Tissue-specific expression of *NANOG* gene in human eye

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The genes associated with multipotency in the eye cells at different stages of differentiation continue to be in the focus of biomedical research. In this study we revealed the changes in the *NANOG* mRNA expression in the human eye tissues at the early developmental stages. Using *in situ* hybridization we have obtained the new evidence for *NANOG* transcriptional activity in the human eye tissues at 8−10.5 weeks of prenatal development. *NANOG* transcriptional activity was detected in ectodermal derivatives tissues (cornea epithelium and lens) as well as in neuroectodermal tissue (neural retina). The highest *NANOG* mRNA concentration has been registered in cornea epithelium. The differences in the *NANOG* mRNA expression pattern could relate to the eye cells properties and their microenvironment. It is known that even in definitive tissue the epithelium retains the self-renew ability, while the retinal cells self-maintenance potential *in vivo* is extremely limited. Our findings confirm the presence of *NANOG* mRNA in tissues derived from different germ layers and clarifies the cellular markers characteristic of various eye cell types. The data obtained could help facilitate the understanding the cell biology and cell differentiation mechanisms.

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Abstract

The genes associated with multipotency in the eye cells at different stages of differentiation continue to be in the focus of biomedical research. In this study we revealed the changes in the *NANOG* mRNA expression in the human eye tissues at the early developmental stages. Using *in situ* hybridization we have obtained the new evidence for *NANOG* transcriptional activity in the human eye tissues at 8−10.5 weeks of prenatal development. *NANOG* transcriptional activity was detected in ectodermal derivatives tissues (cornea epithelium and lens) as well as in neuroectodermal tissue (neural retina). The highest *NANOG* mRNA concentration has been registered in cornea epithelium. The differences in the *NANOG* mRNA expression pattern could relate to the eye cells properties and their microenvironment. It is known that even in definitive tissue the epithelium retains the self-renew ability, while the retinal cells self-maintenance potential *in vivo* is extremely limited. Our findings confirm the presence of *NANOG* mRNA in tissues derived from different germ layers and clarifies the cellular markers characteristic of various eye cell types. The data obtained could help facilitate the understanding the cell biology and cell differentiation mechanisms.

Keywords: Human eye; development, differentiation; *in situ* hybridization

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1. Introduction

In genetic regulation of mammal eye development the most intriguing question is the role of gene associated with pluripotency. The transcription factors Oct4, Sox2, Klf4, c-Myc, Nanog and Lin28 have previously been reported to form the core of a regulatory network controlling pluripotency of the embryonic stem cells (ESCs). ESCs have unlimited capacity for self-renewal and an ability to differentiate into more than 200 cell types (Boyer et al., 2005; Wang et al., 2006; Young, 2011). Stability of *Oct4*, *Sox2*, *Nanog* expression is provided by autoregulatory loops resulting from the activation of their intrinsic promoters, cross-activation of each other's promoters and by binding to the regulatory elements of the specific genes sets (Kallas, Pook, Trei, & Maimets, 2014; Loh et al., 2006; Rodda et al., 2005). Mechanisms regulating stemness are determined by cell properties, cell interactions and influence of the numerous signaling pathways.(Mournetas, Sanderson, Fernig, Murray, & Nunes, 2014) Ectopic *Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, *Lin28* genes expression is well-known to reprogramme the somatic cell genome into embryonic state and alters the differentiation program (Hu, Friedrich, Johnson, & Clegg, 2010; Kim et al., 2009; Wernig et al., 2007). It has been established that the transcriptional factor Oct4 plays a key role in pluripotency induction and reprogramming (Pesce & Schöler, 2004). As have been shown on eye models of various animals the functions of some of these regulators are not limited to the pluripotency/multipotency (Camp et al., 2009; Pauklin, Thomasen, Pester, Steuhl, & Meller, 2011).

Analysis of the undifferentiated cells markers expression in differentiated cells during various periods of vertebrate development is of great importance.

The subject of our interest is the multipotency genes expression, in particular the transcription factor NANOG expression (www.ncbi.nlm.hin.gov; Gene ID: 79923, HGNC: 20857) in human eye ontogenesis. NANOG contains a conservative DNA-binding homeo domain (Chambers et al., 2003; Hart, Hartley, Ibrahim, & Robb, 2004) and has several pseudogenes (Booth & Holland, 2004). The gene is expressed at highly level in the ESCs and testis, where the role of this gene in the cell's pluripotent status maintaining have been proved (Hart et al., 2004; Hyslop et al., 2005; Mitsui et al., 2003). *NANOG* and *OCT4* transcription activity significantly increases in tumor cells lines characterized by abnormal high proliferation, in embryonic carcinoma and human retinoblastoma (Hart et al., 2005; Jeter, Yang, Wang, Chao, & Tang, 2015; Seigel et al., 2007). A dual and sequential knockdown of *OCT4* and *NANOG* in transformed human stem cells with features of cancer cells showed some functional divergence of transcription machinery from the normal SC self-renewing state. Reduction in *NANOG* level expression led to the loss of cancer stem cells (CSCs) self-renewal coupled with apoptosis (Ji, Werbowetski-Ogilvie, Zhong, Hong, & Bhatia, 2009).

As for human eye the information about the multipotency markers in different cell types of definitive tissues *in vivo* is poor and fragmentary. Expression of *NANOG* and other multipotency markers such as *SOX2*, *OCT4*, *KLF4*, *NESTIN*, *PAX6* was revealed in the corneal limbus epithelial cells which retain the ability of self-renewal in the adult eye (Pauklin et al., 2011). However, in the adult corneal epithelium the level of *NANOG* transcriptional activity is extremely lower than in ESC. Earlier, the *NANOG* expression was detected in the human eye at 10.5 week of prenatal development in non-dissected tissues of cornea, total lens, retina, pigment epithelium with choroid, by PCR (Firsova et al., 2008). However, PCR can not show the *NANOG* mRNA distribution in different eye cell types. In this study we focused on a detail analysis of *NANOG* mRNA localization in the human eye tissues during the early prenatal development at 8−10.5 weeks of gestation.

2. Materials and Methods

2.1. Ocular tissues

The objects of the study were the eye tissues obtained from the human abortuses (post-operative material) after legal abortions from medical terminations from licensed institutions of the Ministry of Health of the Russian Federation, the Research Center of Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences, acting within the framework of the law of the Russian Federation about protection of the health of citizens and according to the approved list of medical indications. The age of fetuses was determined by an obstetrician. Foetal human eye tissues were obtained according to ethics regard and appropriate measures to ensure biosafety, and accompanied with medical report on the absence of pathologies. All manipulations on postoperative human material were performed in accordance with European GTP (Good Tissue Practices) II Guidance (European Union Project in the framework of the Public Health Program, 2003-2008) and Directive 2004/23/EC of The European Parliament and of The Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells and approved by the Research Center of Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences; Ethics Committee of the Koltzov Institute of Developmental Biology Russian Academy of Science (Ethical Application Ref: № 22, 15 March 20018). We have analysed the eyes of 8 human embryos at 8−10.5 weeks of gestation (8, 9.5, 10, 10.5 week), two embryos for each stage.

2.2. Histological analysis

The eyes of the embryos from 8 to 10.5 weeks of gestation were fixed in the Buen's solution, washed in 70% ethanol, dehydrated in the series of alcohols of increasing concentrations and were embedded in paraffin according to the method used earlier (Markitantova et al., 2008). Sections of 7 microns were stained with hematoxylin followed by contrasting with eosin.

2.3 cDNA synthesis for RT-PCR and sequencing

Total RNA from the individual human retinas of 9.5 weeks was extracted by TRI Reagent (Sigma, USA). To avoid contamination by genomic DNA the DNase digestion was performed (Fermentas, LTU). The quantity and quality of the RNA were evaluated using NanoDrop system (Thermo Fisher Scientific, USA) and gel electrophoresis. First-strand of cDNA was synthesized with 50 ng total RNA using SuperScript RT (Gibco-BRL, USA) and random hexamer oligonucleotide primers (Sileks, RF). Expression of well-known eye regulator *PAX6* was estimated as a control. Gene-specific primers for RT-PCR were designed on the data of human genes structure from NCBI: *NANOG* (GenBank: AB093576.1), *PAX6* (Gene ID: 5080) using Lasergene (DNAStar software, USA). RT-PCR was performed using a master kit (Sileks, RF). To exclude amplification from genomic DNA primers were synthesized from flank intron/exon junctions and from different exons: for *NANOG*, 5'-CCTCCTCTTCCTCTATACTAA-3' (from the boundary of the exon 1) and 5'-CTGCGTCACACCATTGCTATTC-3' (from exon 4). Primers for *PAX6* were: 5'-gtcatcaataaacagagttcttc-3' (exon 7); 5'-cgattagaaaaccatacctgtat-3' (exon 10). We had only one product 523 bp specific for *NANOG* and one product 424 bp specific for *PAX6*. The cDNA was normalized by housekeeping gene *RPL19* (Gene ID: 6143). Primers for housekeeping *RPL19* gene were: 5'-AGGGTACAGCCAATGCCCGA-3' (exon 4) and 5'-CCTTGGATAAAGTCTTGATGATC-3' (exon 6), the fragment length was 326 bp. RT-PCR was performed in thermocycler (Eppendorf, DE) according to the design. The reaction without cDNA template was performed as the control for primers contamination. PCR products were visualized on 1.5% agarose gel using DNA ladder 100 bp (Sileks M, RF). Gene's expression was estimated by a gel-analyzer Quantity One (BioRad, USA). The identity of the amplified nucleotide sequences to the studied genes was proved by sequencing on ABI PRIZM-3100 (Applied Biosystems, USA) system with BigDye 1.1 reagents (Thermo Fisher Scientific, USA) before cloning.

2.4 ***PCR fragments Cloning and RNA probes synthesis for in situ hybridization***

The PCR fragments of *NANOG* (523 bp) and *PAX6* (424 bp) amplified on template of the human retina of 9.5 week of gestation were cloned into the transcription vector using a TOPO TA cloning kit (Invitrogen, USA). RNA probes were generated and labeled by *in vitro* transcription procedures. We

synthesized antisense RNA probes complementary to *NANOG* mRNA and *PAX6* mRNA and sense RNA probes (negative controls) labeled with digoxigenin using Dig-labeling RNA kit (Roche, CHE). Subsequent colorimetric reaction was performed using Dig RNA detection kit (Roche, CHE). The efficiency of the probes labeled was assessed by direct hybridization signals detection on filters. The probes concentration was 200 ng per 65 mkl hybridization buffer optimal for the clear hybridization signal without the non-specific background staining. Corresponding sense probes used as negative controls gave no hybridization signal.

2.5 ***Preparation of cryosections and in situ hybridization***

Human eyes at 8, 9.5, 10, 10.5 weeks of prenatal development were fixed in 4% paraformaldehyde on the 0.1 M PBS (pH 7.4), at +4°C for cryosections. Six sections were stained for each eye in each experiment. We didn't find out any significant biological variability. The eyes were washed in several changes of 100 mM phosphate buffer (pH 7.5), incubated in 20% sucrose on 100 mM phosphate buffer, at +4°C for 24 hours, embedded and frozen in TissueTec OCT (Leica, DE). Sections of 12 mm thickness were prepared for *in situ* hybridization on a Leica CM1900 UV cryostat and were attached on Super frost-Plus glass slides (Thermo Fisher Scientific, USA). Samples preparation and *in situ* hybridization procedures were performed as described for the fish, and adapted for the human eye (Barthel & Raymond, 2004; Markitantova et al., 2008). The labeled antisense RNA probes were detected with an anti-digoxigenin antibody that has been linked with the enzyme alkaline phosphatase (AP). After incubating the antibody with the samples we detected the mRNA by adding the substrate BCIP (bromo-chloro-indolyl-phosphate) and NBT (nitro blue tetrazolium). Slides were mounted in Immu-Mount (Thermo Scientific™, USA) and then analyzed by microscopes Olympus DP70, DM RXA2 (Leica, DE). All the experiments were repeated at least three times.

3. Results

We took into account the fact that *NANOG* gene has two highly homologous isoforms and 11 pseudogenes to design highly specific primers (Booth & Holland, 2004; Das, Jena, & Levasseur, 2011).

Human retina at the stage of 9.5 week served as a template for cDNA which was used for *NANOG* and *PAX6* RNA probes synthesis for *in situ* hybridization (Figure 1).

We performed cDNA sequencing of PCR fragments completely overlapping the coding part of the gene in order to avoid misinterpretation of the results of PCR analysis. The nucleotide sequence of human *NANOG* gene (Gene ID: 79923) have been confirmed. PCR products of *NANOG* and *PAX6* were cloned into the transcription vector pCRII-TOPO with dual promoters T7 and SP6 for DIG labeled-antisense and sense riboprobes synthesis and were used for *NANOG* mRNA localization in the human eye during 8−10.5 weeks of development by *in situ* hybridization.

Histological analysis showed that at the stages studied the rudiments of all eye tissues had already been represented in the germinative form except the ciliary body (Figure 2A). Cornea epithelium differentiates into the outer layer consisting of flattened cells and the inner layer consisting of cuboidal cells. In the retina, cells from the densely packed outer neuroblastic layer (ONbL) migrated to the newly formed and more loosely packed inner neuroblastic layer (INbL).

We have found for the first time the *NANOG* transcripts localization in the eye tissues of ectodermal origin (cornea and conjunctive epithelium, lens epithelium) and in the tissues of neuroectodermal origin, retinal neuroblast layers in human fetuses of 8−10.5 weeks of development. *NANOG* was not detected in the mesenchymal eye tissues.

According to our data the pattern of *NANOG* mRNA expression in the retina during these stages of the eye development undergoes a change. The *NANOG* transcripts were localized predominantly in the cells of the retina marginal area at 10.5 week of human gestation (Figure 2B,C). It was shown that cells differentiation from this region of neural retina is delayed significantly relative to the central part. Proliferating cells population of the retina marginal region was larger compared with the central area at all time-points examined (Markitantova & Zinovieva, 2012) It has been shown that stem-like cells were localized among the retina peripheral region cells which may be a potential source for retina restoration (Bhatia, 2010). The distinct hybridization signal with *NANOG* mRNA was observed at the stage of 9.5-10, 10.5 weeks in both inner and outer retina neuroblast layers (Figure 2B,C). The data about *NANOG*

mRNA distribution obtained by *in situ* hybridization in this study are consistent with the immunoassay (Firsova et al., 2008).

In parallel we have studied the *PAX6* mRNA localization at the same eye developmental stages as a positive control, because this transcription factor is well-known to be in control of the eye tissues morphogenesis in vertebrates (Gehring, 1996; Nishina et al., 1999). The *PAX6* transcripts were revealed in corneal as well as in lens epithelium of human eye at the all analyzed stages of gestation. We have registered the most intensive hybridization signal to *PAX6* mRNA throughout the retinal neuroblasts at 8-10.5 weeks and in the marginal area of the forming retina (Figure 3A,B). No hybridization signal was observed in the mesenchymal eye cells (corneal endothelium, corneal and iris stroma) at all the studied stages of the human eye development. Earlier PAX6 protein was localized in the same eye tissues of ectodermal (corneal and lens epithelium) and neuroectodermal (retina, ciliary body epithelium) origins, but was not detected in mesenchymal tissues according to the data of fluorecsent and non-fluorescent immunochemistry (Firsova et al., 2009; Nishina et al., 1999). Functions of Pax6 as a main regulator of the multipotent cells proliferation in peripheral retina and of the exit of the retinal progenitor cells from the cell cycle during differentiation of ganglion and amacrin cells in the mammal's eye have been proved (Hsieh & Yang, 2009; Marquardt et al., 2001). Thus, our results on the analysis of the *NANOG* and *PAX6* by in *situ* hybridization are in good agreement with the data obtained previously by others methods (Firsova et al., 2009; Nishina et al., 1999) and confirm the transcriptional activity of the both genes in the eye tissues studied. The transcription activity of the gene was also detected in the adult eye cells. Using the reverse transcription PCR analysis, the minor amount of *NANOG* mRNA transcripts were also found in the retina of the adult human (Firsova et al., 2008). Although, it is logical to assume that in mature cells the level of gene expression can decrease and their role is not exclusively related to the cells multipotency.

The *NANOG* in differentiated human eye cells can perform other function than maintenance of pluripotency state. The previous reports demonstrated that *Oct4* and *Nanog* transcription must be silenced

when human ES cells differentiate, so that the developmentally important genes are activated. These events during the early stages of embryogenesis are accompanied by accumulation of repressive epigenetic marks such as DNA methylation, H3K9me3, and H3K27me3 while the neural genes *Pax6*, *N-Oct3* acquired histone marks H3K4me3 associated with transcriptional activation (Das et al., 2011; Deb-Rinker, Ly, Jezierski, Sikorska, & Walker, 2005).

4. Discussion

Recent researches of the multipotency genes have raised the question about their expression and possibility to perform regulatory functions in the differentiating cells. The transcriptional factor NANOG/Nanog has been considered only as a cell pluripotency marker for a long time. NANOG/Nanog exhibited stage-specific activity in early mammalian embryogenesis similar to OCT4/Oct4 (Chambers et al., 2003; Kallas et al., 2014; Pesce & Schöler, 2004). Blocking the expression of pluripotency factors in early period of mammalian embryos development is considered to be a necessary step for realization of the molecular programs of specific cell type differentiation (Liang et al., 2008). In light of recent studies the range of Nanog functions is much wider (Camp et al., 2009; Hu et al., 2010; Jeter et al., 2015).

In the present study we first have obtained the data on the *NANOG* mRNA distribution in the human eye tissues development at 8−10.5 weeks (Figure 2B,C) in comparison to *PAX6* mRNA (Figure 2D,E). We have found the similar pattern of tissue-specific mRNA distribution for both regulatory genes in the eye tissues. Our results on *NANOG* mRNA localization in the corneal epithelium during the early embryogenesis confirm the data obtained in the adult human eye by other authors (Pauklin et al., 2011). The *Nanog* expression level increases in the epithelium cells of esophagus, oral cavity, skin (Piazzolla et al., 2014), that led to the suggestion about high selective activity *Nanog* in self-renewal epithelial tissues. Functions of *NANOG* gene during the early eye development are not clear yet they may be related to the regulation of the proliferative activity of eye progenitor cells.

However, we have determined the *NANOG* mRNA localization not only in forming human cornea, but also in the neural retina. Previously, the high expression levels of other cells multipotency regulatory gene-marker *GNL3* and *Ki67* (cell cycle marker) in proliferating retinal neuroblasts had been

229 determined at 9−11 weeks (Markitantova & Zinovieva, 2012). *GNL3* controls the proliferative activity
230 and cells multipotent state in ESC, neural SC and some cancer cell lines (Ma & Pederson, 2008; Nomura
231 et al., 2009). In general, there is a similarity in the expression pattern of regulatory genes *NANOG*, *PAX6*,
232 *GNL3* and *Ki67* in the analyzed human eye tissues at the early stages of development. The functional
233 relationship of *Nanog* and the cell cycle machinery in mammal embryogenesis have been established: the
234 high level of *Nanog*/*NANOG* expression in mouse and human ESCs is necessary not only to maintain
235 undifferentiated cell status (Chang, Wang, Knott, Chen, & Cibelli, 2009), but to trigger the G1-S
236 transition in the cell cycle as well (Zhang et al., 2009). Nanog is able to suppress directly the $p27^{KIP1}$
237 expression after transduction of mouse fibroblasts in somatic cells as was shown by high-resolution
238 massive DNA sequencing of chromatin immunoprecipitation (ChIP-seq) (Münst et al., 2016). It is well-
239 known that the continuous ESCs cell cycle is provided by the coordinated operation of signaling
240 pathways STAT3, LIF, PI3 kinase pathway, WNT, TGF, BMP, FGF and others (Kemp, Willems, Abdo,
241 Lambiv, & Leyns, 2005; Li et al., 2004; Loh et al., 2006). The same signaling molecules are involved in
242 the control of specific cell differentiation that can be maintained through alternative molecular
243 components. The increase in the *NANOG* expression level in tumor stem cells and certain types of cancer
244 cells is also accompanied by reactivation of molecular mechanisms leading to cell proliferation increasing
245 (Jeter et al., 2015). Nanog overexpression synergizes with gene *AURKA*, critical mitosis marker, to induce
246 proliferation, neoplastic growth, chromosomal aberrations (Jeter et al., 2015). RNA interference-mediated
247 silencing of *NANOG* reduced cells proliferative activity and *CyclinD1* expression in the same chain by
248 ChIP data (Han et al., 2012). NANOG increases the cell proliferation in liver cancer through the
249 Nodal/Smad3 pathway (Sun et al., 2013). We have found the transcriptional activity of the gene *NANOG*
250 in somatic differentiating cells of the eye tissues of various origins in human ontogenesis (Firsova et al.,
251 2008). Wide pattern localization of *NANOG* mRNA in the eye tissues suggests its multifunctional role in
252 special cellular and molecular niches. The eye tissues at the studied stages are still developing and mostly
253 undifferentiated. The current evidence in the field, together with NANOG's mRNA pattern similar to that
254 of Pax6 as we have indicated, would rather support the hypothesis that *NANOG* and *PAX6* transcripts are

present in proliferating progenitors in the developing ocular tissues and that it is then down-regulated as the cells exit the cell cycle and commit to differentiating into specific cell types.

It should be noticed that each cell type is characterized by a certain epigenetic status of the genome that programs gene expression (Melcer et al., 2012). The analysis of the ESC and tumor stem cells shows that regardless of their origin the promoters of *Nanog* and *Oct4* are hypomethylated, and a high level of expression of these genes is observed. In somatic cells, trophoblast stem cells and NIH/3T3 cells (mouse embryonic fibroblast cells) Nanog promoter is hypermethylated, and transcription is repressed (Hattori et al., 2007). However, histone modification, DNA methylation and acetylation are only some aspects of the epigenetic mechanisms regulating the genes transcription through a complicated step-by-step process. Recent findings have demonstrated the role of endogenous non-coding RNA molecules, including long noncoding RNAs (lncRNAs) in the cell choice to self-renewal or differentiation into any lineage. In mouse embryonic stem cells the specific sets of the lncRNAs modulate developmental state: knockdown and overexpression of these transcripts lead to the substantial changes in *Oct4* and *Nanog* mRNA levels and to the shifts in cellular lineage-specific gene expression and in pluripotency mouse ESCs (Mohamed, Gaughwin, Lim, Robson, & Lipovich, 2010). At least 20 lncRNA inducing the expression of Nanog and Oct4 are known (Ghosal, Das, & Chakrabarti, 2013). In addition to non-coding RNAs, the highly homologous isoforms and pseudogenes may contribute to differential regulation and tissue-specific *NANOG* and *OCT4* expression (Das et al., 2011; Lin, Shabbir, Molnar, & Lee, 2007; Pain, Chirn, Strassel, & Kemp, 2005). For example, cell type-specific *NANOG* and its pseudogene *NANOGP8* expression were revealed in both undifferentiated and differentiated human cells. It is supposed that rapidly dividing cells such as neonatal fibroblasts, cell line human epitheloid cervix carcinoma (HeLa), cell line human neuroblast from neural tissue (SH-SY5Y), human mesenchymal stem cells (MSCs), human ESCs express *NANOG* while relatively slow dividing cells such as adult fibroblasts, heart tissue, human umbilical vein endothelial cells (HUVECs) have both *NANOG* and *NANOGP8* (Ambady et al., 2011). In terminally differentiated smooth muscle cells known as the slowest cycling cells only

NANOGP8 is expressed. Nevertheless, it was reported that all the analyzed cell types were potentially capable of binding a NANOG consensus sequence *in vitro* system (Ambady et al., 2011).

Despite of considerable interest to epigenetic regulation the data for the human eye tissues are fragmentary, contradictory, dealing with a limited number of regulatory genes (Bonnin, Belville, Chiambaretta, Sapin, & Blanchon, 2014; Qu et al., 2010; Rai et al., 2006). Eye cells differentiation is not only accompanied by specific changes in the DNA, histones modification, but also non-coding RNAs expression, nucleolar reorganization. The main aspects of epigenetic regulation in the eye are summarized in detail in review (Cvekl & Mitton, 2010). Unfortunately, there are no comprehensive data on regulation of the multipotency genes in the human eye in this regard.

Data obtained showed that *NANOG* expressed selectively in both ectodermal and neuroectodermal human eye tissues in embryogenesis. The expression of *NANOG* in human eye in development may be related to the cells proliferation and differentiation and can be explained by the cells type peculiarities: cell cycle kinetics, the differentiation state in specific cellular and molecular niche that undergoes dynamics in ontogenesis.

5. Conclusions

The results obtained extend the dataset currently available for the developing human retina (Tian et al., 2015; Hoshino et al., 2017; Mellough et al., 2019) and open up the new prospects for future investigation of the transcriptional factor NANOG role in genetic regulation of human eye tissues differentiation. Data on *NANOG* expression in the eye tissues of different embryonic origins contribute to the clarification of genetic processes in the human eye cells and may be taken into account for the development of differential cell and tissue therapy methods. Such approaches are being developed for other system to differential cancer therapy based on selective regulation *Nanog*, *Oct-4*, *Sox2*, *Klf4* expression in P19 embryonic carcinoma stem cells through transcriptional factor decoys (TFDs) thus downregulating the genes expression in a specific manner (Rad et al., 2015). Knowledge of the multipotency genes expression specificity and their role in regulatory networks in the human eye tissues

is a prerequisite for the successful application of biotechnological approaches to solve a number of problems associated with the eye pathologies.

Conflicts of Interest: The authors report no conflicts of interest.

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Figure Legends

Figure 1. Electrophoresis of PCR fragments, corresponding to the genes *NANOG* and *PAX6*, on the cDNA template from the human retina (9.5 week of gestation). The length of the nucleotide sequences after amplification cDNA with gene-specific primers were: housekeeping gene RPL19, 326 bp (1), NANOG, 523 bp (2), PAX6, 424 bp (3), M – DNA ladder 1000 bp (Sileks M), marker for the PCR products length; RT(-) – negative control without the template of cDNA; bp – base pair.

Figure 2. Localization of the *NANOG* mRNA in human eye at early stages of prenatal development: analysis by chromogenic *in situ* hybridization. Hematoxylin an eosin staining of the human eye sections at the 8 to 10.5 weeks of gestation (column A). Hybridization signals with anti-sense RNA probes show the *NANOG* mRNA trancripts in the corneal and lens epithelium, both outer and inner retinal neuroblastic layers (column B) (column B). No hybridization signals with sense RNA probe used as negative control for *NANOG* mRNA (column C) are observed. Abbreviations: PR, peripheral retina; CR, central retina; LEC, lens epithelium cells; CE, corneal epithelium; ON, optic nerve; GCL, ganglion cell layer; INbl, inner neuroblastic layer; ONbl, outer neuroblastic layer; RPE, retinal pigmented epithelium. Scale bars: 500 µm eye histology (A), 500 µm *NANOG,* 8−10.5 weeks (B), (C).

Figure 3. Localization of the *PAX6* mRNA in human eye at the same stages of early prenatal development: analysis by chromogenic *in situ* hybridization. Strong hybridization signals for *PAX6* mRNA are detected in both corneal and lens epithelium, in inner and outer neuroblastic retinal layers.

Positive staining is more prominent in peripheral area of retina and is absent in optic nerve (column A) No hybridization signals with sense RNA probe used as negative controls for *PAX6* mRNA (column B) are observed. Abbreviations: PR, peripheral retina; CR, central retina; LEC, lens epithelium cells; CE, corneal epithelium; ON, optic nerve; GCL, ganglion cell layer; INbl, inner neuroblastic layer; ONbl, outer neuroblastic layer; RPE, retinal pigmented epithelium. Scale bars: 500 µm *PAX6*, 10.5 weeks (A); 100 µm *PAX6*, 9.5−10 weeks (A), (B).

Figure 1

Electrophoresis of PCR fragments, corresponding to the genes *NANOG* and *PAX6*, on the cDNA template from the human retina (9.5 week of gestation)

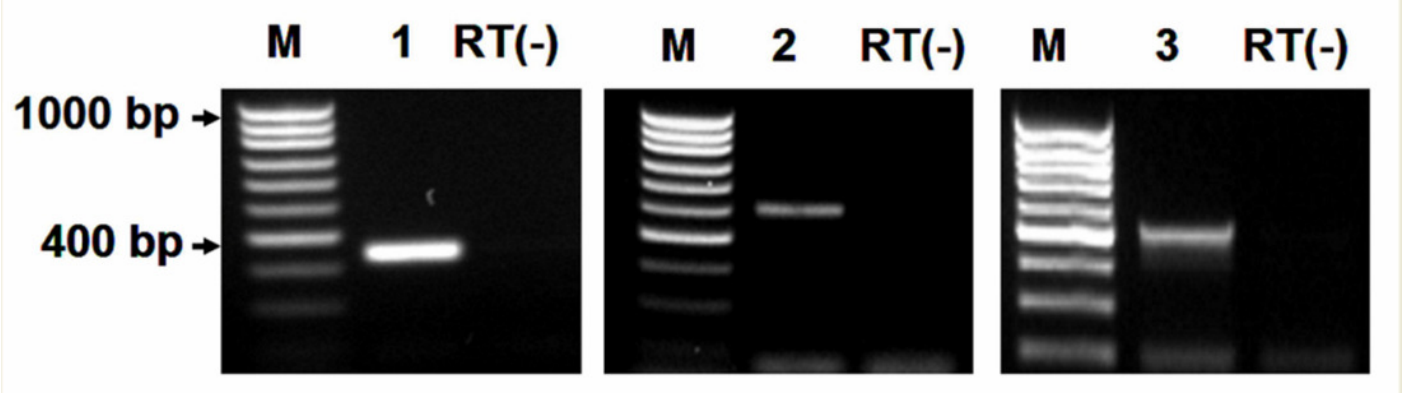

Figure 2

Localization of the *NANOG* mRNA in human eye at early stages of prenatal development: analysis by chromogenic *in situ* hybridization

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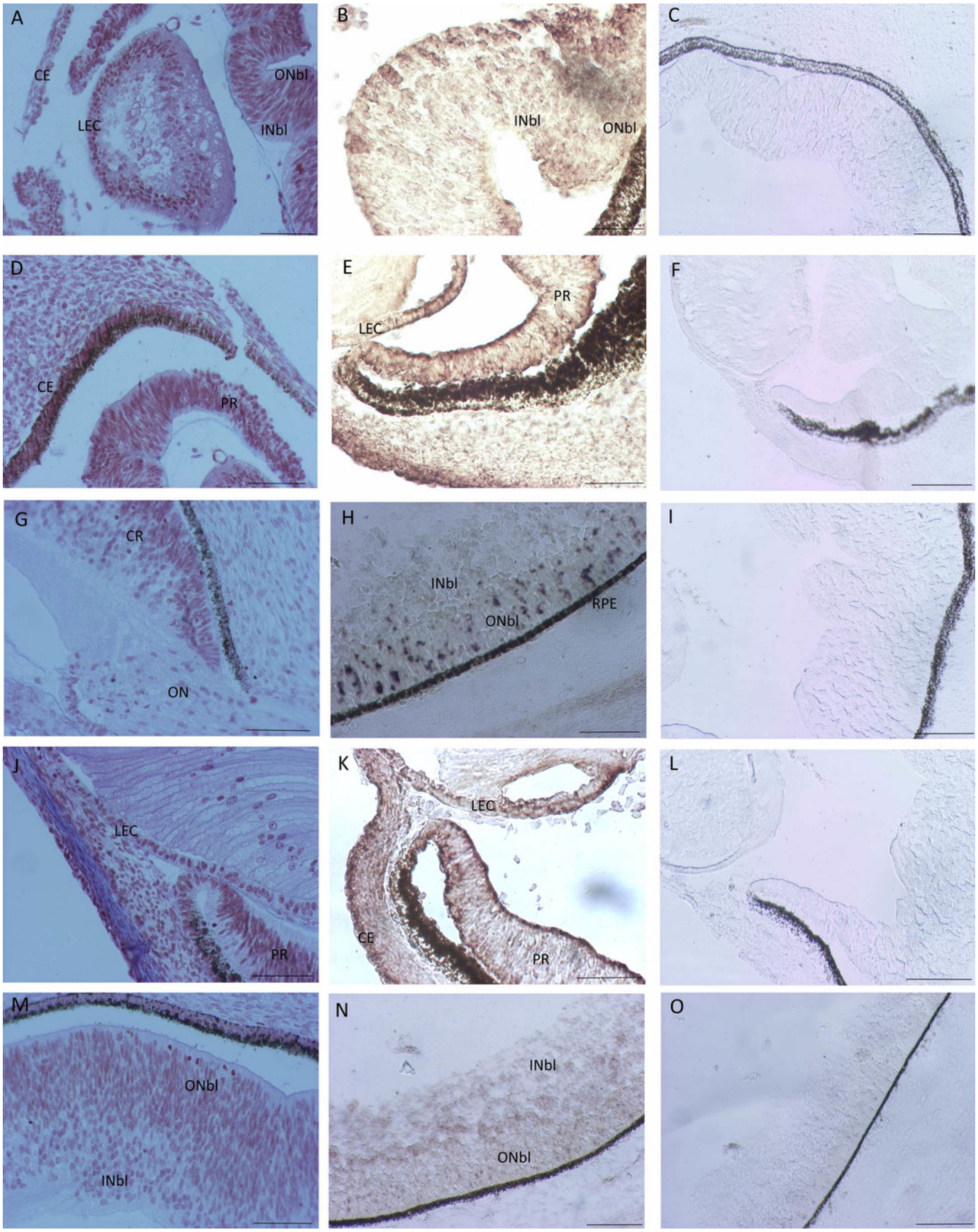
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Figure 3

Localization of the *PAX6* mRNA in human eye at the same stages of early prenatal development: analysis by chromogenic *in situ* hybridization

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