and in the nerve fiber layer (nf). (**e-g**) The negative control showed no Ki-67, alpha-tubulin and Oct-4 signal. Triple immunofluorescence staining to nestin (*green*), Ki-67 (*red*), Oct-4 (*green*), alpha-tubulin (*red*) and DAPI (*blue*). Scale bar 25 μm

and conjunctiva. Those cells could represent a population of neural crest cells that migrated from adjacent mesenchyme to form precursor cells for corneal regeneration in adulthood. Additionally, nestin positive cells in this area co-localized with Ki-67 proliferation marker. Our results confirm previous study performed on human embryos and fetuses of similar developmental stages [14]. McGowan et al. found precursor cells in the limbus, which originated from the mesenchymal neural crest in human adults, as well [8]. This finding supports the concept that the limbus area is a proliferative zone, during both development and adulthood.

Although some studies indicate that choroid first appears during seventh developmental week, our study showed that the beginning of choroid development from the neighboring mesenchyme started already during the fifth developmental week. Strong nestin expression in the walls of blood vessels of the choroid indicates its central role in the angiogenic response.

In our study, nestin positivity was absent in the pigmented layer of retina. However, Davari et al. experimentally showed that the pigmented layer may de-differentiate into retinal neurons and progenitor cells positive to nestin [52]. PGP9.5 was positive in human eye already during the fifth developmental week, especially in the inner neuroblastic layer and marginal zone indicating faster neuronal maturation in comparison to the optic nerve. In contrast, in rat retina it appeared at later developmental stages, in the neuroblastic layer and presumptive horizontal cells [18]. During all investigated periods, PGP9.5 was strongly expressed in the neural structure, with an increase of proportion of the PGP9.5 positive surface

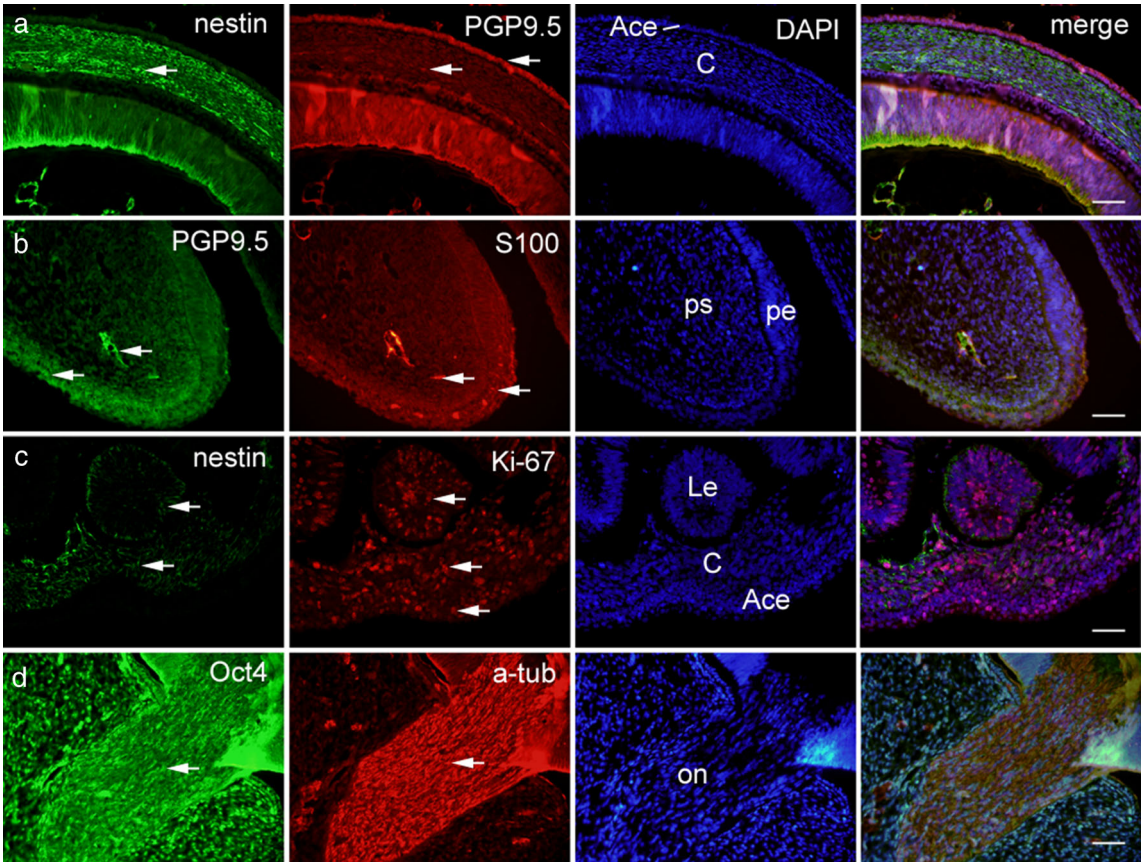

Fig. 5 (**a**) Section through the eye of a 7-week human embryo: nestin (*green*) positive cells (*arrow*) can be seen in the corneal stroma (C) while PGP9.5 (*red*) positive cells (*arrow*) are positive in the anterior corneal epithelium (Ace) and in the nerve fibers of cornea (*arrow*). (**b**) Section through the eye of a 9-week human embryo: PGP9.5 (*green*) and S100 (*red*) positive cells (*arrows*) can be seen in nerve fibers of palpebral stroma (ps) and palpebral epithelium (pe). (**c**) Section through the eye of a 5-week human embryo: nestin (*green*) positive cells (*arrows*) can be seen in the lens (Le), in the nerve fibers of the cornea (C), while Ki-67 (*red*) is additionally positive in the anterior corneal epithelium (Ace). (**d**) Section through the eye of a 8-week human embryo: Oct4 (*green*) and alpha-tubulin (*red*) positive cells (*arrows*) can be seen in the optic nerve (on). Triple immunofluorescence staining to nestin, PGP9.5, S100, Ki-67, Oct4, alpha-tubulin and DAPI (*blue*), scale bar 25 μm

especially in the inner neuroblastic layer and optic nerve, while in the lens fibers and in the retinal pigment epithelium, PGP9.5 was expressed only during late embryonic period. PGP9.5 expression in the retinal pigment epithelium of adult humans was found in age-related eye diseases such as drusen and basal laminar deposits. It was suggested that it might be involved in the degradation and disposal of proteins from these cells [53]. In the anterior corneal and palpebral epithelium PGP9.5 was moderately to mildly expressed from the fifth towards the 12th week of development, which accords with findings on the adult rat cornea [54].

Already during the earliest developmental stages, S100 was positive at the apical surface of the neural retina and in the area of future photoreceptors, suggesting that S100 can be used as an early marker of photoreceptor differentiation. Opposite results were reported for differentiation of pig and mouse photoreceptors, which seem to appear later in development than in humans [49]. In the seventh developmental week, sparse S100 positive cells found deeper in the transient fiber layer of Chievitz of the future macula might represent the first appearance of the Müller cells (based on morphology and localization in this layer), which is in contrast to the study of Iwanaga et al. who found S100 positive Müller cells only in the 10-30 weeks retina [23].

In the early fetal human retina, a higher proportion of the S100 positive surface represented astrocytes, densely distributed in the inner neuroblastic layer (ganglion cell layer) and nerve fiber layer. The first S100 positive horizontal cells appeared in outer neuroblastic layer during the ninth week of development. S100 was also positive in the ciliary epithelium and posterior epithelium of the iris, and temporally in the pigmented retina, which is in accordance with the study of Iwanaga et al. [23]. Co-localization of PGP9.5 and S100 in the inner neuroblastic layer during the 12th week of development was probably caused by superposition of very dense neuroblasts and astrocytes, and the thickness of the section.

Mature photoreceptors have cilia that contain nine outer microtubule-doublets and are important for maintaining the separation of the outer and inner segment compartments [55, 56]. During eye development, following the process of

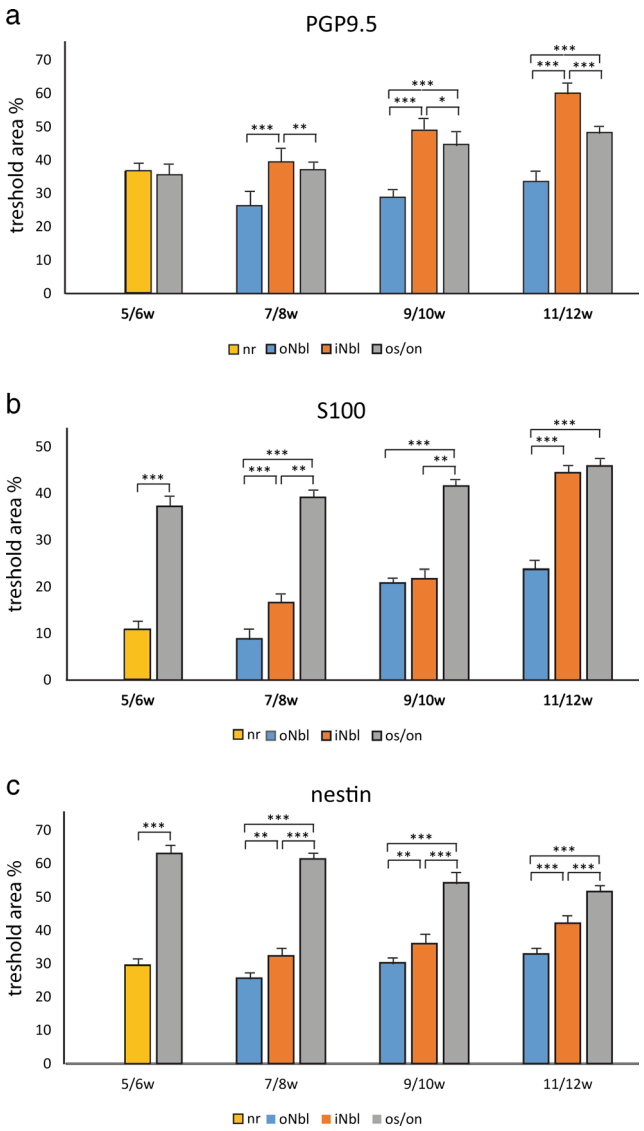

Fig. 6 Threshold area percent of PGP9.5 (**a**), S100 (**b**) and nestin (**c**) in the neural retina (nr), outer (oNbl) and inner (iNbl) neuroblastic layer and optic stalk (os)/optic nerve (on) during 5th-12th developmental week. Data are shown as mean±SD. Significant differences (Mann-Whitney for fifth-sixth week and Kruskal-Wallis for 7th-12th week) indicated by *p<0.01, **p<0.001, ***p<0.0001

proliferation, cells subsequently entered the process of early differentiation, associated with strong to moderate expression of alpha-tubulin at the apical surfaces of the neural retina cells in the area of the future photoreceptors. A similar expression pattern, showing the presence of primary cilia on the surfaces of ependymal cells was detected during the human spinal cord development [44]. The future photoreceptors co-expressed both alpha-tubulin in primary cilia and nestin. Since cilia contain a variety of ion channels, receptors, transporter proteins, and downstream effector molecules [57], our findings indicate possible transfer of signals between the intraretinal space and the pigmented epithelial cells. During early fetal development, cilia seem to be involved in generation of the photoreceptors outer segment domain [58] and in maintaining mature photoreceptor cell polarity [59].

Additionally, we found alpha-tubulin positive primary cilia in the corneal stroma and epithelium during the investigated period, which accords with findings on mouse embryonic cornea [43]. Primary cilia assembly and later disassembly in adult cornea are important to maintain corneal transparency, while dysfunction of primary cilia leads to defective patterning of cornea during postnatal development [43]. In our study, the marginal zone and nerve fiber layer also displayed strong alpha-tubulin expression, possibly indicating the process of differentiation and lengthening of neuroblast axons, which comprise neurotubules and establish the main component of the marginal zone, which accords with our previous studies [60, 29, 44].

Neural stem cells studies and restriction of their stemness revealed that suppression of Oct-4 is important for the transition of undifferentiated neuroblasts into neurons [61]. In our study, strong Oct-4 expression was found in the retinal neuroblasts during the late embryonic period, indicating a possible role of Oct-4 in preventing premature cell differentiation. Co-localization of Oct-4 and Ki-67 (data not shown) in the retinal neuroblasts probably represent the pool of neural stem proliferating cells that were not yet ready for terminal differentiation. In agreement with this observation is a study on Oct-4 knock-out mice, where loss of Oct-4 activity is a prerequisite for terminal cell differentiation [61], while interference of Oct-4 gene expression can cause disturbances in cell differentiation [62]. The expression of Oct-4 positive proliferation cells within developing retina opens new possibilities for the clinical use of these cells, in order to develop new protocols for clinical and experimental research [63].

In late embryonic development, Oct-4 and alpha-tubulin strongly co-localized within cells of outer neuroblastic and nerve fiber layers and the optic nerve, particularly in the area of the future photoreceptors. Eggenschwiler and Anderson showed that primary cilia control the balance of early stem cell proliferation and differentiation, implying their key roles in the embryonic development [42]. In accordance with our results is a study of Schluter et al. who found cilia present in almost all apical cell surfaces of renal tubules expressing the Oct-4 stemness marker [64]. The expression of the pluripotency Oct-4 gene would suggest a broad variety of differentiation options for Oct-4 positive cells, but it remains unknown how these cell become a lineage-specific cell type and how the pluripotent gene such as Oct-4 plays a role in this process [65].

Limitations of this study are the use of only immunohistochemical methods instead of more powerful proteomics, as well as a small sample size due to difficulty in obtaining normal human conceptuses.

In conclusion, the described expression of investigated markers indicates their importance in eye growth and

morphogenesis, while their spatially and temporally restricted pattern coincides with differentiation of initially immature cells into specific retinal cell lineages. Alterations in their spatiotemporal interplay might lead to disturbances of visual function. Some of the keys to future clinical management lie in understanding the molecular mechanisms of eye development. Potential strategies for treatment could include gene therapy and transplantation of stem cells in order to regenerate photoreceptors or replace them. Understanding of the early human eye development will be essential in order to pursue these strategies. Further studies on the differentiation potential of cells with stemness properties during early human eye development are needed to elucidate pluripotency cell fate restrictions.

Acknowledgments This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (grant no. 021-2160528-0507).

Conflict of interests All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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