A distinct effect of transient and sustained upregulation of cellular factor XIII in the goldfish retina and optic nerve on optic nerve regeneration

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Abbreviations : FXIII-A, factor XIII A subunit; cFXIII, cellular factor XIII; CNS, central nervous system; RGC, retinal ganglion cell; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; TG, transglutaminase; TG_R, retinal transglutaminase; RACE, rapid amplification of cDNA ends; RT, reverse transcription;

Running title: Cellular factor XIII in goldfish optic nerve regeneration

Abstract

Unlike in mammals, fish retinal ganglion cells (RGCs) have a capacity to repair their axons even after optic nerve transection. In our previous study, we isolated a tissue type transglutaminase (TG) from axotomized goldfish retina. The levels of retinal TG (TG_R) mRNA increased in RGCs 1–6 weeks after nerve injury to promote optic nerve regeneration both *in vitro* and *in vivo*. In the present study, we screened other types of TG using specific FITC-labeled substrate peptides to elucidate the implications for optic nerve regeneration. This screening showed that the activity of only cellular coagulation factor XIII (cFXIII) was increased in goldfish optic nerves just after nerve injury. We therefore cloned a full-length cDNA clone of FXIII A subunit (FXIII-A) and studied temporal changes of FXIII-A expression in goldfish optic nerve and retina during regeneration. FXIII-A mRNA was initially detected at the crush site of the optic nerve 1 h after injury; it was further observed in the optic nerve and achieved sustained long-term expression (1–40 days after nerve injury). The cells producing FXIII-A were astrocytes/microglial cells in the optic nerve. By contrast, the expression of FXIII-A mRNA and protein was upregulated in RGCs for a shorter time (3–10 days after nerve injury). Overexpression of FXIII-A in RGCs achieved by lipofection induced significant neurite outgrowth from unprimed retina, but not from primed retina with pretreatment of nerve injury. Addition of extracts of optic nerves with injury induced significant neurite outgrowth from primed retina, but not from unprimed retina without pretreatment of nerve injury. The transient increase of cFXIII in RGCs promotes neurite sprouting from injured RGCs, whereas the sustained increase of cFXIII in optic nerves facilitates neurite elongation from regrowing axons.

Key words: cellular factor XIII; transglutaminase; CNS regeneration; retina; optic nerve;

goldfish

1. Introduction

Central nervous system (CNS) neurons in fish can regrow their axons and restore their functions even after nerve transection, while CNS neurons in mammals lose the capacity to regenerate. Therefore, during the recent decade we have been searching for regeneration-associated genes involved in fish optic nerve regeneration using fish visual systems (Liu et al., 2002; Matsukawa et al., 2004; Sugitani et al., 2006; Koriyama et al., 2007; Nagashima et al., 2011). We succeeded in cloning a tissue type transglutaminase (TG), a cross-linking enzyme from axotomized goldfish retina (Sugitani et al., 2006). Retinal tissue type TG (TG_R) mRNA levels increased in the retina 1–6 weeks after optic nerve injury. We further reported that TG_R promoted optic nerve regeneration both in fish and rats (Sugitani et al., 2006).

In humans, the TG family (EC 2.3.2.13) is subdivided into at least eight members (TG 1–7 and coagulation factor XIII). TG1 is found in keratinocytes, TG2 is distributed in all tissues, TG3 is found in skin epithelial cells, and TG4 is found in prostate. The distributions and functions of the other types of TG (TG 5–7) have not been elucidated. In contrast, coagulation factor XIII (FXIII) is one of the most studied TG members. FXIII has two major functions. It is a plasma TG that acts as a blood coagulation factor, participates in the process of cross-linking between fibrin molecules, and contributes to the stabilization of clot formation. FXIII exists in plasma as A_2B_2 heterodimers comprised of two catalytic A subunits (A_2) and two carrier B subunits (B_2) of the inactive form (Muszbek et al., 1996; Lorand 2001). In addition, a number of reports have demonstrated that FXIII exists as homodimers of A subunits (A_2) in various types of cells, such as hepatocytes,

monocytes/macrophages and platelets (Kiesselbach and Wagner, 1972; Muszbek et al., 1985; Adány and Antal, 1996; Adány and Bárdos, 2003). The intracellular type of FXIII, known as “cellular factor XIII (cFXIII)”, has been considered to be the source of coagulation FXIII in plasma. Further studies demonstrated that cFXIII is widely distributed in various types of tissue, including lung, kidney, stomach, skin, esophagus, liver, testis, bone and spleen (Derrick et al., 1993; Quatresooz et al., 2008). The variety of tissue distribution of cFXIII, therefore, suggests that active FXIII A subunit (FXIII-A) has another important function in addition to its role in clot formation and wound healing (Muszbek et al., 2011).

Since the enzymes of the TG family have a common active site, it is very difficult to determine which type of TG is involved in various biological functions. To solve this problem, Hitomi et al. developed new FITC-labeled TG subtype-specific substrate peptides by screening a random library of phage-displayed peptides (Sugimura et al., 2006; Hitomi et al., 2009a, 2009b). In the present study, by monitoring these TG-specific substrate peptides, we observed FXIII-A gene upregulation in injured goldfish optic nerves at an early stage of the regeneration process. The FXIII-A gene was rapidly expressed as cFXIII in the optic nerve and the retina after optic nerve injury. Sustained long-term (1–40 days after nerve injury) expression of cFXIII was detected in the optic nerve, while the expression of cFXIII in the retina was transient (3–10 days after nerve injury). Here, we offer an explanation of the sustained and transient expression of cFXIII in the optic nerve and retina after nerve injury, respectively.

2. Experimental procedures

2.1. Animals and surgery

Adult common goldfish (*Carassius auratus*; body length about 7–8 cm) were used in this study. Goldfish were anesthetized with ice-cold water. The optic nerve was crushed 1 mm away from the posterior of the eyeball with forceps. After surgery, goldfish were kept in water tanks at 22 ± 1°C for 1–40 days. Animals were used and handled according to the guidelines for animal experiments of Kanazawa University. We paid specific attention to minimize the number of experimental animals and their pain and suffering.

2.2. In situ TG activity assay in optic nerves after nerve injury

Frozen tissue sections of goldfish optic nerves were prepared without fixation. Briefly, optic nerves were removed and embedded in optimum cutting temperature (OCT, Tissue Tek; Miles, Eikhart, IN, USA) compound immediately at 4°C. Following freezing with liquid nitrogen, the tissues were sectioned at a thickness of 14 μm and stored at −80°C until use. According to the method described by Itoh et al. (2011), following washing and blocking, sections were incubated with 1 μM FITC-conjugated peptides pepK5, pepT26 and pepF11, which have specific affinities for individual TG isozymes TG1, TG2 and FXIII, respectively. The incubation continued for 2 h at 28°C. To avoid non-specific reactions, mutant peptides pepK5QN, pepT26QN and pepF11QN, in which each reactive glutamine residue had been replaced by asparagine, were used as negative controls. Following washing, bound FITC signals were detected using a fluorescence microscope (VB-7000; Keyence, Tokyo, Japan).

2.3. Cloning of goldfish FXIII-A gene

To identify the genes whose expression was specifically upregulated during the early stage of optic nerve regeneration, a cDNA library was prepared from goldfish optic nerves 1 day after nerve injury. Positive clones were selected by screening the cDNA library using a 600 bp cDNA probe for FXIII-A generated referring to zebrafish and blowfish FXIII-A cDNA. Sequencing analysis was performed using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the Big Dye Terminator V.1.1 Cycle Sequencing Kit (Applied Biosystems). To obtain full-length cDNA clones of FXIII-A, 5'-RACE was performed using the 5'-Full RACE Core Set (Takara Bio Inc., Otsu, Japan) using total RNA isolated from intact optic nerves under the conditions recommended by the manufacturer. The FXIII-A sequences were analyzed using the FASTA program of the DNA Data Bank of Japan.

2.4. RT-PCR analysis of FXIII-A mRNA

Total RNA was isolated from intact or lesioned retinas or optic nerves on various postoperative days using Sepasol RNA I (Nacalai Tesque Inc., Kyoto, Japan). First-strand cDNA was synthesized with oligo-dT primer and Thermo Script Reverse Transcriptase (Invitrogen, CA, USA). The fragment of FXIII-A cDNA was amplified using Taq polymerase (Takara Bio Inc., Otsu, Japan) and specific primers as follows: 5'-GTCACAATGGGCATCACAAC-3' (sense) and 5'-CGTTTATTGCTGGATGGTGA-3' (antisense). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control.

2.5. Tissue preparation

At specific times following optic nerve injury, retinal and optic nerve samples were

prepared for histological analysis. Briefly, eyes were enucleated, bisected and fixed in 4% paraformaldehyde solution containing 0.1 M phosphate buffer (pH 7.4) and 5% sucrose for 2 h at 4°C. After infiltration with increasing concentrations of sucrose (5–20%), followed by an overnight incubation in 20% sucrose at 4°C, the tissues were embedded in OCT compound and sectioned at a thickness of 12 μm.

2.6. In situ hybridization

In situ hybridization was carried out as previously described (Sugitani et al., 2006). Briefly, tissue sections were rehydrated and treated with 5 μg/ml proteinase K (Invitrogen, CA, USA) at room temperature for 5 min. After acetylation and prehybridization, hybridization was performed with cRNA probes labeled with digoxigenin in hybridization solution overnight at 42°C. On the following day, the sections were washed and treated with 20 μg/ml RNase A at 37°C for 30 min. To detect the signals, the sections were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Germany) overnight at 4°C and visualized with tetrazolium-bromo-4-chloro-3-indolyl-phosphate (Roche) as the substrate.

2.7. Immunohistochemistry

Goldfish retinal sections were incubated at 121°C for 15 min in 10 mM citrate buffer. Following washing and blocking, the sections were incubated with anti-FXIII-A rabbit polyclonal antibody (1:200, Lab Vision, CA, USA) overnight at 4°C. Following incubation with a biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, bound antibodies were detected using horseradish peroxidase-conjugated streptavidin and 3-amino-9-ethylcarbazole (Dako Cytomation,

Denmark).

For double immunofluorescent staining of optic nerve sections and retina, anti-FXIII-A rabbit polyclonal antibody (1:200, Lab Vision, CA, USA) or mouse monoclonal antibody (1:200, Lab Vision, CA, USA) were used as the first primary antibodies. Following incubation with the first secondary antibody labeled with Alexa Fluor 488 anti-rabbit or anti-mouse IgG (Invitrogen, CA, USA) for 2 h at room temperature, incubated with the second primary antibodies of GFAP mouse monoclonal antibody (1:400, SIGMA, St. Louis, MO, USA) or Iba1 rabbit polyclonal antibody (1:500, Wako, Osaka, Japan) or βIII tubulin (TUJ1) mouse monoclonal antibody (1:200, Covance, Madison, WI, USA) overnight at 4°C. Alexa Fluor 594 anti-rabbit or anti-mouse IgG (Invitrogen, CA, USA) was used as the second secondary antibody for 2 h at room temperature. Cell nuclei were then stained with 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) solution in phosphate-buffered saline (PBS; pH 7.4) for 20 min at room temperature. Positive fluorescence signals were detected using a fluorescence microscope.

2.8. Western blot analysis

Retinal proteins were separated by SDS-PAGE in 8.5% acrylamide slab gels and then transferred to a nitrocellulose membrane for 1 h at 50 mA. The membrane was incubated with anti-FXIII-A rabbit polyclonal antibody (1:200, Lab Vision) overnight at 4°C and then alkaline phosphatase-conjugated anti-rabbit IgG antibodies (1:1000 dilution; Santa Cruz Biotechnology). Signals were detected using the BCIP/NBT Kit (KPL, USA).

2.9. Addition of optic nerve extracts prepared from injured optic nerves to retinal explant cultures

Two types of retina, unprimed (naïve retina without optic nerve crush) or primed, in which the optic nerve had been crushed 3 days before, were used for culture. The optic nerve samples were removed, cut into small pieces and washed three times with PBS (pH 7.4) followed by homogenizing in L15 (Leibowitz-15, Invitrogen, CA, USA) medium using an ultrasonic homogenizer (VP-050, TAITEC, Koshigaya, Japan) in an ice cold incubator. To obtain optic nerve extracts, the homogenate solution was separated by centrifugation at 10,000 × *g* for 10 sec. Retinal explants were cultured in 0.5 ml L15 medium containing 1.5 mg of optic nerve extract protein. Control retinal explants were cultured without optic nerve extracts. To test the effect of cFXIII in optic nerves, anti-FXIII-A rabbit polyclonal antibody was added to the optic nerve extracts and incubated for 15 min at room temperature before culture explants being performed (final concentration of 1.0 μg/ml IgG).

2.10. Construction of a plasmid vector for overexpression of FXIII-A

For FXIII-A gene transfection into goldfish retinal explants, we constructed expression plasmid vectors with inserted FXIII-A cDNA. Full-length goldfish *FXIII-A* cDNA fragments were amplified by polymerase chain reactions (forward primer: 5′-CGGAATTCCGCTGCAGTTTAAGTGGTCA-3′, reverse primer: 5′-TGGTCGACCGTTTATTGCTGGATGGTG-3′) and inserted into the *EcoR*I-*Sal*I sites of the multiple cloning site of the plasmid vector pEGFP-C1 (Clontech, CA, USA). The successful insertion of *FXIII-A* cDNA into pEGFP-C1 (FXIII-A-pEGFP-C1) was confirmed by DNA sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.11. Overexpression of FXIII-A in retinal explant cultures

Goldfish retinal explants were prepared as previously described (Sugitani et al., 2006). Two types of retina, unprimed (naïve retina without optic nerve crush) or primed, in which the optic nerve had been crushed 3 days before, were used for retinal explant cultures. Briefly, retinas were isolated under sterile conditions and cut into 0.5-mm squares using scissors. After washing three times with PBS (pH 7.4), the retinal explants were resuspended in L-15 medium without fetal calf serum (FCS) or antibiotics (2 retinas in 0.9 ml L-15 medium). For overexpression of FXIII-A in retinal explant cultures, pEGFP-C1 containing full-length FXIII-A cDNA (FXIII-A-pEGFP-C1) were transfected using Lipofectamine 2000 (Invitrogen).

For each transfection, 2 μl Lipofectamine 2000 diluted in 48 μl L-15 medium was mixed with 1 μg of FXIIIA-pEGFP-C1 plasmid diluted in 4 μl PLUS reagent (Invitrogen) and 46 μl L-15 medium (FCS and antibiotics free). A total of 100 μl of reaction mixture was incubated for 20 min at room temperature to allow complex formation and then added to 0.9 ml of the explant re-suspended culture medium and incubated for 3 h at 37°C with gentle agitation. Following centrifugation, the supernatant was removed and the pellet was resuspended in L15 medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Then, retinal explants were cultured with 0.5 ml medium per dish in poly-ornithine-coated 35-mm dishes at 28°C.

2.12. Overexpression of FXIII-A in RGC-5 cells

RGC-5 cells were originally produced by Dr. N. Agarwal at University of North Texas Health Science Center and were received from a line maintained by Dr. Hara at Gifu Pharmaceutical University. RGC-5 cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 U/ml penicillin and 100 μg/ml

streptomycin in a humidified atmosphere of 5% CO_2 at 37°C for 24 h prior to use (Koriyama et al., 2009a). For overexpression of FXIII-A in RGC-5 cells, full-length FXIII-A cDNAs in pEGFP-C1 (FXIII-A-pEGFP-C1) were transfected into RGC-5 cells using Lipofectamine 2000 (Invitrogen) and PLUS reagent (Invitrogen) for 3 h at 37°C according to the manufacturer's protocol. After treatment with staurosporine (final concentration of 400 nM) for 30 min at room temperature, cells were washed and resuspended with DMEM. 1×10^4 of RGC-5 cells per 35-mm dish were incubated with 5% CO_2 at 37°C for 24 h.

2.13. Immunohistochemical study of explant cultures and RGC-5 cells

At specific times after culturing, retinal explants and RGC-5 cells in 35-mm dishes were washed gently with PBS (pH 7.4) and treated with 0.1% glutaraldehyde in PBS for 30 min at room temperature for fixation. Following washing and blocking, retinal explants or RGC-5 cells were incubated with anti-FXIII-A rabbit polyclonal antibody (1:200) overnight at 4°C. Following incubation with Alexa Fluor 594 anti-rabbit IgG (Invitrogen, CA, USA) for 2 h at room temperature, bound positive fluorescence signals were detected using a digital microscope.

2.14. Measurement of neurite outgrowth

Neurite outgrowth from the retinal explants was assayed by measuring the length and density of neurites in each explant of a total of 40–50 explants in a 35-mm dish. All explants in the dish were counted depending on the number and length of neurite outgrowth from the explant. In this study, positive neurite outgrowth was defined by neurite length (>100 μm). The percentage of explants showing positive neurite outgrowth was compared

under various culture conditions. The length of each neurite of the retinal explants or RGC-5 cells was measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.15. Statistics

Neurite outgrowth from the retinal explants under various culture conditions was expressed as a percentage of the number of neurite-bearing explants per 40–50 explants in a 35-mm dish. Differences between conditions in the number of explants that produced neurite outgrowth were analyzed by Chi-square test, followed by Bonferroni's multiple comparison. The effects of overexpression of FXIII gene in RGC-5 cells on neurite outgrowth were also analyzed by Chi-square test. Neurite length from RGC-5 cells was expressed as the mean ± SEM and analyzed by one-way ANOVA, followed by Scheffe's multiple comparison. Statistical significance was set at $p < 0.05$.

3. Results

←Fig. 1

3.1. Increase of TG enzymatic activity in injured optic nerves

To examine how TG activity is altered and which member of the TG family changes in goldfish optic nerves after nerve injury, we performed an *in situ* assay of TG activity using FITC-conjugated substrate peptides that have a high affinity for an individual TG isozyme (Akiyama et al., 2010; Itoh et al., 2011). In control longitudinal optic nerve sections, no significant activity could be seen in any type of TG isoforms (Fig. 1, 0 h). Next, to analyze TG activity in the acute phase after optic nerve injury, we examined the change in individual TG isozyme activities at 3 h after optic nerve injury. As a result, strong FXIII activity was detected at the crush site of the optic nerve (Fig. 1, FXIII: 3 h) compared with TG1 and TG2 activities (Fig. 1, TG1 & TG2: 3 h), although weak TG1 activity was seen diffusely around the optic nerve. Three days after optic nerve injury, stronger FXIII activity was detected around the crush site (Fig. 1, FXIII: 3 d). In contrast, TG1 activity was not detected (Fig. 1, TG1: 3 d) and TG2 activity was first detected at the crush site (Fig. 1, TG2: 3 d). Peptides, in which each reactive glutamine residue had been replaced by asparagine, were used as negative controls and showed no FITC signals (data not shown). Therefore, FXIII was activated more rapidly and strongly than other types of TGs after optic nerve injury.

←Fig. 2

3.2. Cloning of FXIII-A cDNA isolated from injured goldfish optic nerves

To identify the FXIII-A gene that was specifically upregulated during the early stage of optic nerve regeneration, a cDNA library was constructed from goldfish optic nerves 1 day after injury. Out of 200,000 clones, positive clones were selected by screening the cDNA

library using the 600 bp FXIII-A cDNA probes generated referring to zebrafish and blowfish FXIII-A cDNA. A positive clone with a 2,560 bps fragment was identified as the full-length cDNA clone of FXIII-A (DNA Data Bank of Japan, Mishima, Japan; accession no. AB622931) encoding a protein of 744 amino acid residues (Fig. 2) with a predicted molecular mass of 83.8 kDa. Goldfish FXIII-A exhibited very high homology with zebrafish FXIII-A (85% identical) and moderate homology with human and mouse FXIII-A (48 and 49% identical, respectively).

←Fig. 3

3.3. Increase of FXIII-A gene expression in optic nerves following optic nerve injury

We examined the changes in FXIII-A expression in optic nerves using RT-PCR. As shown in Fig. 3A, upregulation of FXIII-A mRNA was observed 1 day after optic nerve crush compared with the control. The increased level of FXIII-A mRNA was maintained for 30 days after optic nerve injury. By contrast, the level of GAPDH mRNA that was used as an internal standard did not change during this period.

In *in situ* hybridization of optic nerve crush samples, the increase of FXIII-A mRNA was already detected 1 h after nerve injury at the crush site of optic nerves (Fig. 3B, 1 h) compared with the control (Fig. 3B, 0 h). Furthermore, a lot of FXIII-A mRNA-positive cells were shown to be in the optic nerve 3–7 days after injury (data not shown), and this expression became much stronger 10 days after injury (Fig. 3B, 10 d left panel). In optic nerve transverse sections 10 days after injury, FXIII-A positive cells spread inside the nerve (Fig. 3B, 10 d right panel).

←Fig. 4

3.4. Non-neuronal cells produce cellular FXIII in damaged optic nerves

In control optic nerves, no positive signals for FXIII-A protein could be seen when

labeled with anti-FXIII-A antibody (Fig. 4A, 0 d). In optic nerves injured 3 days previously, strong positive signals for FXIII-A protein could be seen around the crush site of optic nerves (Fig. 4A, 3 d). Nuclear staining of the same sections with DAPI 3 days after injury (Fig. 4A, DAPI) showed that DAPI positive cells coincided with FXIII-A expressing cells. These findings indicate that FXIII-A expression in injured optic nerves was produced by non-neuronal cells. In order to identify what type of non-neuronal cells produced FXIII-A, we performed double immunofluorescence staining with three antibodies of glial marker protein as follows: 1) GFAP (glial fibrillary acidic protein), which is an astrocyte marker (Sofroniew and Vinters, 2010), 2) Iba1 (ionized calcium binding adapter molecule 1), which is a calcium binding protein specifically expressed by macrophages and microglia (Imai and Kohsaka, 2002), and 3) galactocerebroside, which is found in oligodendrocytes (Ankerhold and Stuermer, 1999). In optic nerves injured 3 days previously, GFAP positive astrocytes clearly expressed FXIII-A (Fig 4B upper panels). On the other hand, only a weak FXIII-A signal could be observed in Iba1 positive microglia/macrophages (Fig 4B lower panels). Galactocerebroside positive cells were localized to optic nerve lesion sites; however, they did not coincide with FXIII-A signals (data not shown).

←Fig. 5

3.5. Increase of FXIII-A mRNA and protein expression in retina after optic nerve injury

We next analyzed the change in FXIII-A mRNA levels in retina after optic nerve injury. As shown in Fig. 5A, the levels of FXIII-A mRNA increased at 3–10 days, peaking at day 5 and then decreasing by day 20 after optic nerve injury. The level of GAPDH mRNA did not change during this period. *In situ* hybridization with a FXIII-A antisense RNA probe (Fig. 5B) showed increased levels of FXIII-A mRNA at day 3, peaking at day 7, then declining at day 10 and returning to control levels by day 20. The use of the sense RNA probe showed

no hybridization signals (Fig. 5B, Sense). These findings show that the increase in FXIII-A mRNA was mainly localized to the RGC layer with the levels peaking at 3–10 days after optic nerve injury.

←Fig. 6

To confirm the change in FXIII-A expression at the protein level, we performed western blot analysis. As shown in Fig. 6A, a single 84 kDa band of FXIII-A protein was identified from retinal samples of fish with injured optic nerves. The size of this band was equal to the estimated molecular weight of goldfish FXIII-A. Immunohistochemical staining revealed that an increased amount of FXIII-A protein was mainly localized to RGCs (Fig. 6B). Weak immunoreactivity for FXIII-A protein was seen in all nuclear layers of the control retinas (Fig. 6B, 0 d). Three days after optic nerve injury, the signal started to increase in the ganglion cell layers (Fig. 6B, 3 d), peaking at day 7 (Fig. 6B, 7 d) and then decreasing at day 10 after optic nerve injury (Fig. 6B, 10 d). The same changes in FXIII-A mRNA and protein levels in the retina were observed in five to six samples.

Furthermore, we performed immunohistochemical staining using a RGC marker TUJ1 (βIII tubulin) and FXIII-A antibodies. As a result, in Fig. 6C, TUJ1 positive RGCs merged perfectly with FXIII-A positive cells.

←Fig. 7

3.6. Cellular FXIII from injured optic nerves has amplifying effects on neurite outgrowth in retinal explant cultures

Optic nerve injury induced upregulation of cFXIII expression in optic nerves. To investigate the functional role of upregulation of cFXIII in optic nerves, we performed retinal explant cultures in the presence of optic nerve extracts and examined neurite outgrowth under various conditions by measuring the number of explants with long and

dense neurites (Fig. 7). In unprimed (naïve) retinal cultures, extracts from any types of optic nerve preparations were not effective (Fig. 7A, E). In primed retinas, where the optic nerve had been crushed 3 days earlier, extensive neurite outgrowth was induced in 57% of explants in 3-day cultures with extracts from injured optic nerves (+3d-N) (Fig. 7C, E). In contrast, addition of anti-FXIII-A IgG (final concentration 1μg/ml) to the culture medium significantly inhibited extract-induced neurite outgrowth to < 37% (Fig. 7D, E). Addition of extracts from intact optic nerves (+0d-N in primed retina) did not have any effect on neurite outgrowth, which was similar to the control ((-) in primed retina, Fig. 7B, E).

←Fig. 8

3.7. Overexpression of FXIII-A in the retina and RGC-5 cells in cultures

To investigate the role of increased cFXIII expression in RGCs, we induced overexpression of the FXIII-A gene in the retina, and then neurite outgrowth was measured in retinal explant cultures. The cDNA encoding goldfish FXIII-A was cloned into a plasmid vector (pEGFP-C1) in order to express cFXIII as a fusion protein with EGFP (enhanced green fluorescent protein) and transfected into retinal explants using Lipofectamine 2000. In this study, we compared the effect of neurite outgrowth using two types of retina. One was unprimed (naïve retina without optic nerve lesion), and the other was primed, where the optic nerve had been crushed 3 days before. As seen in Fig. 8A-1, unprimed retinal explants expressing the EGFP-FXIII-A fusion protein induced neurite outgrowth with long neurites. When viewed in dark field, the same explants also expressed the reporter protein EGFP, being green fluorescence (Fig. 8A-2). These results indicated that cFXIII-EGFP fusion cDNA cloned into a plasmid vector was successfully transfected into retinal explants, and overexpression of cFXIII was confirmed by immunohistochemical staining using anti-FXIII-A antibody (data not shown). In contrast, addition of anti-FXIII-A IgG (final

concentration 1 μg/ml) to the culture medium significantly inhibited transfection-induced neurite outgrowth (Fig. 8C, D). Explants expressing only EGFP (Mock) showed no significant neurite outgrowth (Fig. 8B). Fig. 8D clearly shows that overexpression of FXIII-A induced neurite outgrowth only from unprimed retina, but not from primed retina. These results indicated that endogenous cFXIII in RGCs was already upregulated in primed retina.

Similarly, Fig. 8E demonstrates the effect of overexpression of FXIII-A in RGC-5 cells, murine retinal precursor cells. Overexpression of FXIII-A clearly induced neurite outgrowth in RGC-5 cells (red, Fig. 8F) compared with EGFP expressing cells (green, Fig. 8E). FXIII-A induced neurite outgrowth in RGC-5 cells in both cell number (Fig. 8G) and length levels (Fig. 8H).

4. Discussion

4.1. Sustained increase of cellular FXIII expression in optic nerves after nerve injury

In our previous study (Sugitani et al., 2006), we cloned a full-length cDNA for retinal tissue type TG (TG_R) as a gene upregulated during fish optic nerve regeneration. TG_R mRNA levels were increased in goldfish RGCs at 1–6 weeks after optic nerve injury. From the experiments of gain and loss of function of this gene, we concluded that TG_R was involved in facilitation of regrowing optic axons both *in vitro* and *in vivo* (Sugitani et al., 2006). This work encouraged us to study the involvement of other types of TG in goldfish optic nerve regeneration after nerve injury. For this purpose, we used a new method developed by Hitomi et al. with FITC-labeled TG-specific substrate peptides (2009a, 2009b). As shown in Fig. 1, strong cFXIII activity could be detected at the crush site of optic nerves within 3 h after nerve injury, and this cFXIII activity was concentrated at the optic nerve scar by 3 days after nerve injury (Fig. 1). To confirm the exact cellular origin of FXIII production, we performed double immunofluorescence staining with some antibodies for glial markers. As shown in Fig. 4B, the candidate cells for FXIII production were primarily GFAP positive astrocytes and secondarily microglial cells. Cellular FXIII was produced by such DAPI positive non-neuronal cells at the optic nerve lesion site.

In the TG family, only cFXIII activity was increased so rapidly (within 3 h) at the nerve crush site after injury (Fig. 1). At a transcriptional level, sustained increase of FXIII-A mRNA in optic nerves at 1–40 days after nerve injury (Fig. 3) matched well with the period of axon elongation (Kato et al., 2007). Furthermore, Monsonego et al. (1998) reported that crushed fish optic nerves released FXIII exhibiting TG enzymatic activity. Our retinal explant culture experiments clearly showed that FXIII derived from injured optic nerves induced neurite outgrowth from primed retina with optic nerve injury from 3 days

beforehand, but not from unprimed (naïve) retina whose optic nerve was transectioned just before removing the eye in order to make the explants (Fig. 7). These results strongly indicate that the sustained cFXIII expression in optic nerves promotes neurite elongation from regrowing axons for a long time. The distribution of FXIII-A mRNA in optic nerves further supports this idea. At present, we are not sure what molecules are substrates of cFXIII for the neurite outgrowth or elongation. Fibronectin, collagen, myosin, vinculin and filamin are estimated as putative substrates (Ariëns et al., 2002; Hsieh et al., 2008). These substrates may also play an important role in mediating the cross-linking action of cFXIII in optic nerve regeneration.

4.2. Transient increase of cellular FXIII expression in goldfish retina after nerve injury

The levels of FXIII-A mRNA and protein increased in the retina 3–10 days and rapidly returned to the control level by 20 days after nerve injury. The cellular localization of FXIII-A mRNA and protein was limited to the RGCs (Figs. 5 and 6). In our previous study (Kato et al., 2007), four periods of goldfish optic nerve regeneration were classified: i) the first period of preparation for neurite sprouting (1–6 days after nerve injury); ii) the second period of axonal elongation (1–6 weeks after nerve injury); iii) the third period of synaptic refinement in the tectum (2–5 months after nerve injury); and iv) the last period of restoration of visual function (~6 months after nerve injury). The transient increase of cFXIII 3–10 days after nerve injury corresponds to the period of neurite sprouting during the optic nerve regeneration process. The experiments of FXIII-A overexpression showed that cFXIII induced neurite outgrowth from unprimed (naïve) retina but not from primed retina (Fig. 8A, D). It is considered that the levels of endogenous cFXIII in the primed retina already start to increase in RGCs at this time (Figs. 5 and 6). Furthermore, the neurite

sprouting effect of FXIII-A overexpression could be seen in the murine RGC-5 cells compared with mock EGFP-overexpressing cells (Fig. 8E-G). Addition of anti-FXIII-A antibody to the explants culture medium completely blocked the neurite sprouting action of FXIII-A (Fig. 8C, D). These results further indicate that cFXIII has a potential to stimulate neurite sprouting and/or outgrowing action(s) to RGCs and it works stage-specifically during optic nerve regeneration.

Followed by the decrease of cFXIII expression in RGCs, levels of TG_R mRNA and protein increased in RGCs 10–40 days after optic nerve injury, corresponding to the second period of axonal elongation. In contrast to cFXIII, recombinant TG_R protein enhanced neurite outgrowth only from primed retina, but not from unprimed retina (Sugitani et al., 2006). Just before TG_R upregulation in retina, neural nitric oxide synthetase (nNOS) was recognized as an upregulation molecule in fish RGCs 7–20 days after optic nerve injury (Koriyama et al., 2009b). Interestingly, it is reported that NO liberated by NOS inhibits FXIII activity (Catani et al., 1998; Bernassola et al., 1999). This study showed that the timing of cFXIII decrease in RGCs coincided with upregulation of nNOS in RGCs. These reciprocal changes of cFXIII and TG_R expressions in fish injured RGCs suggest that these two types of TGs are important for neurite sprouting and axonal elongation during the optic nerve regeneration process.

4.3. Possible explanation for the distinct effect of retinal and optic nerve cFXIII on optic nerve regeneration in fish

In the present study, we cloned coagulation factor XIII A subunit (FXIII-A) cDNA from injured goldfish optic nerves, and for the first time, we report here that cellular FXIII expression was identified in neural cells. We also demonstrate for the first time that cFXIII

is involved in optic nerve regeneration and directly stimulates neurite sprouting from injured RGCs in the early stage of nerve regeneration. Thereafter, cFXIII derived from glial cells in injured optic nerves stimulates neurite elongation over 40 days after nerve injury.

The difference in expression time (transient and sustained) and location (RGCs and glial cells) strongly indicates a distinct function of cFXIII in the optic nerve regeneration process. Since anti-FXIII antibody completely blocked the effects of the optic nerve extracts or the recombinant FXIII protein on primed retina or unprimed retina, respectively, the working site of FXIII on neurite outgrowth might be the surface of growing axons or injured RGCs. Although the exact reason why FXIII works differently in the RGCs and the optic nerves is not known, one possibility is thought to be the various interactions with different above-mentioned substrates for FXIII enzymatic action in the injured RGCs and the injured optic nerves. The different time course and different localization of cFXIII expression further suggest a distinct effect of cFXIII on neurite sprouting and axonal elongation. Further studies are needed to elucidate the mechanisms responsible for neurite outgrowth and elongation.

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References

Adány, R., Antal, M., 1996. Three different cell types can synthesize factor XIII subunit A in the human liver. Thromb. Haemost. 76, 74-79.

Adány, R., Bárdos, H., 2003. Factor XIII subunit A as an intracellular transglutaminase. Cell. Mol. Life Sci. 60, 1049-1060.

Akiyama, M., Sakai, K., Yanagi, T., Fukushima, S., Ihn, H., Hitomi, K., Shimizu, H., 2010. Transglutaminase1 preferred substrate peptide K5 is an efficient tool in diagnosis of lamellar ichthyosis. Am J Pathol. 176, 1592-1599.

Ankerhold, R., Stuermer, C.A., 1999. Fate of oligodendrocytes during retinal axon degeneration and regeneration in the goldfish visual pathway. J Neurobiol. 41, 572-584.

Ariëns, R.A., Lai, T.S., Weisel, J.W., Greenberg, C.S., Grant, P.J., 2002. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood 100, 743-754.

Bernassola, F., Rossi, A., Melino, G., 1999. Regulation of transglutaminases by nitric oxide. Ann. N Y Acad. Sci. 887, 83-91.

Catani, M., V., Bernassola, F., Rossi, A., Melino, G., 1998. Inhibition of clotting factor XIII activity by nitric oxide. Biochem Biophys Res Commun. 249, 275-278.

Derrick, E.K., Barker, J.N., Khan, A., Price, M.L., Macdonald, D.M., 1993. The tissue distribution of factor XIIIa positive cells. Histopathology 22, 157-162.

Hitomi, K., Kitamura, M., Alea, M.P., Ceylan, I., Thomas, V., El. Alaoui, S., 2009a. A specific colorimetric assay for measuring transglutaminase 1 and factor XIII activities. Anal. Biochem. 394, 281-283.

Hitomi, K., Kitamura, M., Sugimura, Y., 2009b. Preferred substrate sequences for transglutaminase 2: screening using a phage-displayed peptide library. Amino Acids. 36, 619-624.

Hsieh, L., Nugent, D., 2008. Factor XIII deficiency. Haemophilia 14, 1190-1200.

Imai, Y., Kohsaka, S.,2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. Glia. 40, 164-174.

Itoh, M., Kawamoto, T., Tatsukawa, H., Kojima, S., Yamanishi, K., Hitomi, K., 2011. In situ detection of active transglutaminases for keratinocyte type (TGase 1) and tissue type (TGase 2) using fluorescence-labeled highly reactive substrate peptides. J. Histochem. Cytochem. 59, 180-187.

Kato, S., Koriyama, Y., Matsukawa, T., Sugitani, K., 2007. Optic nerve regeneration in goldfish. In: Becker, C.G., Becker, T. (Eds), Model Organisms in Spinal Cord Regeneration. Wiley-VCH, Germany, pp. 355-372.

Kiesselbach, T.H., Wagner, R.H., 1972. Demonstration of factor XIII in human megakaryocytes by a fluorescent antibody technique. Ann. NY. Acad. Sci., 202, 318-328.

Koriyama, Y., Homma, K., Sugitani, K., Higuchi, Y., Matsukawa, T., Murayama, D., Kato, S., 2007. Upregulation of IGF-I in the goldfish retinal ganglion cells during the early stage of optic nerve regeneration. Neurochem. Int. 50, 749-756.

Koriyama, Y., Ohno, M., Kimura, T., Kato, S., 2009a. Neuroprotective effects of 5-S-GAD against oxidative stress-induced apoptosis in RGC-5 cell. Brain Res. 1296, 187-195.

Koriyama, Y., Yasuda, R., Homma, K., Mawatari, K., Nagashima, M., Sugitani, K., Matsukawa, T., Kato, S., 2009b. Nitric oxide-cGMP signaling regulates axonal elongation during optic nerve regeneration in the goldfish in vitro and in vivo. J. Neurochem. 110, 890-901.

Liu, Z. W., Matsukawa, T., Arai, K., Devadas, M., Nakashima, H., Tanaka, M., Mawatari, K., Kato, S., 2002. Na,K-ATPase alpha3 subunit in the goldfish retina during optic nerve regeneration. J. Neurochem. 80, 763-770.

Lorand, L., 2001. Factor XIII: structure, activation and interactions with fibrinogen and fibrin. Ann. N.Y. Acad. Sci. 936, 291–311.

Matsukawa. T., Sugitani, K., Mawatari, K., Koriyama, Y., Liu, Z., Tanaka, M., Kato, S., 2004. Role of purpurin as a retinol-binding protein in goldfish retina during the early stage of optic nerve regeneration: its priming action on neurite outgrowth. J. Neurosci. 24, 8346-8353.

Monsonego, A., Mizrahi, T., Eitan, S., Moalem, G., Bárdos, H., Adány, R., Schwartz, M., 1998. Factor XIIIa as a nerve-associated transglutaminase. FASEB J. 12, 1163-1171.

Muszbek, L., Adány, R., Szegedi, G., Polgár, J., Kávai, M., 1985. Factor XIII of blood coagulation in human monocytes. Thromb. Res. 37, 401-410.

Muszbek, L., Ádány, R., Mikkola, H., 1996. Novel aspects of blood coagulation factor XIII. I. Structure, distribution, activation, function. Crit. Rev. Clin. Lab. Sci. 33, 357–421.

Muszbek, L., Bereczky, Z., Bagoly, Z., Komáromi, I., Katona, É., 2011. Factor XIII: a coagulation factor with multiple plasmatic and cellular functions. Physiol Rev. 91, 931-972.

Nagashima, M., Fujikawa, C., Mawtari, K., Mori, Y., Kato, S., 2011. HSP70, the earliest-induced gene in the zebrafish retina during optic nerve regeneration: Its role in cell survival. Neurochem. Int. 58, 888-895.

Quatresooz, P., Paquet, P., Hermanns-Lê, T., Piérard, G.E., 2008. Molecular mapping of Factor XIIIa-enriched dendrocytes in the skin. Int. J. Mol. Med. 22, 403-409.

Sofroniew, M.V., Vinters, H.V., 2010. Astrocytes: biology and pathology. Acta Neuropathol. 119, 7–35.

Sugimura, Y., Hosono, M., Wada, F., Yoshimura, T., Maki, M., Hitomi, K., 2006. Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library: identification of peptide substrates for TGase 2 and Factor XIIIA. J Biol Chem. 281, 17699-17706.

Sugitani, K., Matsukawa, T., Koriyama, Y., Shintani, T., Nakamura, T., Noda, M., Kato, S., 2006. Upregulation of retinal transglutaminase during the axonal elongation stage of goldfish optic nerve regeneration. Neuroscience 142, 1081-1092.

Figure Legends

Fig. 1

***In situ* activity of cFXIII, TG1 and TG2 in goldfish optic nerves after nerve crush, stained with FITC-labeled TG-specific substrate peptides.**

(0 h) No treatment (control). (3 h) Optic nerve 3 h after nerve crush. (3 d) Optic nerve 3 days after nerve crush. (TG1, upper panels) TG1 staining. Weak TG1 activity was diffusely seen in the optic nerve at 3 h after nerve injury compared with the control, but disappeared 3 days after nerve injury. (TG2, middle panels) TG2 staining. A weak increase of TG2 activity could be first seen at the crush site of the optic nerve 3 days after nerve injury compared with the control or optic nerve at 3 h after nerve injury. (FXIII, lower panels) cFXIII staining. Strong cFXIII activity could be seen at the crush site (marked with white arrowheads) of the optic nerve 3 h after nerve injury compared with the control. Increased cFXIII activity was detected in the surrounding areas 3 days after nerve injury. The experiments were done in triplicate. The white arrowheads indicate the crush sites of optic nerve. Scale bar = 200 μm.

Fig. 2

Deduced amino acid sequence of a cDNA clone for FXIII-A isolated from goldfish optic nerves.

The cDNA clone for FXIII-A contains a full-length open reading frame consisting of 744 amino acid residues. The active site (329–350) is underlined and the predicted Ca^{2+}-binding site is indicated by asterisks.

Fig. 3

Sustained increase of FXIII-A mRNA in goldfish optic nerves after nerve injury.

(A) Semi-quantitative analysis of FXIII-A mRNA in optic nerves after injury by RT-PCR. Levels of FXIII-A mRNA started to increase 1 day after nerve injury. The increased levels were maintained for 20 days and then gradually declined 30–40 days after nerve injury. The levels of GAPDH mRNA did not change during this period. (B) *In situ* hybridization of FXIII-A mRNA in optic nerves. The first signals of FXIII-A mRNA were detected just at the crush site of the optic nerve 1 h after nerve injury compared with the control optic nerve. At 10 days after optic nerve injury, positive signals became stronger in the optic nerve (Fig. 3B, 10 d left panel) and were further detected in a transverse section of the optic nerve with higher magnification (Fig. 3B, 10 d right panel). The arrows indicate optic nerve crush sites. Scale bar = 500 μm (0 h, 1 h, 10 d left panel), 50 μm (10 d right panel).

Fig. 4

FXIII-A-producing cells in goldfish optic nerves after nerve injury.

(A) Immunohistochemical staining of the optic nerve with anti-FXIII-A antibody. 0 d; No immunoreactive signals for FXIII-A could be seen in the control (intact) optic nerve. 3 d; FXIII-A protein expression could be seen mainly at the crush site of the optic nerve 3 days after nerve injury. The white arrowheads indicate optic nerve crush sites. Scale bar = 100 μm (0 d, 3 d). DAPI; Nuclear staining with DAPI at a high magnification of the area indicated by a square shown in 3 d. Note that most cells accumulating at the crush site expressed both FXIII-A and DAPI. Scale bar = 50 μm. (B) Double fluorescence staining of FXIII-A and glial marker, GFAP (upper panels) and Iba1 (lower panels). Note that GFAP positive cells clearly merged with FXIII-A expressing cells, whereas Iba1 positive microglial cells showed weak FXIII-A expression in optic nerves injured 3 days previously.

Scale bar = 20 μm.

Fig. 5

Transient increase of FXIII-A mRNA in goldfish retina after nerve injury.

(A) FXIII-A mRNA was semi-quantified in retinas for 30 days after nerve injury by RT-PCR. Levels of FXIII-A mRNA in retina transiently increased during 3–10 days after nerve injury. The level of GAPDH mRNA did not change during this period. (B) *In situ* hybridization of FXIII-A mRNA in goldfish retina after nerve injury. In the control retina (0 d), faint signals of FXIII-A mRNA could be seen in the INL and GCL. FXIII-A mRNA expression was slightly increased in the GCL at 3 days and peaked at 7 days after nerve injury compared with the control. The signals started to decrease at 10 days and returned to the control level by 20 days after nerve injury. No positive signals could be seen with the sense probe (Sense). Scale bar = 40 μm. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.

Fig. 6

Immunohistochemistry of FXIII-A protein in goldfish retina after optic nerve injury.

(A) Immunoblotting analysis of FXIII-A protein in goldfish retina with anti-FXIII-A antibody. A single band of an 84-kDa protein was detected in retinal samples. R, retinal samples of fish with injured optic nerves; M, molecular-weight marker. (B) Immunohistochemical staining of FXIII-A protein in goldfish retina after optic nerve injury. FXIII-A protein expression was weakly detected in the INL and GCL in the control retina (0 d). FXIII-A protein expression started to increase only in the GCL at 3 days after nerve injury compared with the control retina. The expression peaked in the GCL at 7 days and

then rapidly decreased at 10 days after nerve injury. The experiments were repeated four to five times. Scale bar = 50 μm. (C) Double immunofluorescence staining of TUJ1 (retinal ganglion cell marker) and FXIII-A in retina 3 days after optic nerve injury. TUJ1 positive RGCs merged perfectly with FXIII-A stained cells. The experiments were repeated four to five times. Scale bar = 20 μm.

Fig. 7

Neurite outgrowing effect of optic nerve extracts prepared 3 days after nerve injury in retinal explant cultures.

(A-D) Photomicrographs of neurite outgrowth in retinal explant cultures. (A) Addition of 3d-N optic nerve extracts to unprimed (naïve) retina. (B) Control culture (-) in primed retina. (C) Addition of 3d-N optic nerve extracts to primed retina. (D) Addition of 3d-N optic nerve extracts to primed retina plus anti-FXIII-A IgG. Scale bar = 200 μm. (E) Graphical representation of neurite outgrowth at 3 days of culture. In unprimed (naïve) retina, no overinduction of neurite outgrowth could be seen after the addition of extracts prepared from optic nerves injured 3 days earlier (+3d-N) compared with no addition (-) or addition of extracts prepared from control optic nerves (+0d-N). In primed retina with the optic nerve crushed 3 days earlier, the extract of injured 3d-N optic nerves clearly induced neurite outgrowth in retinal cultures compared with the (-) or (+0d-N) group. The neurite outgrowth with the addition of 3d-N optic nerve extracts in primed retina was completely blocked by anti-FXIII-A IgG (1 μg/ml). (**$p < 0.01$).

Fig. 8

Effect of overexpression of FXIII-A on neurite outgrowth from retina and RGC-5 cells

in culture.

(A) Overexpression of FXIII-A in goldfish retinal explants. Cells overexpressing FXIII-A in retinal explants without pretreatment of the optic nerve (unprimed retina) induced long neurite outgrowth (A-1) as compared with mock (B). (A-2) Successful transfection was confirmed by a significant increase of enhanced green fluorescent protein (EGFP) levels in retinal explants. (C) Effects of neurite outgrowth were completely blocked by anti-FXIII-A IgG (1 μg/ml) (D, +Ab). Overexpression of FXIII-A and EGFP was carried out by transfection of the FXIII-A and EGFP fusion gene with lipofectamine. (D) The number of explants showing neurite outgrowth was significantly higher in the FXIII-A-overexpressing explants derived from unprimed (naïve) retina for 5 days as compared with the control (Ctrl) or the EGFP (Mock) overexpressing explants. The experiments were repeated four to five times. Scale bar = 100 μm. (E, F) The effect of overexpression of FXIII-A (red) and EGFP (green) in RGC-5 cells on neurite outgrowth. FXIII-A overexpression (F) induced significantly longer neurites than that of EGFP (E). Scale bar = 100 μm. (G, H) Graphical representation of the number of cells showing neurite outgrowth (G) and length of neurites (H) in FXIII-A-overexpressing cells and EGFP-overexpressing cells (Mock, E). (*$p < 0.05$, **$p < 0.01$).

Fig.1.

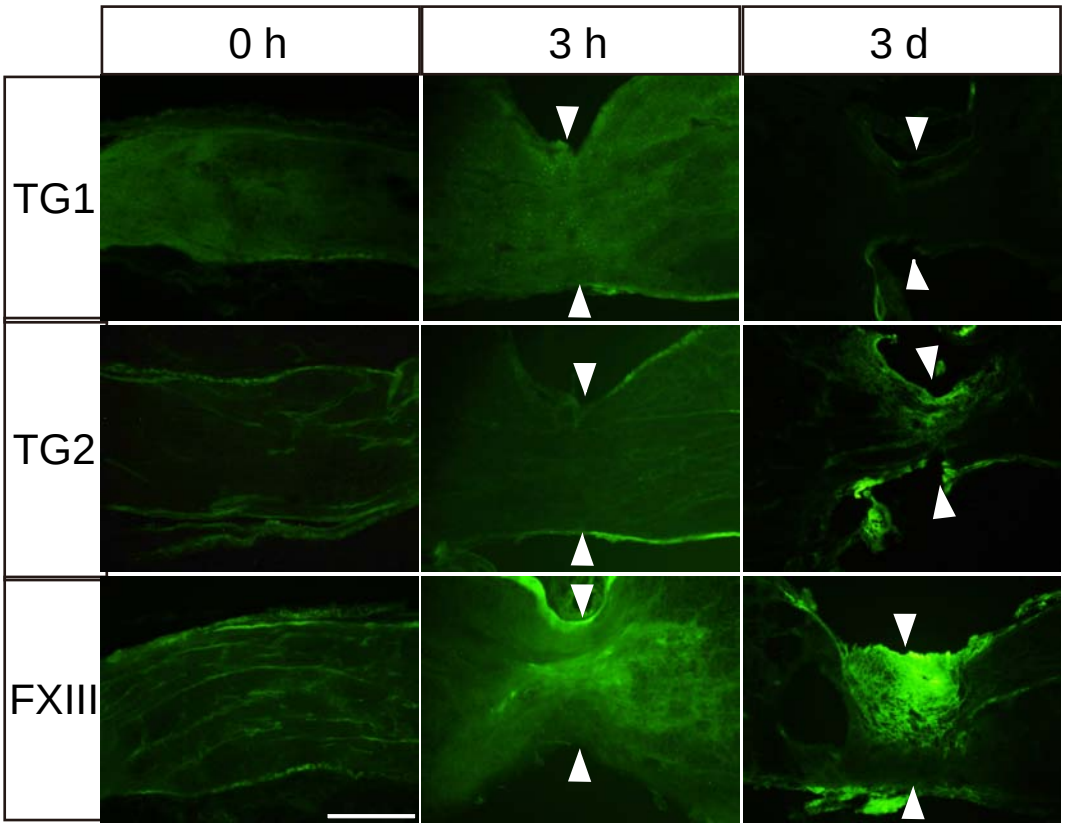
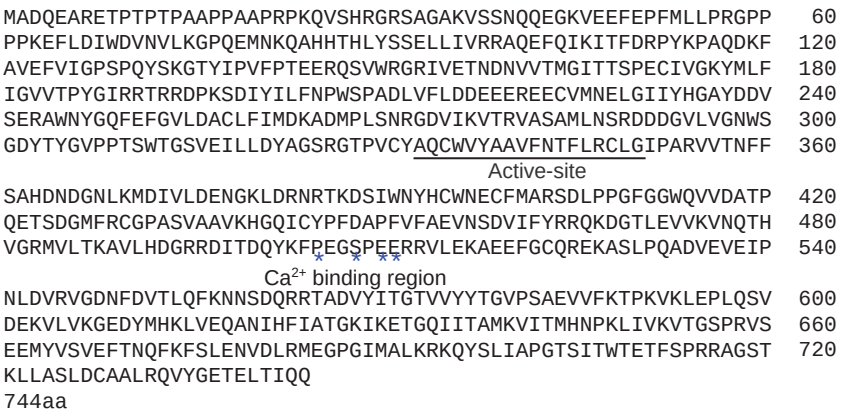


Fig.2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Active-site
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VGRMVLTKAVLHDGRRDITDQYKFPEGSPEERRVLEKAEEFGCQREKASLPQADVEVEIP 540
Ca2+ binding region
NLDVRVGDNFDVTLQFKNNSDQRRTADVYITGTVVYYTGVPSAEVVFKTPKVKLEPLQSV 600
DEKVLVKGEDYMHKLVEQANIHFIATGKIKETGQIITAMKVITMHNPKLIVKVTGSPRVS 660
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744aa

Fig.3

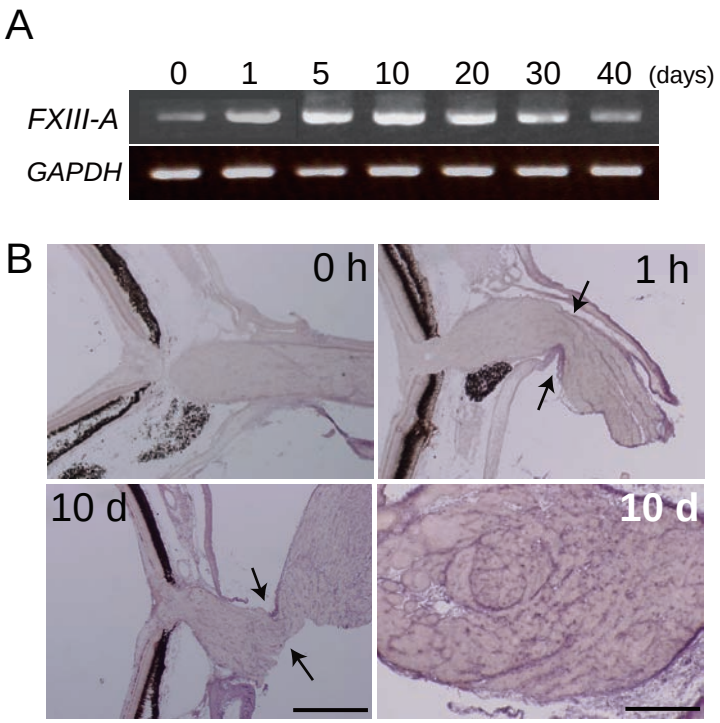


Fig.4

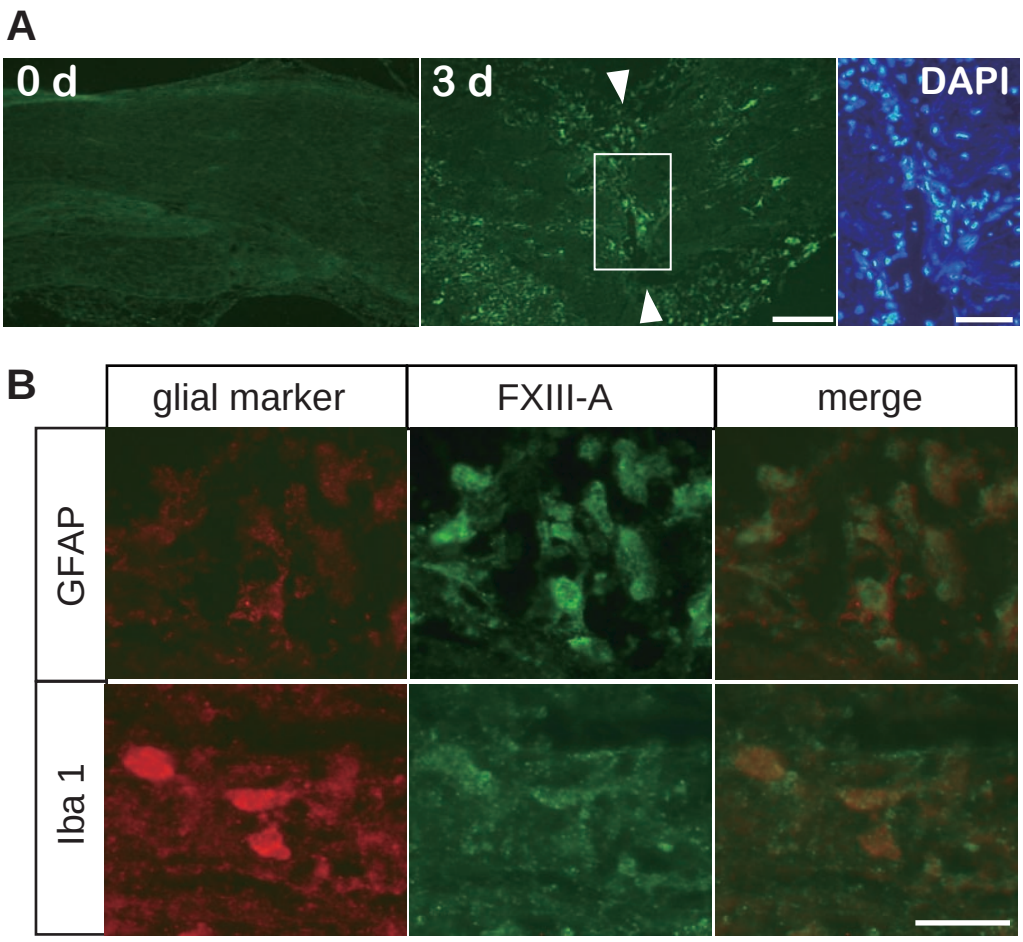
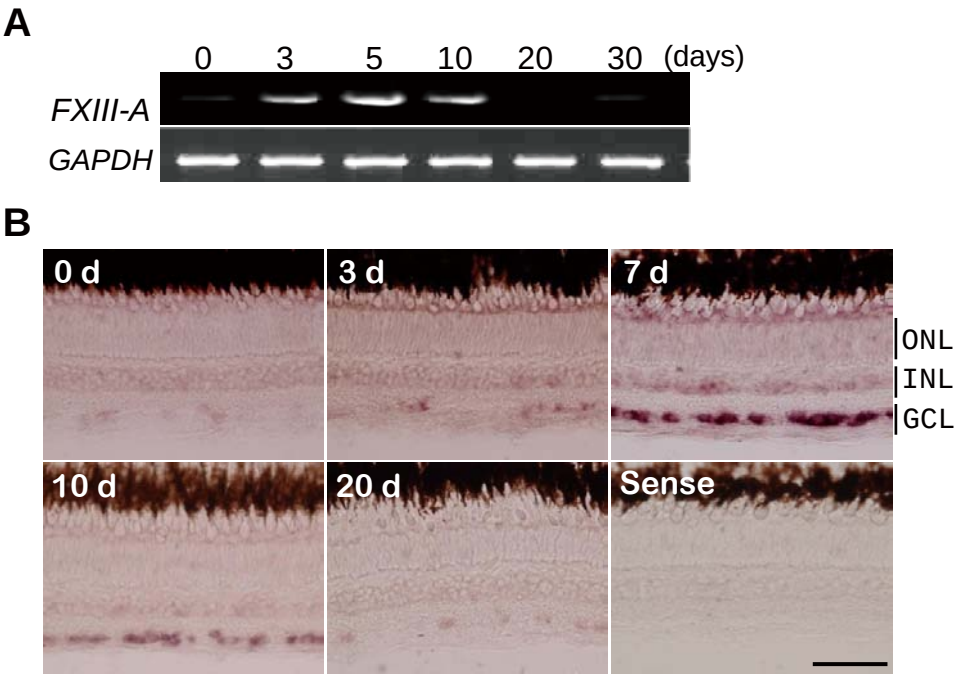


Fig. 5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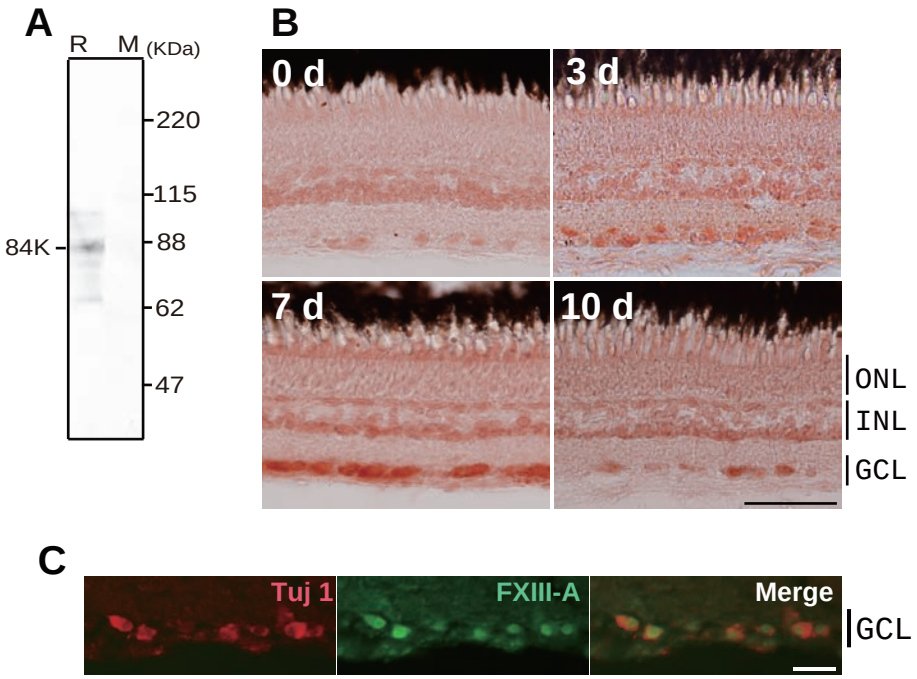
0
3
5
10
20
30
(days)
FXIII-A
GAPDH
B
0 d
3 d
7 d
ONL
INL
GCL
10 d
20 d
Sense

Fig.6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R
M
(KDa)
220
115
84K
88
62
47
B
0 d
3 d
7 d
10 d
ONL
INL
GCL
C
Tuj 1
FXIII-A
Merge
GCL

Fig.7

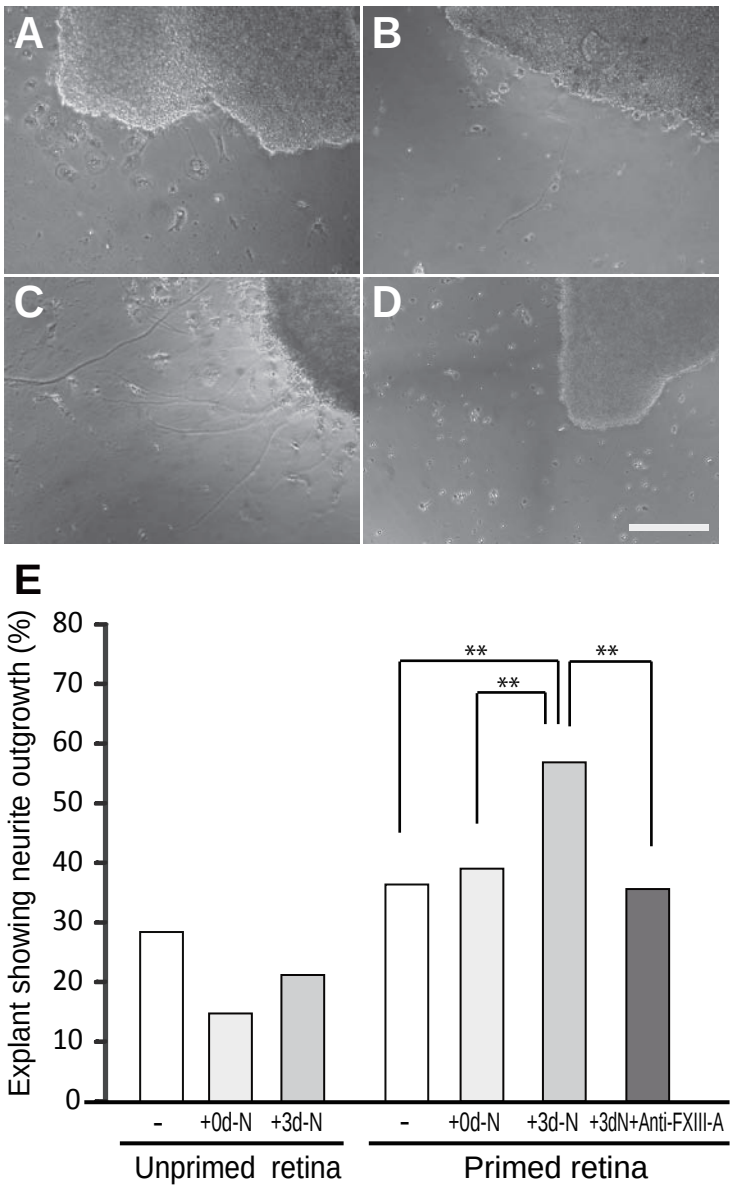
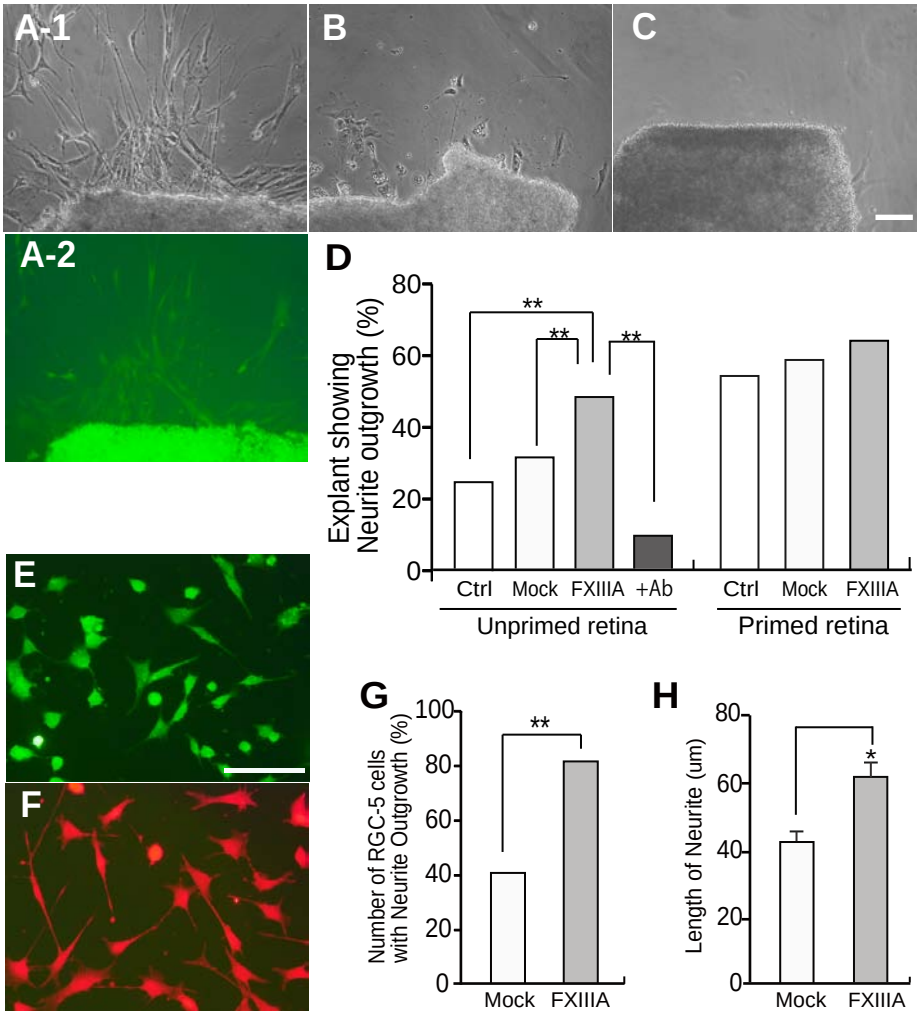

A
B
C
D
E
Explant showing neurite outgrowth (%)
80
70
60
50
40
30
20
10
0
**
**
**
-
+0d-N
+3d-N
-
+0d-N
+3d-N
+3dN+Anti-FXIII-A
Unprimed retina
Primed retina

Fig.8


A-1
B
C
A-2
D
Explant showing Neurite outgrowth (%)
80
60
40
20
0
**
**
**
Ctrl
Mock
FXIIIA
+Ab
Ctrl
Mock
FXIIIA
Unprimed retina
Primed retina
E
F
G
Number of RGC-5 cells with Neurite Outgrowth (%)
100
80
60
40
20
0
**
Mock
FXIIIA
H
Length of Neurite (um)
80
60
40
20
0
*
Mock
FXIIIA