Experimental Eye Research 116 (2013) 109–128

Contents lists available at ScienceDirect

Experimental Eye Research

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

The structure and development of *Xenopus laevis* cornea

Wanzhou Hu [a,1], Nasrin Haamedi [a,1], Jaehoon Lee [b], Tsutomu Kinoshita [b], Shin-ichi Ohnuma [a,*]

[a] UCL Institute of Ophthalmology, UCL, 11-43 Bath Street, London EC1V 9EL, UK
[b] Department of Life Science, Faculty of Science, Rikkyo University, 3-34-1 Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan

ARTICLE INFO

Article history:
Received 18 March 2013
Accepted in revised form 17 July 2013
Available online 27 July 2013

Keywords:
Xenopus
cornea
eye
embryo
development
amphibian
neural crest
metamorphosis
cell death

ABSTRACT

The African clawed frog, *Xenopus laevis*, is a widely used model organism for tissue development. We have followed the process of corneal development closely in *Xenopus* and examined the corneal ultrastructure at each stage during its formation. *Xenopus* cornea development starts at stage 25 from a simple embryonic epidermis overlying the developing optic vesicle. After detachment of the lens placode which takes place around stage 30, cranial neural crest cells start to invade the space between the lens and the embryonic epidermis to construct the corneal endothelium. At stage 41, a second wave of migratory cells containing presumptive keratocytes invades the matrix leading to the formation of inner cornea and outer cornea. Three-dimensional electron microscopic examination shows that a unique cell mass, the stroma attracting center, connects the two layers like the center pole of a tent. After stage 48, many secondary stromal keratocytes individually migrate to the center and form the stroma layer. At stage 60, the stroma space is largely filled by collagen lamellae and keratocytes, and the stroma attracting center disappears. At early metamorphosis, the embryonic epithelium gradually changes to the adult corneal epithelium, which is covered by microvilli. Around stage 62 the embryonic epithelium thickens and a massive cell death is observed in the epithelium, coinciding with eyelid opening. After metamorphosis, the frog cornea has attained the adult structure of three cellular layers, epithelium, stroma, and endothelium, and two acellular layers between the cellular layers, namely the Bowman's layer and Descemet's membrane. After initial completion, *Xenopus* cornea, in particular the stroma, continues to thicken and enlarge throughout the lifetime of the animal. In the adult, a p63 positive limbus-like wavy structure is observed at the peripheral edge of the cornea. Proliferation analysis shows that the basal corneal epithelial cells actively divide and there are a small number of proliferating cells among the stroma and endothelial cells. This study shows that the development and structure of *Xenopus* cornea is largely conserved with human although there are some unique processes in *Xenopus*.

1. Introduction

The cornea, as the anterior-most part of the eye, directly interacts with the external environment. It maximizes light transmittance into the eye and, with an even higher refractive power than lens, accounts for 70–75% of the eye's total refractive power. In addition, the cornea functions as a physical barrier to protect deep ocular structures from potentially harmful organisms and substances. The cornea meets these critical roles by employing a unique complex three-layered structure comprising a corneal epithelium layer, a stromal layer, and an endothelium layer without any pigmentation or vasculature. As one of the most sensitive tissues of the body, the cornea is also densely innervated with sensory nerve fibers. Studies of formation of the cornea, mainly using mammals and chick, have revealed complex mechanisms of corneal development and maturation (Linsenmayer et al., 1998; Soules and Link, 2005; Zhao et al., 2006; Zieske, 2004). Briefly, the vertebrate cornea begins its development as simple ectoderm tissue, which proliferates extensively under external signals to give rise to the primitive lens. Almost immediately after lens detachment, waves of neural crest cells (NCCs) migrate into the space between lens and epithelium. In chick, it is believed that neural crest invasion occurs in two steps. The first wave develops into corneal endothelium, followed by the second wave, which differentiates into keratocytes.

Abbreviations: NCC, neural crest cell; SAC, stroma-attracting center; CMZ, ciliary marginal zone.

* Corresponding author. Tel.: +44 20 7608 4062.
E-mail address: s.ohnuma@ucl.ac.uk (S.-i. Ohnuma).

[1] These authors contributed equally to this work.

0014-4835
http://dx.doi.org/10.1016/j.exer.2013.07.021

In contrast, studies in rodents described a single migration of cells giving rise to both cell types. In addition, a recent report (Gage et al., 2005) provided evidence that in mouse, keratocytes and endothelial cells are derived from a mixture of NCCs and head mesoderm.

Defects of corneal development or maintenance are a major cause of blindness. Also, slight changes in corneal properties such as contour, smoothness, thickness, transparency or architecture result in visual distortion and loss of clarity. Recently, many genes that are associated with corneal diseases have been identified (Aldave, 2011; Nielsen et al., 2013; Schmedt et al., 2012). Human corneal diseases often manifest their associated defects in specific layers of the cornea. For example, Meesmann's juvenile dystrophy manifests as a defect in the corneal epithelium, while granular dystrophy and Fuchs' dystrophy manifest as defects in the stroma and endothelium layers respectively. Some diseases are in contrast associated with multiple layers. Interaction between the layers is likely to play a critical role in corneal pathogenesis. However, an understanding of the molecular interactions underlying disease pathology, necessary for prevention of disease and the development of novel treatments, is at present almost entirely lacking. This is largely due to a lack of suitable assay models. There is no good *in vitro* model of this three-dimensional complex structure of epithelium, stroma, and endothelium. Also, mammalian models are limited (Koizumi et al., 2012; Stuart and Keadle, 2012) and are not ideal for the investigation of detailed mechanisms because of the cost, time and limits on animal number. Therefore, development of suitable animal models using more accessible lower animals are required in order to gain better understanding of the pathogenesis of human corneal diseases.

The African clawed frog, *Xenopus laevis/tropicalis*, has been used extensively as a model organism. Its rapid development and ease of manipulation have made it particularly suitable for studying some complicated developmental processes. Recently, a new technology of gene knockout, transcription activator-like effector nucleases (TALENs) (Mussolino and Cathomen, 2012), has proved to be effective in *Xenopus* (Ishibashi et al., 2012; Lei et al., 2012). This technology was developed by genetic engineering of bacterial proteins, transcriptional activator-like effectors, which recognize specific nucleotide sequences (Boch and Bonas, 2010). Simple injection of two engineered TALENs into *Xenopus* blastomeres results in gene knockout with almost 100% efficiency in the F0 embryos, which can produce a further generation. The F1 frogs can produce a few thousand in the next generation, indicating that *Xenopus* may be an ideal organism to construct corneal disease models.

Despite extensive studies on lens regeneration using *Xenopus* cornea (Filoni, 2009; Henry and Tsonis, 2010), our knowledge of its structure and formation is very limited. In 1979, Bard and Abbott briefly described its development. They found that at stage 32, periocular mesenchymal cells start to migrate into the space between the corneal epithelium and the lens to form the monolayer structure of the prospective endothelium, connected to the outer cornea in the center. Around stage 43, additional mesenchymal cells were observed to colonise the space between the two corneal layers. By stage 63, outer cornea and inner cornea are completely joined with corneal stroma in between (Bard and Abbott, 1979).

In human, cornea formation is largely complete at 5 months gestation. At birth, all components of the cornea are already present in their proper proportions. After the fetal period, the cornea maintains its structure without significant changes in thickness, as the human eye does not change much in size during its lifetime. However, amphibian and fish eyes grow during the whole life of the animal. Reflecting this, adult amphibian and fish have functional retinal stem cells in the peripheral retina, called the ciliary marginal zone, which continue to produce retinal neurons and glial cells (Ohnuma et al., 2002). It is known that the human cornea has limbal stem cells at the periphery, which contribute to the maintenance and repair of the cornea. However, nothing is known about *Xenopus* corneal stem cells.

Metamorphosis, a unique feature of the amphibian lifecycle, adds more complexity to this process. *X. laevis* tadpoles start metamorphosis at stage 48 through activation of thyroid hormone (Nieuwkoop and Faber, 1967). They first develop hind limbs and fore limbs, followed by gradual degeneration of the tail. All processes are complete by stage 66. Along with changes in external appearance, the structures and functions of many internal organs are also altered to be better suited for a new lifestyle and living environment, such as a functional lung that develops to breathe air directly (Pronych and Wassersug, 1994). Dramatic changes also occur in the eye. Before metamorphosis, all retinal ganglion cell axons project to the contralateral tectum in the diencephalon. However, during metamorphosis, they start to project to the ipsilateral side, providing a wider view to cover both front and lateral sides, which is advantageous for catching prey while remaining aware of the surrounding environment (Nakagawa et al., 2000). So far, nothing is known about changes of the *Xenopus* cornea during metamorphosis.

Therefore, this paper investigates details of *Xenopus* corneal development, including embryonic cornea formation, the changes that occur during metamorphosis, the maturation process and the structure of the adult frog cornea. It clearly reveals the potential of using the *Xenopus* cornea as a new system to investigate the detailed molecular mechanisms of human corneal disorders.

2. Experimental procedures

2.1. Xenopus eye tissue processing

Xenopus embryos were obtained by *in vitro* fertilization, and staged according to Niewkoop and Faber (Nieuwkoop and Faber, 1967). Prior to processing, embryos younger than stage 45 were anaesthetised by immersion in 0.1× Modified Barth's Saline (MBS) containing 0.2 mg/ml tricaine methanesulfonate (MS222). Tadpoles staged between 50 and 66 were immersed in MS222 at a concentration ranging from 0.5 to 2 mg/ml. Older and bigger tadpoles require a higher dosage of MS222 for full anaesthetisation. Adult *Xenopus* frogs were anaesthetised by injecting 600 μl of 0.4 g/ml MS222. For *Xenopus* older than stage 50, the tadpoles were decapitated and only heads were processed. In the case of adult frogs, eyes were carefully dissected out and processed.

2.2. Electron microscopy (EM)

Tissues were embedded in epoxy resin for examination by both light and electron microscopes. Tissues were fixed in Karnovsky fixative comprising 3% (v/v) glutaraldehyde, 1% (v/v) paraformaldehyde in 0.08 M sodium cacodylate pH 7.4, followed by secondary fixative 1% (w/v) osmium tetroxide for 2 h. Following osmication, tissues were rinsed in distilled water and then passed through ascending alcohols: 50%, 70%, 90%, 100% for 10–15 min. After ethanol dehydration tissues were passed via propylene oxide (epoxy propane) then a 1:1 mixture of propylene oxide and Araldite to 3–6 h in full resin. Samples were embedded in fresh resin, labeled and placed in a 60 °C oven overnight to polymerize as reported (Coulter, 1967).

After polymerization, the resin was trimmed away from the tissue surface using a Reichert Jung OM-3 ultra-microtome. For light microscopy, thick sections (1 μm) were cut by diamond knife and collected on a glass slide. For EM, thin sections (70–90 nm) were cut and picked up onto mesh grids.