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Effect of BCL6 in Xenopus Eye Development

Nilin Gupta

THE FLORIDA STATE UNIVERSITY

COLLEGE OF MEDICINE

EFFECT OF BCL6 IN XENOPUS EYE DEVELOPMENT

BY

NILIN GUPTA

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The members of the committee approve the thesis of Nilin Gupta defended on 10/18/2010.

_____
Yoichi Kato Professor Directing Thesis

_____
Choogon Lee Committee Member

_____
Branko Stefanovic Committee Member

_____
Jamila I Horabin Committee Member

_____
Wu-Min Deng Committee Member

Approved:

_____
Richard Nowakowski, Chair, Biomedical Sciences

_____
John P Fogarty, Dean, College of Medicine

The Graduate School has verified and approved the above-named committee members.

I dedicated this to my family

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ABSTRACT

BCL6, a protooncogene, is most commonly involved in diffuse large cell lymphomas (DLCL) which is the most common form of non-Hodgkin lymphoma. BCL6 recruits a number of transcriptional corepressors including BCL6 corepressor (BCoR) to function as a transcriptional repressor. BCoR mutation causes two X-linked allelic disorders termed as Oculofaciocardiodental (OFCD) and Lenz microphthalmia syndrome, which are characterized by ocular, dental, cardiac and skeletal anomalies. The involvement of BCL6 in the development of the eye has not been reported so far. Here, we report that BCL6 is essential for eye development in Xenopus embryo prior to the neurula stage. We found BCL6 function is required for eye development prior to stage 11 and loss-of-function studies of BCL6 showed a reduction in expression of *Rx*, *Pax6* and *Otx2*, which play vital roles at the early stage of eye development, in the anterior neural plate. These findings suggest that BCL6 regulates the expression of major eye field transcriptional factors (EFTFs) and controls eye development during the early stages of *Xenopus* development.

CHAPTER 1

INTRODUCTION

1.1.   Development of the eye in vertebrates:

Eye development is a multistep process, which requires specific inductive signals and precise morphogenetic movements. The eye is derived from three types of tissue during embryogenesis: the neural ectoderm gives rise to the retina and the retinal pigment epithelium (RPE), the mesoderm produces the cornea and sclera, and the lens originates from the surface ectoderm (epithelium) (Wong, 2006). The first morphological sign of eye development is the bilateral evagination of diencephalon during the early neurula stage. This evagination of diencephalon is marked as the appearance of the optic pit in mammals, whereas it is called as optic primordial in fish and amphibians. The continued evagination of optic primordial leads to the formation of optic vesicles. The optic vesicles are later connected to the developing central nervous system by a stalk that later becomes the optic nerve (Chow and Lang, 2001). The optic vesicle subsequently makes contact with the head ectoderm and initiates signaling for the pseudostratified thickening of the ectoderm called the lens placode (Wawersik and Maas, 2000). The lens placode subsequently invaginates forming the lens pit, whereas the optic vesicle invaginates to form the optic cup. The optic cup will give rise to the neural retina (NR) and retinal pigmented epithelium (RPE) (Hever et al. 2006). The iris and ciliary body develops from the peripheral edges of the retina. The sclera is derived from mesenchymal cells of neural crest origin, which also migrate to form the cornea and trabecular meshwork of the anterior chamber of the eye (Wong, 2006). There is a deepening of the lens pit, which occurs loosing the connection between the lens pit and the overlying surface ectoderm to form a lens vesicle. The overlying surface ectoderm differentiates into the corneal epithelium and the cells at the posterior of the lens vesicle elongate towards the anterior to form the primary fiber cells. The lumen of the lens vesicle is filled by the primary fiber cells. The peripheral invaginating cells of the lens placode develop into the anterior half of the lens vesicle forming the lens epithelium. The mature lens comprises of an anterior epithelial layer composed of non-proliferating central lens

epithelial cells and a narrow band of proliferating cells known as the germinative zone. The genes important for fiber cell differentiation are expressed in the transitional zone, which lies just posterior to the germinative zone. The post-mitotic region of lens epithelial cells in the transitional zone develops into secondary fiber cells. Immediately posterior to the transitional zone (at the lens equator) cells line up into columns called meridional rows and elongated into secondary fiber cells. In this way, the lens grows continuously throughout the life of the vertebrate organism, progressively adding layer upon layer of secondary fiber cells onto the lens nucleus (Robinson, 2006).
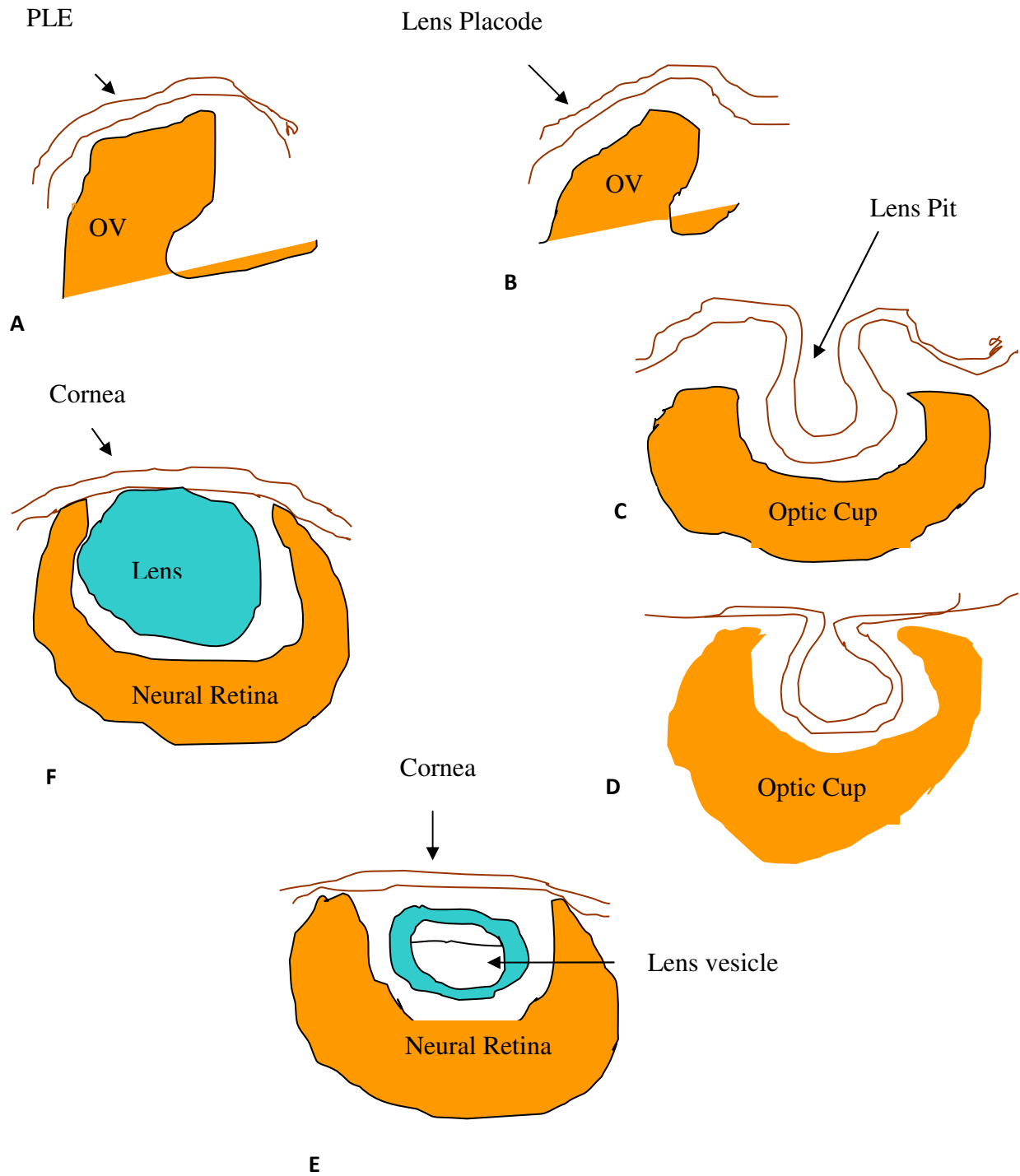
PLE

Lens Placode

OV

OV

Lens Pit

A

B

Cornea

Optic Cup

C

Lens

Neural Retina

Optic Cup

D

F

Cornea

Lens vesicle

Neural Retina

E

Figure 1. Vertebrate eye development

A.The development of eye begins with the invagination of optic vesicle (OV) in the presumptive lens ectoderm. (PLE). B. After physical contact of optic vesicle with PLE, cells within the PLE becomes elongated to form the lens placode. C. The invagination of lens placode gradually forms the lens pit and OV invaginates to form the optic cup. D. The lens pit gradually become enlarges and the contact between the lens pit and overlying surface ectoderm become poor which forms lens vesicle. E. The overlying surface ectoderm differentiated to form the corneal epithelium and cells at the posterior of lens vesicle elongate to form primary fiber cells. The optic cup also differentiated to form neural retina. F. Finally, the primary fiber cells completely fill the lumen of lens vesicle and they make lens epithelium.

1.2. Cascade of events during Xenopus eye development:

Vertebrate eye development needs a series of steps, including specification of the anterior neural plate, evagination of the optic vesicles from the ventral forebrain and the cellular differentiation of the lens and retina (Mathers et al. 1997). Zuber et al. (2003) have proposed a molecular cascade for eye development in *Xenopus*. In this cascade, they propose that the ectoderm is converted into neural plate in response to neural inducers. Later on presumptive forebrain is specified by the regulated expression of *Otx2* for the initiation of eye development. Noggin, which acts as a neural inducer by inhibiting BMP4 signaling (Zimmerman et al. 1996), induces the expression of all the eye field transcription factors such as *Pax6, Six3, Rx, Lhx2, tll, Otx2* and *Optx2*. It is interesting to note that *Otx2* did not have any effect on the induction of other transcription factors. However, *Otx2* plays a significant role in the anterior neural plate by preventing the inhibitory effect of noggin on *ET* which induces the expression of the remaining eye field transcription factors. At stage 12 and 13, there is a loss of *Otx2* expression in the eye field caused by *ET* and *Rx,* but not other eye field transcription factors. *ET* comes on first in the molecular cascade of eye development and induces *Rx,* which inhibits the expression of *Otx2* in the later stages of eye development. The gain-of-function of *ET* induces *Rx* in most of *Xenopus* embryos but not all of the embryos. However, the gain-of-function of *ET* inhibits *Otx2* expression in almost all embryos, which indicates *ET* can suppress *Otx2* in a *Rx* dependent or independent fashion. *ET* lies at the head of the circuit which induces *Rx*, which activates a crossregulatory network of *Pax6*, *Six3*, *Lhx2* and *tll*, followed by *Optx2*, which is induced by *Pax6* (Zuber et al. 2003).
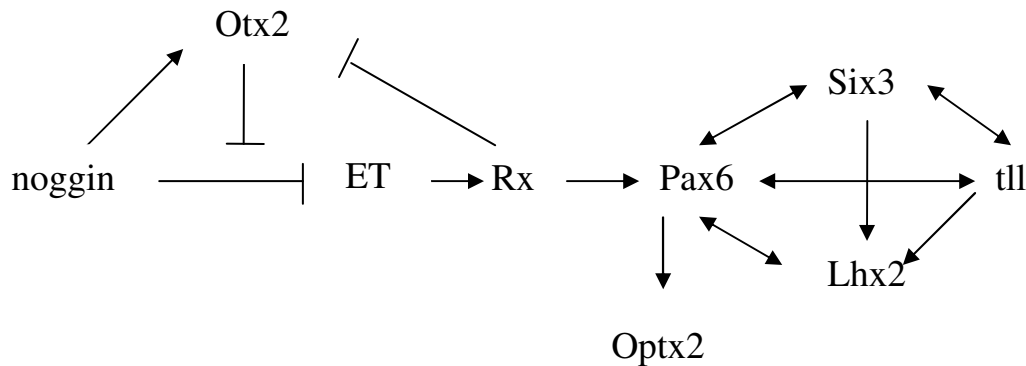
Otx2

noggin ⟶⊣ ET → Rx ⟶ Pax6 ⟷ Six3 ⟷ tll

Pax6 ⟷ Lhx2

Optx2

Figure 2. Model of eye field induction in the anterior neural plate in *Xenopus*

1.3. The major eye field transcription factors in eye development:

1.3.1. Rx: The *Rx* homeobox genes were first isolated as genes involved in anterior head formation in *Xenopus* by Mathers et al. (1997). It contains a highly conserved paired –like homeodomain, octapeptide sequences and conserved motif in the carboxy terminal end of their proteins. The homeodomain regions of these genes are conserved between *Xenopus*, *Drosophila*, *zebrafish, mouse*, *medaka* and *human* (Bailey et al. 2004).

Two *Rx* genes (*Xrx1* and *Xrx2*) have been reported in *Xenopus laevis* so far. The expression pattern of *Xrx1* and *Xrx2* are similar in nature but not identical (Zilinski et al. 2004). The *Xenopus Rx* genes can be first detected at stage 12 (late gastrula stage). The anterior border of *Rx* expression is sharply delineated from the cells of the cement gland anlage, which is the most anterior dorsal structure in *Xenopus* (Mathers et al. 1997). The posterior border of *Rx* expression is in the proximity of the forebrain midbrain boundary. It indicates that the *Rx* early expression domain is primarily localized to the putative forebrain. The expression domain of *Rx* is divided into two fields, which give rise to the optic cups and the primordia of the retina. At the early neurula stage, *Rx* transcripts can be detected in the presumptive retina, diencephalon and

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telencephalon territories. At later stages, its expression is limited to the evaginating eye vesicles, diencephalon floor, pituitary and pineal gland (Lupo et al. 2000). When the optic cups are developed, both presumptive neural retina and pigmented epithelium express *Rx* while no expression is detected in the developing lens and cornea at any stage. In later stages of eye development, *Rx* expression is limited to the retinal ciliary margin, which contains the multipotent retinal stem cells that are required for the generation of retinal cells throughout life. During the later stages of development, *Rx* is reactivated in the photoreceptor cells (Bailey et al. 2004). Loss of function studies using *Rx* dominant negative repressor constructs *(Rx-EnR)* in *Xenopus* resulted in severe impairment of eye and anterior brain development (Andreazzoli et al. 1999).

1.3.2. Otx2: *Otx2* is a member of the orthodenticle-related family of transcription factors, which contain a homeodomain of the *bicoid* class. In *Xenopus*, one of the earliest genes to be expressed in the presumptive anterior neuroectoderm is *Otx2*. *Otx2* expression begins at a very early stage, stage 8, but is clearly visible at stage 9.5. At stage 10.25 *Otx2* shows expression in the dorsal bottle cells and in cells of the dorsal deep zone fated to give rise to the prechordal mesoendoderm, which suggests its role in the specification of anterior structures. At stage 10.5, *Otx2* starts to express in presumptive anterior neuroectoderm, where it persists in subsequent developmental stages (Pannese et al. 1995). *Otx2* shows a dynamic expression pattern during early developmental stages in the anterior neuroectoderm. *Otx2* is expressed in all of the presumptive anterior neuroectoderm at stage 12 but subsequently its expression appears to be repressed in most of the anterior part of the neural plate during stage 12.5-13 (Andreazzoli et al. 1999). At stage 14, *Otx2* expression is restricted to the mesoendodermal and ectodermal cells of anterior dorsal regions (Pannese et al.1995). Gain-of-function studies of *Rx* in *Xenopus* shows that *Rx* inhibits *Otx2* expression during the early neurula stage till the tail bud stage of the *Xenopus* embryo. During the tail bud stage *Otx2* expression again becomes activated and shows expression in the optic vesicles and diencephalon in *Xenopus* (Andreazzoli et al. 1999). Similar results of *Otx2* expression have also been discussed by Zuber et al. (2003). Zuber et al. (2003) suggested that the presumptive forebrain is specified by the regular expression of *Otx2* before eye development initiates in the forebrain. *Otx2* blocks the inhibitory function of noggin in the expression of *ET*. However, at early neurula stage (stage 12.5-13), *ET* induces the expression of

Rx, which in turn suppresses the expression of *Otx2* in the anterior neural plate as mentioned above. This loss of *Otx2* expression between stages 12 and 13 is also synchronized by the induction of several EFTFs in the eye field. Furthermore, the induction of *Rx* by *ET* in the absence of *Otx2* expression was only restricted in the anterior neural plate at the early neurula stage, therefore it was assumed that *Otx2* is not the inducer of EFTFs itself but may provide an environment that primes the anterior neuroectoderm for eye field formation (Zuber et al. 2003).

1.3.3. Pax6 : *Pax6* is a highly conserved family member of transcription factors containing the paired box domain (PD) and homeobox DNA-binding domains followed by a proline-serine-threonine rich domain (Simpson and Price et al. 2002, Treisman et al. 1991). It consists of 422 amino acids. The PD binds with DNA in a bipartite fashion using the N-terminal and C-terminal subdomains. It is involved in the development of the central nervous system and eye development. Walther et al. (1991) isolated the *Pax* genes from mouse on the basis of sequence homology to the *Drosophila gsb-d* paired box. *Pax6* was found to be expressed in the anterior neural plate, optic vesicles, lens and nasal placodes during the early stages of mouse development (Walther et al. 1991, Grindley et al. 1995). In *Xenopus*, *Pax6* has been shown to be expressed in the eye, forebrain, hindbrain and spinal cord and its expression has been observed in two phases of eye development. In the early phase, from stage 12.5 to stage 33/34, *Pax6* is expressed throughout the developing retina. In the late phase, after stage 33/34, *Pax6* is expressed throughout the mature cells in the outer half of the retina, in the cells of the ciliary marginal zone, amacrine and ganglionic cells (Hirsch and Harris, 1997). In *Xenopus*, *Pax6* plays a vital role in formation of the lens. At mid-gastrula stages in *Xenopus*, the ectoderm is transiently competent to respond to lens-inducing signals. During late gastrula stages and commencement of neural tube stages, the presumptive lens ectoderm acquires a lens-forming bias, becomes specified to form lens and begins differentiation, which needs the activation of several genes in particular cascade to regulate the crystalline gene for normal lens development in the eye (Zygar et al. 1998). *Pax6* induces expression of the lens specific marker β1 crystalline without inducing the general neural marker NCAM. Gain-of-function studies of *Pax6* revealed the formation of ectopic lens in whole embryo as well as induction of β1 crystallin in animal cap explants, indicating that *Pax6* plays a pivotal role in lens formation (Altmann et al. 1997, Zygar et al. 1998).

1.3.4. Sox2: *Sox 2* is a member of the *Sox* genes which encode Sry-related transcription factors (Nitta et al. 2006). They contain a single HMG (high mobility group) domain that binds to the DNA in a sequence- specific manner (Kamachi et al. 2000). The *Sox* family genes are further grouped into several subfamily groups classified as *Sox1, Sox2* and *Sox3* on the basis of structural similarity (Kishi et al. 2000). The expression of *Sox2* can first be detected at the early gastrula stage (stage 10.5), where it is expressed in the dorsal ectoderm but not in the deep mesoderm. The expression pattern of *Sox2* is pan-neural throughout embryonic stages, including the central nervous system (CNS), neural crest, placodes and lateral line. The expression of *Sox2* persists till stage 32 in *Xenopus* embryos (Mizuseki et al. 1998). Loss-of-function of *Sox2* inhibited expression of NCAM and other regional neural markers, which indicated *Sox2* plays an important role in the neural differentiation of early *Xenopus* ectoderm (Kishi et al. 2000). *Sox2* has been also speculated to be important for lens development, since *Sox2* regulates the expression of δ-crystallin in chicken and γ-crystallin in mouse (Kamachi et al. 1995).

1.3.5. ET: *ET* is reported to be the initiator of eye field specification. *ET* belongs to the T-box family of genes and is expressed very early in the eye field in *Xenopus* (Li et al. 1997). The T-box genes encode a family of transcription factors sharing a characteristic sequence similarity within the DNA-binding domain (T-domain) (Showell et al. 2004). Furthermore, *ET* is an orthologue of human *Tbx3*. In the *Xenopus* embryo, *ET* expression in the retina primordia begins as a single band across the midline in the anterior neural plate at stage 12.5, the *ET* band of expression persists till stage 15. At stage 16, the band of expression decreases gradually and completely disappears at stage 18. In the later stages, the expression of *ET* in the retina is clearly localized to the dorsal part of the retina but not in the lens or the ventral half of the retina. (Li et al. 1997). *ET* is expressed in the dorsal retina but not in the ventral retina at later stages of eye development. Ectopic expression of *ET* in the *Xenopus* embryo suppressed the molecular markers of ventral retina such as *Pax2* and netrin, and led to inhibition of formation of morphologically visible ventral retina, which indicates its role in dorsal/ventral patterning of the retina at later stages of *Xenopus* development (Wong et al. 2002).

1.4. BCL6 as a transcription repressor:

The proto-oncogene B cell leukemia/lymphoma (BCL6) encodes a nuclear transcriptional repressor, which is frequently translocated in lymphomas. It is responsible for formation of germinal center during normal B cell development (Shaffer et al. 2000, Jardin et al. 2007). BCL6 is located on chromosome 3q27 and was originally identified by virtue of its involvement in chromosomal translocation in diffuse large cell lymphoma (DLCLs) which is an important feature of non-Hodgkin lymphoma (Baron et al.1993, Kerckaert et al. 1993 and Ye et al. 1997). Previous studies had reported that rearrangement of the BCL6 gene can be found in 30-40% of the DLCLs and in a few (5-10%) follicular lymphoms (LoCoco et al. 1994, Bastard et al. 1994 and Otsuki et al. 1995). These rearrangements juxtapose heterologous promoters, derived from the rearranged chromosomes, to the BCL6 coding domain, causing its deregulated expression by a mechanism called promoter substitution (Ye et al. 1997). The BCL6 protein encodes nuclear phosphoprotein transcription repressor having 706 amino acids. It consists of an amino-terminal BTB domain (bric-a-bric, tramtrack, broad complex), also called Pox virus zinc (POZ finger) domain, a middle repression domain (RDII) domain and six zinc finger (ZF) domains at the carboxy terminus (Dhordain et al. 1995). These domains regulate transcription of target genes via distinct interactions. The BTB/POZ domain has a conserved protein-protein interaction motif, by which BCL6 can homodimerize or heterodimerize with other proteins to perform its role in transcriptional repression. The BTB/POZ domain interacts directly with the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor (or its relative N-CoR), which in turn associates with both mSIN3A (a mammalian ortholog of the yeast SIN3 corepressor) and histone deacetylases (HDACs) to constitute a large repressing complex (Jardin et al. 2007). In addition to N-CoR, the BTB domain interacts with several other proteins, which are members of the BTB/POZ –zinc finger family (BAZF, LRF, PLZF). The middle RD II domain contains an additional autonomous *trans* repression domain, which is also responsible for the interaction with mSIN3A. The carboxy terminal BCL6 zinc finger domain binds with the DNA in a sequence specific manner leading to the transcription repression of target genes. This domain has the highest affinity for the core sequence of 9 bp (TTCCT A/CGAA) which has perfect homology with the consensus signal transduction and activators of transcription (STAT) binding site (Jardin et al. 2007). Whole mount in situ hybridization studies to observe the BCL6

expression profile in *Xenopus* embryos revealed that BCL6 is expressed in the nervous system including the eye at different stages of development which suggested that BCL6 may be essential for development of the eye from early to later stages (Sakano et al. 2010).

1.5. Hypothesis:

The role of BCL6 in diffuse large B cell lymphomas and in development of normal B cells has been well studied, but the function of BCL6 in embryogenesis is poorly understood. Recent study describing the expression profile of BCL6 in *Xenopus* embryos demonstrated that BCL6 is expressed in several ectodermal and mesodermal tissues including the eye and nervous system (Sakano et al. 2010). The role of the BCoR, a transcriptional corepressor, in the development of the eye has already been shown in *Xenopus* (Hilton et al. 2007), zebrafish (Ng et al. 2004) and human (Ng et al. 2004. Hilton et al. 2009). Loss-of-function of BCoR leads to development of different types of eye disorders such as microphthalmia (small eye) and anophthalmia (absence of eye) in *Xenopus tropicalis* embryos (Hilton et al. 2007). BCoR was originally identified by its ability to interact with the site specific transcriptional repressor BCL6 with its BTB/POZ domain and serves as a transcriptional corepressor (Wamstad et al. 2008). Furthermore, the BCoR expression from E13.5 in eye till later developmental stages of eye in mouse has already been characterized (Wamstad and Bardwell, 2007). This literature led us to investigate the role of BCL6 in the development of the eye in *Xenopus* embryos, which has not been studied so far. Therefore, my work is intended to investigate the role of BCL6 in development of the eye in *Xenopus*.

CHAPTER 2

MATERIAL AND METHODS

2.1. Collection and fertilization of eggs: The female frogs are superovulated by injecting human chorionic gonadotropin hormone (hCG) for approximately 12 hours before the time of use. The eggs were collected from a female frog into a petridish. The male frog was sacrificed by giving transdermal injection of 3-aminobenzoic acid ethyl ester for collection of testis. The testis was stored in 1X Modified Bath solution (MBSH) buffer at 4 ° C. A fragment of testis is mixed with freshly obtained eggs for *in vitro* fertilization. Fertilized embryos were kept in 0.1X Marc's Modified Ringer's solution (MMR) at 14 ~ 23 °C for microinjection and other experiments (Peng, 1991). Embryos were staged according to the method described by Nieuwkoop and Faber (1967).

2.2. Preparation of Synthetic RNA and Morpholino Antisense Oligo for microinjection: Capped synthetic mRNAs were generated by *in vitro* transcription with SP6 polymerase, using mMessage mMachine *in vitro* transcription kits (Ambion, Inc.). The *X. laevis* BCL6 (IMAGE ID: 5513995) clone was purchased from Open Biosystems. The fragment of mBCL6 (mutant BCL6) was sub-cloned into pCS2 + 6myc vector and pCS2 + 6myc-GR vector, which contains the glucocorticoid receptor binding domain (GR). The zinc finger domain (ZF) of *BCL6* was sub-cloned into pCS2 + 6 myc-nls-GR vector as described by Kolm and Sive (1995). BCL6 MO and Control MO were designed and produced by Gene Tools, LLC. BCL6 MO was designed according to the sequence of *X. laevis* BCL6 (accession number: NM_001093558). The sequences of BCL6 MO and Control MO were:

BCL6 MO: 5'-TTGAGTTTGAGATGCCATAGTGCCC -3'

Control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

2.3: Microinjection in embryos: Microinjection of embryos was accomplished using an IM-300 Microinjector (Narishige International USA, Inc). For functional assays, the embryos were injected with RNA or MO in the left dorsal blastomere at the 4 cell stage. The injections

were performed in 3% Ficoll 400 in 0.1 X MMR. Injected embryos were transferred into 0.1X MMR and then cultured until they reached the desired stages. Dexamethasone solution (10 µM dexamethasone in 0.1 X MMR) was used for induction of the hormone fused protein construct in the injected embryos. Dexamethasone was changed daily when embryos were cultured longer than 24 hours. A hundred picograms of β-gal RNA was injected with other desired RNAs for functional assays and used as a tracer of injection or an injection control.

2.4: β-Galactosidase Staining and Whole-Mount in Situ Hybridization:

Two different methods were used for β-galactosidase staining for different probes:

1. Embryos were fixed with gal fix solution (2% formaldehyde, 0.2% glutaraldehyde, 0.02% Triton X-100, 0.01% sodium deoxycholate in PBS) on ice for 30 min. followed by β-gal staining.

2. Embryos were fixed for 30 minutes at room temperature in 0.02 % Triton X-100 in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) followed by β-gal staining.

Galactosidase activity was visualized with the Red Gal substrate (Research Organics) in staining buffer (5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2mM $MgCl_2$ in PBS). The embryos were refixed in MEMFA solution for 30 minutes after staining, and subjected to Whole-mount in situ hybridization (WISH) as described earlier (Harland, 1991; Takada et al., 2005) by using Digoxigenin (Roche Applied Science)-labeled antisense RNA probes and BM purple (Roche Applied Science) for the chromogenic reaction. RNA probes for *Rx* (Mathers et al. 1997), *Pax6* (Hirsch and Harris, 1997), *Otx2* (Pannese et al. 1995), *ET* (Takabatake et al. 2000) and *Sox2* (Mizuseki et al. 1998) were used for WISH.

CHAPTER 3

RESULT

3.1: BCL6 is essential for normal development of the eye:

In order to examine the role of BCL6 in eye development, we first performed loss-of-function studies of BCL6 in *Xenopus* embryos by injecting the antisense Morpholino oligo against BCL6 (BCL6MO) in the left dorsal blastomere of 4 cell stage embryos. The BCL6MO was constructed in such a manner so that it binds with the ATG site of mRNA and thus inhibits translation of the endogenous target protein (Heasman et al., 2000). The effect of BCL6MO has already been confirmed in our previous work (Sakano et al. 2010). Forty nanograms of BCL6 MO were injected into a left dorsal blastomere of 4 cell stage embryos (Figure 4) to observe the effect of depletion of endogenous BCL6 in eye development. The results showed that 58.33 % of injected embryo had eye anomalies (microphthalmia and anophthalmia) (Figure 5 and Table 1). To show the specificity of these eye phenotypes with the depletion of endogenous BCL6, we co-injected BCL6MO and 2 ng of mutated BCL6 RNA (mBCL6) into a left dorsal blastomere of 4 cell stage embryo. mBCL6 has a having Myc tag in the translation initiation site so that it is not been recognized by BCL6 MO. The co-injection of BCL6MO and mBCL6 RNA rescued the eye phenotype (58.33% to 25.58 %) (Table 1). It indicates that BCL6 plays an important role in the development of the eye.

3.2. BCL6 is essential for eye development during the early developmental stages:

Now, the question arises that at what stage BCL6 is necessary for development of the eye. To determine the stage at which BCL6 is essential for *Xenopus* eye development, we injected the hormone-inducible dominant negative construct of BCL6-GR RNA (dn- BCL6-GR RNA) at the same site mentioned above. This dominant negative BCL6-GR construct comprises of Zn finger domain of BCL6 so that it competitively inhibits DNA-binding function of endogenous BCL6 (Sakano et al.2010). The hormone-inducible dn-BCL6-GR allows us to

activate dn-BCL6 at different developmental stages by adding dexamethasone in the medium at desired developmental stages. Earlier studies mention that EFTFs such as *Otx2* and *Sox2* are starting to express during the gastrula stage in *Xenopus* for eye development. In addition, the previous study has shown the expression of BCL6 during stage 7 (late blastula stage) at the animal hemisphere (Sakano et al. 2010). In this context, we initially chose stages 6, 9, 11.5, 15, 19 for activation of dn-BCL6-GR to investigate the stage at which BCL6 is essential for eye development. Two nanograms of dn-BCL6-GR RNA was injected at the same site as mentioned above at the 4-cell stage and treated with dexamethasone at respective developmental stages. We observed eye defect phenotypes (62.16 % and 39.50 %) when dn-BCL6-GR was activated at stage 6 and 9, respectively (Figure 6 and Table 2). However, we did not get any eye phenotypes when dn-BCL6-GR was activated at stages 11.5, 15 and 19 (Table 2 and data not shown). To show specificity of dn-BCL6-GR on eye phenotype, we co-injected 2 ng of dn-BCL6-GR and 2 ng of BCL6-GR into the left dorsal blastomere of 4 cell stage embryos and treated them with dexamethasone at stage 9. This co-injection rescued the eye phenotype (65.30 % to 32.65%), which ensures that the eye defect by dn-BCL6-GR is specific to BCL6 function (Table 3). These results indicated that BCL6 is essential for eye formation prior to the neurula stage.

3.3. BCL6 regulates the expression of some EFTFs in eye development:

Previous studies revealed that the eye anlagen is specified by a group of eye field transcription factors (EFTFs) at the anterior neural plate. In order to determine how BCL6 regulates early eye development, the expression of some EFTFs including *Rx*, *Pax6*, *Otx2* and *Sox2* at early neurula stage was examined. We injected 2 ng of dn-BCL6-GR RNA into a left dorsal blastomere of 4 cell stage embryos and added dexamethasone to the medium for activation of the dn-BCL6-GR right after injection. At stage 14, the injected embryos were subjected to WISH for the EFTFs mentioned above. The expression of *Rx* (29.63%), *Pax6* (26.88%) and *Otx2* (73.91%) in the anterior neural plate was suppressed by dn-BCL6-GR in *Xenopus* embryos (Figure 7 and Table 4). However, *Sox2* expression was not reduced by dn-BCL6-GR in the anterior neural plate (Figure 7 and Table 4). To show the specificity of dn-BCL6 effect on the suppression of Rx, *Pax6* and *Otx2* expression, we co-injected dn-BCL6-GR along with 2ng of mBCL6-GR at the same site of injection as mentioned above and treated the injected embryos with dexamethasone right after injection. The co-injection of dn-BCL6-GR and mBCL6-GR

restored the expression level of *Rx* (29.63 to 11.62%), *Pax6* (26.88 to 8.57%) and *Otx2* (73.91 to 0 %) to normal (Table 4). These results indicate that BCL6 may be located upstream of *Otx2* in the molecular cascade of eye development (Figure 3) and maintains the expression of EFTFs for eye development. However, BCL6 does not appear to play a significant role in the regulation of *Sox2*.

CHAPTER 4

DISCUSSION

In this study, we found that BCL6 is essential for eye development prior to the neurula stage of the developing *Xenopus* embryo. BCL6 plays a vital role to regulate the expression of *Otx2*, *Rx*, and *Pax6* but not *Sox2* in the anterior neural plate at the early neurula stage.

BCL6 is essential at the early neurula stage for development of *Xenopus* eye. There were 73.91% of embryos with a reduction in the expression of *Otx2* on depletion of endogenous BCL6. Therefore, we assume that BCL6 is upstream of *Otx2* and is responsible for maintaining *Otx2* expression in the neural plate at stage 14. However, the molecular mechanism of how BCL6 maintains the expression of Otx2 still remains unclear. A previous study mentioned that BMP4 and *Otx2* are expressed together in the anterior region of embryos at stage 13.5 (Gammil and Sive, 2000).The primary function of the BMPs is to act as mesoderm ventralizers and epidermal inducers at the expense of the dorsal mesoderm and neural tissue. BMP signaling is a simple linear cascade that involves the BMP ligands, two types of receptors (type I and type II) and the signal transducers, Smads. BMP signaling is activated by binding of BMP ligands to BMP receptors (serine/threonine kinase receptors) followed by the activation of BMP-Smad and/or BMP-MAP kinase pathways. After binding of BMP ligands to the BMP receptors, the serine/threonine kinase domains of the type II receptors are constitutively active, and phosphorylate Gly-Ser (GS) domains in the type I receptors, which activates type I receptor kinases. The activated type I receptors phosphorylate the receptor Smad (R-Smad) such as Smad1/5/8. Smad1 forms a complex with Smad4 (Co-Smad) after phosphorylation. The complex of Smad1 and Smad4 is then translocated to the nucleus (Hata et al. 1998, Chang and Harland, 2007) and initiates transcription of the downstream target genes such as *Xvent* (Onichtchouk et al. 1998) and *Xmsx1*(Ishimura et al.2000). Since both *Xvent and Xmsx1* are expressed at the early gastrula stage in *Xenopus* and act as a transcriptional repressor for anterior neural markers for neural induction ((Hata et al. 1998, Chang and Harland, 2007), BMP signaling possibly inhibits

Otx2 expression by inducing the *Xvent* and *Xmsx1* genes through Smad1 in the anterior neural plate. Interestingly, BCL6 inhibits BMP signaling by competing with Smad1 for Smad4 in B lymphoma cells (Wang et al. 2008). Based on these studies and our data, BCL6 may maintain *Otx2* expression by inhibiting BMP signaling through making a complex with Smad4 (BCL6-Smad4) and interfering with Smad1/Smad4 complex translocation to nucleus.

In loss-of-function studies of BCL6, we found the inhibitory effect of BCL6MO in expression of *Rx* (29.63%) and *Pax6* (26.88%) is not as severe as that in expression of *Otx*2 (73.91%). According to the molecular cascade of EFTFs in the development of the *Xenopus* eye (Zuber et al. 2003), *Otx2* maintains the expression of *ET*, which is a master gene in eye development and induces the expression of *Rx* and *Pax6* in the anterior neural plate at the neurula stage. But the question still arises why inhibitory effect of BCL6MO in expression of *Otx2* was detected in higher frequency of embryos in comparison to *Rx* and *Pax6*. The previous study reported that the loss-of -function of Wnt4, an inducer of non-canonical Wnt signaling in vertebrates, in *Xenopus* resulted in the downregulation of *Rx* and *Pax6* at the injected site whereas no effect on *Otx2* was detected at stage 13/14. It indicates that Wnt4 regulates the expression of *Rx* and *Pax6* in an *Otx2*-independent manner (Maurus et al. 2005). *Rx* and *Pax6* expression are likely regulated by both *Otx2*-dependent and Wnt4-dependent pathways during the early stages of *Xenopus* eye development. Therefore, milder inhibitory effect of BCL6MO in expression of *Rx* and *Pax6* were observed in our loss-of-function studies of BCL6. In addition, it still remains unsolved if the expression of *ET* is suppressed in BCL6-depleted embryos. According to Zuber et al. (2003), the expression of *ET* is maintained by *Otx2*, so it should be suppressed in the BCL6-depleted embryos. To support our findings on BCL6 function in eye development, a study to investigate the effect of BCL6 on *ET* is also needed. There are several promising transcription factors still unidentified which could regulate *Otx2*, *Rx* and *Pax6*. Therefore, experiments aimed at analyzing the *Xenopus Rx*, *Otx2* and *Pax6* promoter are currently underway.

While *Sox2* and Otx2 are induced by BMP inhibitors such as noggin (Lamb and Harland, 1995; Monsoro-Burq et al. 2005), we did not observe any change in expression of *Sox2* in the anterior neural plate of *Xenopus* embryo by injecting BCL6 MO, which might be due to different additional factors required for regulation of *Sox2* versus *Otx2*. *Sox2* is expressed widely in the

dorsal ectoderm and its expression is pan-neural throughout embryonic stages including in the central nervous system, neural crest, and placodes (Mizuseki et al. 1998). The previous studies investigated that loss-of-function of FGF signaling causes a significant reduction in the expression of *Sox2* during the neurula stage but not gastrula stage, which indicated that FGF signaling is essential for maintenance of *Sox2* but not for its induction in *Xenopus* during neural differentiation (Rogers et al. 2008). It might be possible that the induction of *Sox2* needs the inhibition of BMP signaling and the activation of FGF signaling but that of *Otx2* requires the inhibition of BMP signaling and other unknown factors. Therefore, BCL6 does not affect the expression of *Sox2* due to difference of regulatory mechanisms of their transcription. Understanding the regulatory mechanism of *Sox2* and *Otx2* expression by characterizing their promoters is necessary to further address this important question.

BCoR interacts with BCL6 in a specific manner, with the BCL6 BTB/POZ domain and serves as a transcriptional corepressor (Wamstad et al. 2008). Previous studies reported that mutation in BCoR caused eye defects such as microphthalmia, anophthalmia, colobama and pigmented optic nerve in *Xenopus tropicalis* (Hilton et al. 2007). However, we still do not know if BCL6 recruits BCoR as a co-repressor in eye development. To address whether BCL6 and BCoR are working together prior to the neurula stage of *Xenopus* during eye development, future studies are needed. These studies will complete our understanding of the molecular mechanism of BCoR associated eye disorders which are included in human syndromes, OFCD and Lenz microphthalmia syndrome.

Here, we reported that BCL6 is essential prior to the neurula stage in *Xenopus* eye development. However, BCL6 also shows expression in the *Xenopus* eye at a later stage of embryonic development (stage 25) (Sakano et al. 2010). Further studies are required to investigate the functional role of BCL6 during the later stages of eye development in *Xenopus*. This study will contribute to complete understanding of BCL6 role in eye development.
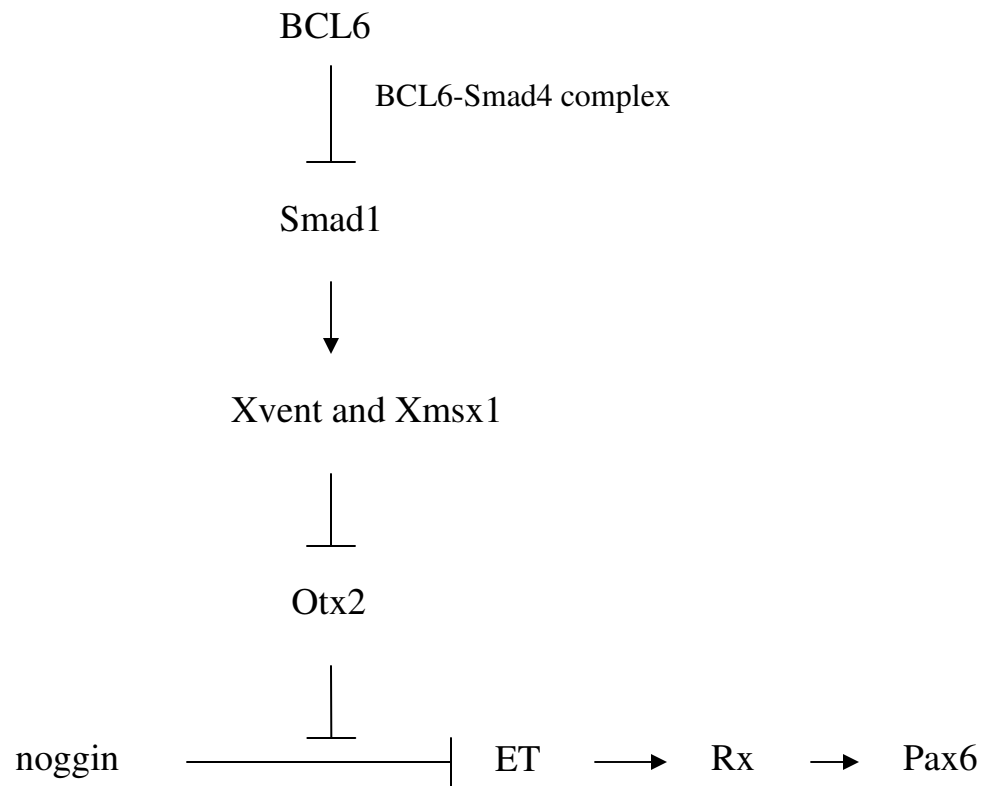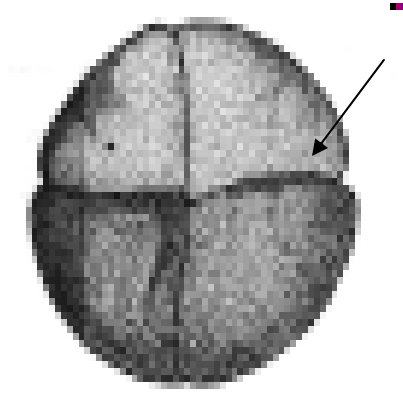
BCL6

$\perp$ BCL6-Smad4 complex

Smad1

$\downarrow$

Xvent and Xmsx1

$\perp$

Otx2

$\perp$

noggin ————————⊣ ET ⟶ Rx ⟶ Pax6

Figure 3. A working model for the regulation of EFTFs by BCL6 for eye development in *Xenopus*.
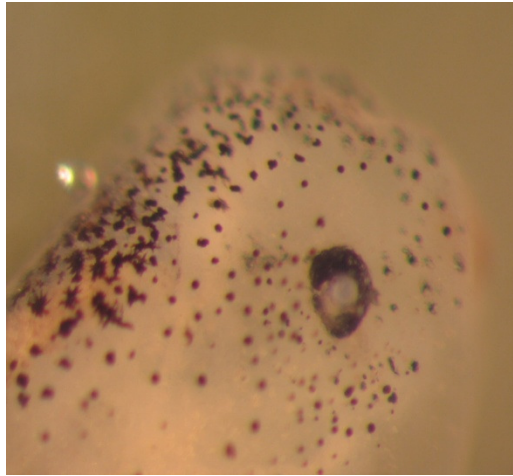
Dorsal side

Ventral side

Figure 4. Site of injection in 4 cell stage *Xenopus* embryo

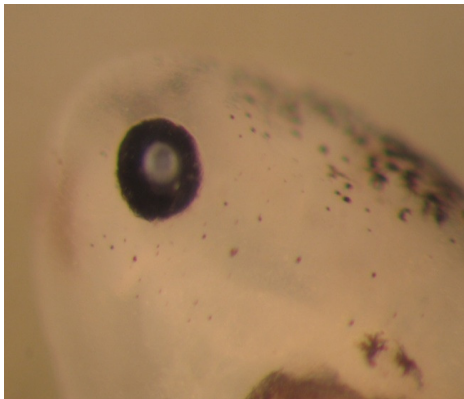This is an animal view and an arrow indicates the site of injection.
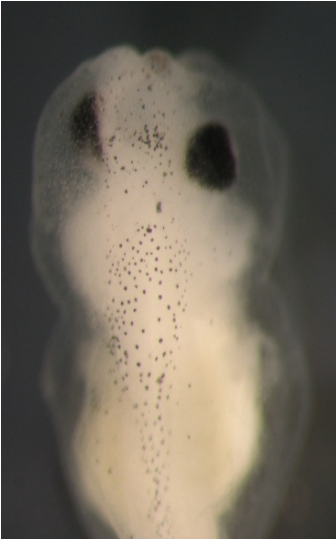
A.

B.

C.

Figure 5. The depletion of BCL6 leads to the defect of eye development.

A. Microphthalmia in the side of injection. B. Microphthalmia in injected eye along with defect
in retinal pigmentation. C. Normal eye of opposite side of same embryo.
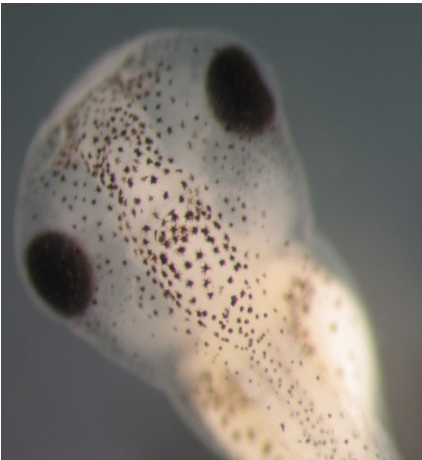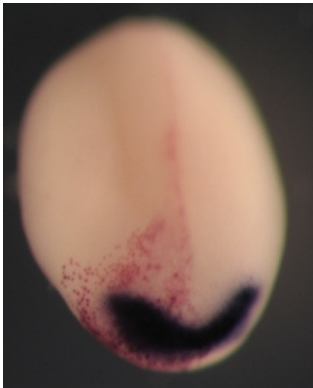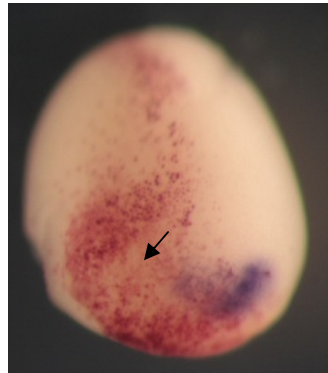
A. B.

C.

Figure 6. Inhibition of BCL6 function by dn-BCL6-GR causes microphthalmia.

A. Injection of dn-BCL6-GR resulted into microphthalmia in injected side. B. Injection of dn-BCL6-GR resulted into microphthalmia along with abnormal retinal pigmentation, absence of lens and other tissues of eye. C. Normal eye of uninjected *Xenopus* embryo.
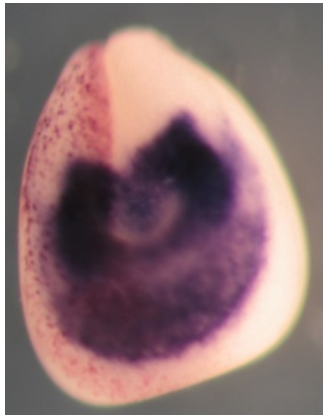
A.

B.

C.

D.
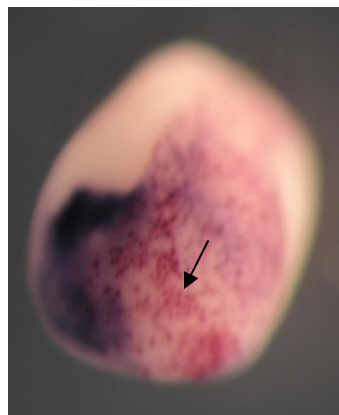
E.
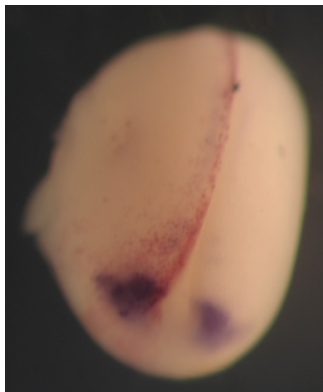
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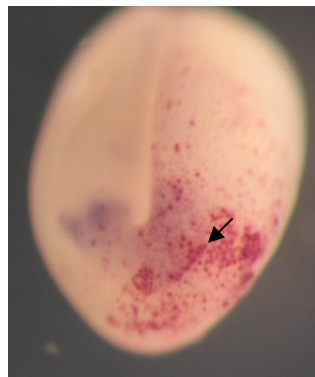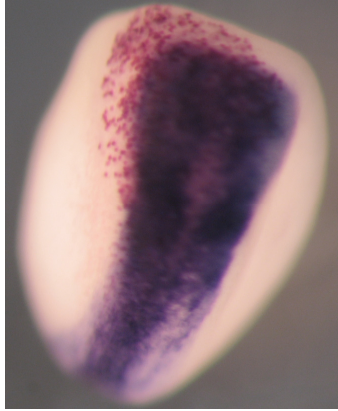
G.   **H.**
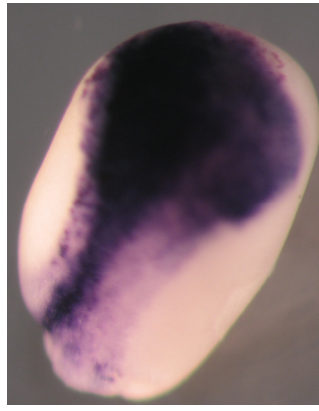
Figure 7. Dysfunction of BCL6 leads to the suppression of Rx, Otx2 and *Pax6* but not *Sox2* in the anterior neural plate of *Xenopus* embryo.

A. Normal expression pattern of *Rx* in the anterior neural plate at stage 14 in β-gal injected embryos. β-gal staining shows overlapping with Rx at the injection site. B. An arrow indicating reduction of *Rx* expression at site of injection in the anterior neural plate. C. Normal expression of *Otx2* in the anterior neural plate at stage 14 in β-gal injected embryos. D. An arrow indicating the reduction in *Otx2* expression in the anterior neural plate at stage 14. E. Normal *Pax6* expression pattern in the anterior neural plate at stage 14 in β-gal injected embryos. F. An arrow indicating reduction in expression of *Pax6* in the anterior neural plate. G. Normal *Sox2* expression at stage 14 in β-gal injected embryos. H. No change in *Sox2* expression in dn-BCL6-GR injected embryos.

Table 1. BCL6 is essential for normal development of *Xenopus* eye .

Type of inj.	Micropthalmia	Anopthalmia	Total Phenotype	Normal	Total	%
BCL6 MO	19	9	28	20	48	58.33
BCL6 MO + BCL6 RNA	9	2	11	32	43	25.58
Uninjected	--	--	--	57	57	0%

Table 2.   BCL6 is essential for development of *Xenopus* eye prior to the neurula stage.

Stage at which Dexamethasone added	Micropthalmia	Anopthalmia	Total Phenotype	Normal	Total	%
6	10	13	23	14	37	62.16
9	12	5	17	26	43	39.50
11.5	--	--	---	15	15	0 %
15`	--	--	---	37	37	0%

Table 3. To show the specificity of dn-BCL6-GR for BCL6 function in *Xenopus* eye.

Type of inj	Micropthalmia	Anopthalmia	Total Phenotype	Normal	Total embryos	%
Dn-BCL6 - GR	15	17	32	17	49	65.30
Dn-BCL6 - GR +BCL6 GR	8	8	16	33	49	32.65
Uninjected	--	--	--	55	55	0

Table 4. Loss of function studies of BCL6 on *Rx*, *Otx2*, *Pax6* and *Sox2* along with rescue experiment.

Probe	Type of Injection	Reduction in expression of EFTFs	Normal embryos	Total embryos	%
Otx2	dn-BCL6GR	17	6	23	73.91
	dn-BCL6 GR + BCL6 GR	---	11	11	0
Rx	dn-BCL6-GR	24	57	81	29.63
	dn-BCL6 GR + BCL6GR	5	38	43	11.62
Pax6	dn-BCL6-GR	25	68	93	26.88
	dn-BCL6 GR + BCL6GR	6	64	70	8.57
Sox2	dn-BCL6-GR	0	21	21	0
	dn-BCL6 GR + BCL6GR	0	39	39	0

APPENDIX A

THE FLORIDA STATE UNIVERSITY
LABORATORY ANIMAL RESOURCES

certifies that

Nilin Gupta

has completed the

FSU Animal Care and Use Committee
Mandated Video Training

November 6, 2007

Dr. Marc Freeman, Chair
Animal Care and Use Committee

Joelle Constant, Secretary
Animal Care and Use Committee

Dr. Kathleen M. Harper, Interim Director
Laboratory Animal Resources
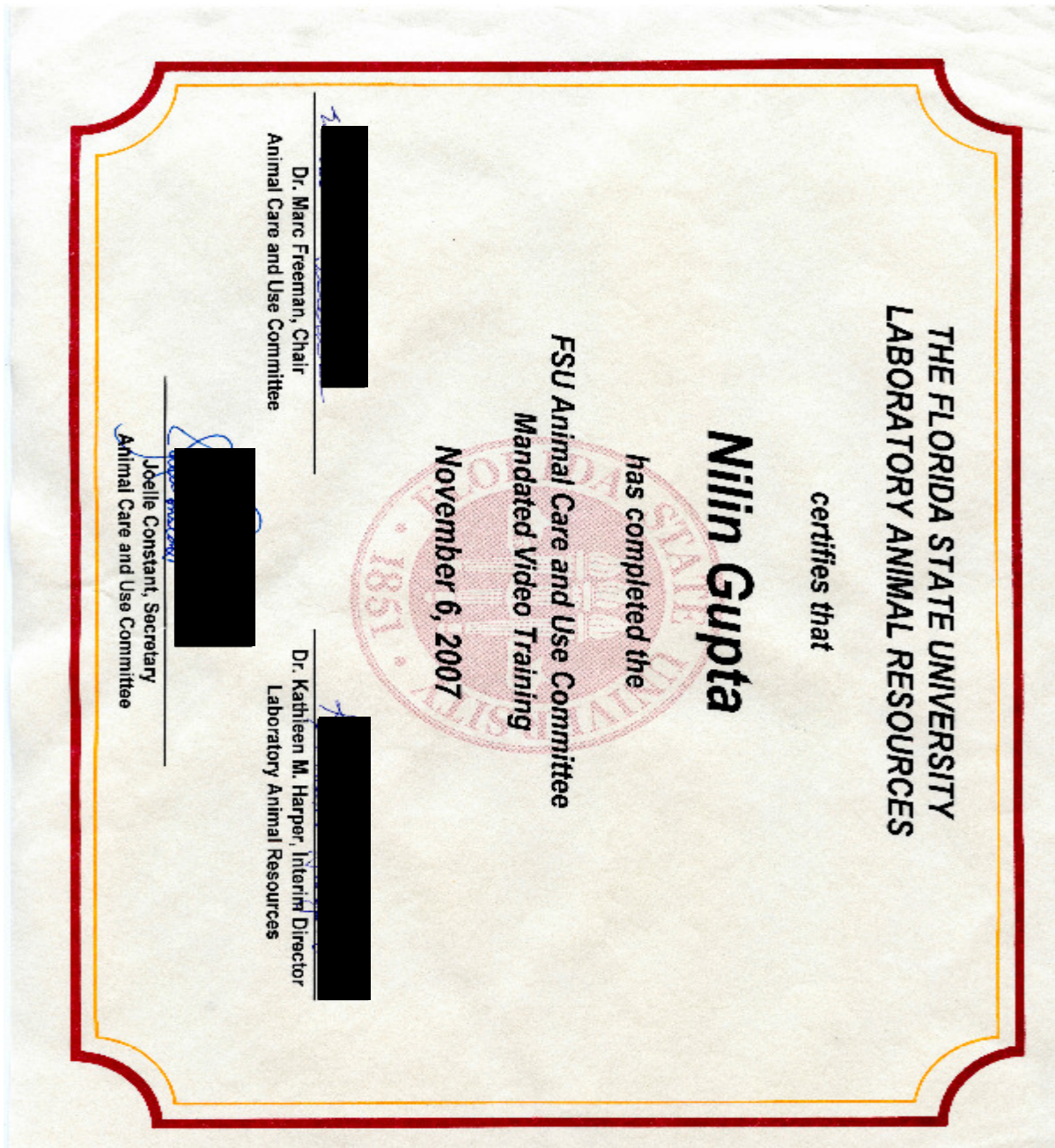
APPENDIX B

Animal Care and Use Committee (ACUC)
101 Biomedical Research Facility
P.O. Box 3064341
Tallahassee, FL 32306-4341
Telephone: 644-4262   Fax: 644-5570
Mail Code: 4341 Email: acucsecretary@mailer.fsu.edu

M E M O R A N D U M

TO: Dr. Yoichi Kato
             Department of Biomedical Sciences
             College of Medicine

FROM: Dr. Elaine Hull, Chair
             Animal Care and Use Committee

SUBJECT: Protocol #0915

DATE: **June 10, 2009**

"YOUR TRIENNIAL PROTOCOL REVIEW HAS BEEN APPROVED"

The Animal Care and Use Committee confirmed approval of the triennial review of **Protocol #0915 (previously #0310), "The Mechanism of Notch Signaling Pathway in Radial Glial Development"** at the **May 28, 2009** ACUC meeting. You are approved for the following species and numbers for the proposed protocol approval period.

Species	Number Animals Approved	Protocol Approval Expiration Date	Rewrite Due
Rats	90		
Xenopus laevis	500	May 31, 2012	April 1, 2012

Enclosed for your records are:
- ✓ A copy of the **Committee Comments**
- ✓ A copy of the **Protocol**
- ✓ The **original of page one for signature and return in the enclosed envelope**

When you order animals on this protocol, please remember to convey the ACUC number to the LAR at 644-4262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Auter at kauter@fsu.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the LAR Facility Manager.

:

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

EMH/kmh
Enclosures

Animal Care and Use Committee (ACUC)
PROTOCOL REVIEW
COMMITTEE COMMENTS AND ACTION

Reviewed by the Animal Care and Use Committee on May 28, 2009

Comments:

Rewrite

#0915, Dr. Yoichi Kato, Approved, non-USDA covered species
Regarding the triannual rewrite of protocol #0915, "The mechanism of Notch signaling pathway in radial glial development" a summary overview was provided by Dr. Lee. This research proposes to use Xenopus and rats to examine the molecular mechanism of radial glial development. Understanding of molecular mechanism of radial glial development will contribute to the identifying genes responsible for congenital disorders of the nervous system and brain cancer as well as the development of treatment for those diseases. There were no questions or comments.

It was moved and seconded to approve the rewrite of protocol #0915. Approved unanimously.


OFFICIAL ACTION

APPROVED	REQUIRES MODIFICATIONS	DISAPPROVED

Dr. Elaine M. Hull, ACUC Chair

June 10, 2009
Date

Dr. Kathleen Harper, LAR Director and
Attending Veterinarian

June 10, 2009
Date

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BIOGRAPHICAL SKETCH

EDUCATION

December 2010   M.S. in Biomedical Sciences
                       Florida State University, Tallahassee, FL
                       GPA: 3.78/4.00

October 2003   M.V.Sc in Animal Nutrition
                       G.B. Pant University of Agriculture & Technology
                       Pantnagar, India
                       GPA:3.58/4.00

December 2000   B.V.Sc. & A.H. in Veterinary Sciences
                       I.G. Agriculture University, Raipur, India.
                       GPA: 3.94/4.00

AWARD

1. Received University Fellowship 2006-2007 from Florida State University, Tallahassee, Florida.
2. Received merit based annual scholarship consecutively for 3 years during session 1996-97; 1997-98; 1998-99 by I.G.A.U., Raipur.

PUBLICATIONS

1. Gupta, N., Kumar, A. and Tiwari, D. P. (2006). Effect of herbs as feed additive on haemato-biochemical constituents in growing crossbred heifers fed paddy straw based ration. Indian Journal of Animal Sciences, *76* , 528-31.
2. Gupta, N., Kumar, A. and Tiwari, D. P. (2005). Effect of herbs as feed additive on nutrient utilization and growth in crossbred heifers fed paddy straw based ration. Indian Journal of Animal Sciences, 75, 52-55.
3. Kumar, A., Pandey, I., Gupta, N. and Tiwari, D. P. (2006). Effect of high roughage diet on enzyme profile and biochemical changes in the rumen of crossbred bullocks. Indian Journal of Animal Sciences, *76* , 633-37.
4. Kumar, A., Gupta, N. and Tiwari, D.P. (2006). Effect of herbs as feed additive on *in vitro* and *in sacco* dry matter digestibility of paddy straw. Indian Journal of Animal Sciences 76, 847-850.
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