The Bdkrb2 Gene Family Provides a Novel View of Viviparity Adaption in Sebastes schlegelii

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Abstract

Background:
Black rockfish (Sebastes schlegelii) is a viviparous teleost. In the prior study, we reported a chromosome-level black rockfish genome assembly and proposed that the rockfish ovarian wall has a similar function to the uterus of mammals. In the present study, the well-developed vascular system was observed in the ovary wall and the exterior surface of the egg membrane. Adaptation of the ovary vasculature to the rising needs of the embryos occurs through both vasodilation and neovascularization. Bdkrb2 encodes a receptor for bradykinin. The two play a critical role in the control of vasodilatation by regulating NO production.

Results:
Eight Bdkrb2 genes were identified in the black rockfish genome. These genes are located on chromosome 14, which are arranged in a tandem array, forming a gene cluster spanning 50 kb. Protein structure prediction, phylogenetic analysis, and tissue expression pattern analysis was done to clarify the relationship of the Bdkrb2 genes and a preliminary exploration of function was conducted. The results show that the eight Bdkrb2 genes evolved two kinds of protein structure and three kinds of tissue expression pattern. Furthermore, some have a relatively high expression in ovarian wall, especially in stages of pre-fertilization and pre-hatching.

Conclusions:
Our study characterizes eight Bdkrb2 genes in the black rockfish, which have a regulatory role in the preparation for fertilization and hatching. This research provides a novel view of viviparity adaption and lays the groundwork for future research into vascular regulation of ovarian in the breeding cycle in black rockfish.

Key words: Bdkrb2, Viviparity, Ovarian wall, Vasodilatation, Adaption
Background

Black rockfish (Sebastes schlegelii) is a viviparous teleost, whose embryos develop in the maternal reproductive system from fertilization to birth. Several studies have revealed the viviparity of black rockfish from the aspect of annual reproductive cycle and sperm storage in the ovary [1-3]. The time course of copulation up to birth spans about 8 months. When fertilization occurs in April, it is already about 6 months after mating [1, 4]. The gestation period is about 50 days [5, 6], during which the embryos develop in the ovary. Study on energetics of embryonic development has shown that offspring need to receive nutrition in addition to that supplied in the yolk and uptake of nitrogenous substance occurs through ingestion and absorption of ovarian fluid in the hindgut [5]. The structures in the ovarian system to supply nutrients to the developing embryos were not clear so far.

Adaptation of the ovary vasculature to the rising needs of the embryos occurs through both vasodilation and neovascularization. In the previous study, we reported a high-quality genome assembly of black rockfish and revealed gene expression patterns related to viviparity through the RNA-seq and ATAC-seq data set. We found expression of genes related to placental development, cell adhesion, trophoblast invasion, calcium-sensing receptors, the NO-sGc-cGMP signalling pathway, and blood vessel function in ovarian wall co-expressed module [4]. We thus hypothesized that the ovarian wall of black rockfish has the function similar to the mammalian uterus.

In mammals, mother shares her bloodstream with the fetus via the placenta to exchange substance and gas. During the whole of gestation period, nutrition of the fetus is ingested by the blood circulation between maternal and fetus in typical viviparous animals. It was pointed out that fetal growth may be limited by uterine blood flow and by function of the uteroplacental, particularly in late gestation [7]. The chemical messenger nitric oxide (NO), a key part of NO-sGc-cGMP pathway, is widely known as endothelium derived relaxing factor, which plays a role in the regulation of fetoplacental circulation in human [8, 9] and oocyte maturation in zebrafish [10]. NO production is stimulated by a variety of mechanical forces such as shear stress [11] and cyclic strain [12] and humoral factors, including Acetylcholine [13], VEGF (Vascular Endothelial Growth Factor) [14], Bradykinin [15, 16], Estrogen [17], S1P (Sphingosine-1-Phosphate) [18, 19], H2O2 (Hydrogen Peroxide) [20], and Angiotensin [21]. Particularly, we are interested in Bradykinin, which regulates NO production by binding to their cognate receptor. Moreover, we found that bradykinin B2 receptor (Bdkrb2) gene family expands in the black rockfish.

BDKRB2, a component of kallikrein-kinin system (KKS), which participates in a wide spectrum of physiological and pathological process, such as vasodilation, glucose homeostasis, inflammation, gastric cancer [9, 22-25]. It is reported that the Bdkrb2 polymorphism is associated with athletic performance [26] and many diseases, including knee osteoarthritis, diabetic nephropathy, hypertension [22, 23, 27]. The vasodilation effect of Bdkrb2 is the result of the synthesis of NO catalyzed by the enzyme nitric oxide synthase (NOS). Normal pregnancy is estrogen-dominated physiological states that are characterized by elevations in uterine blood flow and endothelial nitric oxide synthase (eNOS) [28]. In addition, the expression of Bdkrb2 is
regulated by estrogen [29]. It is unknown that the role of Bdkrb2 in pregnancy or viviparity. We screened and characterized the Bdkrb2 genes in black rockfish and executed phylogenetic analysis, protein structure prediction and tissue expression pattern analysis to clarify the relationship among the members of Bdkrb2 gene family. Besides, we figured out the primary function of Bdkrb2 genes in vascular development and the expression pattern during gestation. These results suggest that the expansion of Bdkrb2 gene family is related to viviparity, providing a new view of viviparity adaption in the black rockfish.

Results

1. Well-developed vascular system in the ovary

In view of evidence that the black rockfish is viviparous, particular attention was paid to the observation of adaptive changes in the physiological structure of ovary. The extrinsic and intrinsic ovarian blood vessels were observed in a group of females at different stages of breeding season (see in Fig.1). Macroscopic observations show that a main vessel is located in the middle of ovary wall. Branch vessels extend laterally and more capillaries extend into the ovary (see in Fig.1A-C). Microscopic observations show that the capillaries spread over the ovary. It is important to note that more intrinsic vessels were observed with the process of final oocyte maturation and gestation (see in Fig.1D-H). Highly vascular membranous tissue was found adjacent to the egg membrane, especially when ready for fertilization and the following stages until hatching (see in Fig.1F-H). Moreover, many genes involved in angiogenesis and regulation of vasoconstriction have a high expression in ovarian wall or eggs (see in Fig S1 and Fig S2). Tissue expression pattern analysis provides molecular basis for vascular changes in ovary and the well-developed vascular system provides physiological basis for material and gas exchanges between the female and embryos. As an aside, it is interesting to note that three of five vasoconstriction regulating genes in ovarian wall were Bdkrb2 genes (see in Fig S2). Bdkrb2 gene encodes a receptor for bradykinin, regulating the production of NO via ligand-receptor binding [15-16]. Tissue transcriptomes of mammals also show that Bdkrb2 gene has relatively high expression in uterus and placenta (see in Fig S3). These data suggest that Bdkrb2 genes in the black rockfish may have an impact on ovarian wall function in the reproductive process.

Fig.1 Macroscopic and microcosmic observation of the ovary at different stages of reproductive process. A) Ovary at the stage of post-mating. Its overall color is yellow, because of fewer blood vessels in the ovary. B) Ovary after fertilization, at the early stage of gestation. Its overall color is pink or red, because the well-developed blood
vessels spread over the surface of egg membrane. C) Ovary at the final stage of gestation. Its overall color is dark, because of developed pigment. The silvery reflections are the individual eyes of the larvae within the ovary. As time goes by, the ovary will enlarge the volume and the ovarian wall will become very thin until the finish of parturition. D) Ovary post parturition. Most embryos came out from cloacal orifice via abdomen extrusion. E) Ovary before mating. Oocytes are opaque. Only a few of blood vessels were observed. F) Ovary after mating. Oocytes develop further, becoming transparent. G) Ovary before fertilization. Oocytes are fully developed and ready for fertilization. The highly vascular membranous tissue adjacent to the egg membrane. H) Ovary during gestation. The eyes of embryo formed.

2. **Bdkrb2 genes in black rockfish**

A total of eight **Bdkrb2** genes were identified in black rock fish genome, including Ssc_10023113, Ssc_10023114, Ssc_10023115, Ssc_10023116, Ssc_10023117, Ssc_10023118, Ssc_10023119 and Ssc_10023120. They are located on chromosome 14, which are arranged in a tandem array, forming a gene cluster spanning 50 kb (see in Fig 2A). Amino acid alignments of **Bdkrb2** genes in black rock fish revealed that Ssc_10023116 was highly homologous to Ssc_10023117 (82%), Ssc_10023118 (84%), Ssc_10023119 (81%) and Ssc_10023120 (78%). Whereas the similarity among Ssc_10023113, Ssc_10023114 and Ssc_2310023115 tended to be around 35% (see in supplemental Fig 3 and supplemental Fig 4). The open reading frame (ORF) of Ssc_10023113 is 1092 bp long, encoding 363 amino acids. The ORF of Ssc_10023114 is 1110 bp long, encoding 369 amino acids. The ORF of Ssc_10023115 is 1029 bp long, encoding 342 amino acids. Each one contains seven transmembrane (TM) domains, forming a typical G protein (see in Fig 2B). The ORF of Ssc_10023120 is 858 bp long, encoding 285 amino acid. The rest four genes, Ssc_10023116, Ssc_10023117, Ssc_10023118, and Ssc_10023119, each contains a 900 bp long ORF and encodes 299 amino acids. Each one contains only six TM domains, resulting in protein conformational changes (see in Fig 2B, 2C). Changes were detected by 3D structure prediction. The proteins with seven TM domains (Ssc_10023113, Ssc_10023114 and Ssc_10023115) have both intracellular and extracellular terminus region, however, the proteins with six TM domains (Ssc_10023116, Ssc_10023117, Ssc_10023118, Ssc_10023119 and Ssc_10023120) only have intracellular terminus region (see in Fig 2C). The conformational changes may cause functional changes.
Fig.2 Schematic diagram of gene location and protein structure. A) Schematic diagram of gene location. B) Schematic diagram of conserved domains. Domain analysis was performed by SMART online tool based on amino acid sequence. TM, transmembrane domain; LC, low complexity domain. C) Schematic diagram of 3D structure. Structure prediction analysis was performed via Phyre2 online tool and modified by PyMol software. Models in line a were shown as cartoon style. Models in line a’ were shown as dot style.

3. Phylogenetic relationship between Bdkrb2 genes

To elucidate the evolutionary relationship of the eight Bdkrb2 genes in black rockfish, a phylogenetic tree was constructed based on amino acid sequences of the Bdkrb2 or Bdkrb2-like genes from 14 species. Major vertebrate groups (cartilaginous fish, ray-finned fish, tetrapods) were roughly recovered, and this is supporting evidence for our correct ortholog identification. The phylogenetic analysis demonstrated all Bdkrb2 genes of fish were clustered into one group and showed an elevated rate of molecular evolution compared with its paralogs in mammals. The eight Bdkrb2 genes in black rockfish were clustered into three different clades. It’s worth noticing that Ssc_10023113 had a closer evolutionary relationship with all the bdkrb2 genes with six TM domains in black rockfish, which suggests that Bdkrb2 genes in the black rockfish may have functional differentiation.
Fig. 3 Phylogenetic analysis of Bdkrb2 paralogs and orthologs in vertebrate. The phylogenetic tree was constructed by MrBayes software. The length of the branch represents genetic distance, and bootstrap percentages are shown as numbers on the branches.

4. Bdkrb2 genes have similar functionalities and dissimilar tissue expression patterns

To understand the functional differentiation of Bdkrb2 genes in black rockfish, the expression pattern was analyzed in different tissues. The result shows that eight Bdkrb2 genes can be roughly classified into three patterns (see in Fig 4). Ssc_10023113 and Ssc_10023119 are highly expressed in the gills, Ssc_10023114, Ssc_10023115 and Ssc_10023116 are highly expressed in the intestines, regardless of gender. Ssc_10023117, Ssc_10023118 and Ssc_10023120 are highly expressed in the ovarian wall in females and genitalia in males (see in Fig 4A). Among the eight genes, Ssc_10023113 has a wider tissue expression, such as the brain, gill, intestine, ovarian wall and genitalia. Particularly attention was paid to the ovarian wall. Expressions of Bdkrb2 genes in the ovarian wall were analyzed by TPM in detail. The result shows that Ssc_1023117 had the highest expression and Ssc_10023113 had a relatively higher expression among the three Bdkrb2 genes with seven TM domains (see in Fig 4B).

Capped mRNA of two genes were next synthesized in vitro. Transgenic zebrafish strain (Flila: EGFP) was used for functional study due to technical limitations in the black rockfish. Both overexpression of Ssc_10023113 and Ssc_10023117 resulted in different degrees of malformation in 24 h post-fertilization (hpf) embryos, including rostral-caudal axis, edema of the pericardial cavity (see in Fig 5A). Deformation rates were 55.77% and 35.65%. Role of Bdkrb2 in vasodilation was well studied, however, the role in vascular development has been rarely reported. Bdkrb2 was identified among the differentially expressed genes associated with development of cerebrovascular dysfunction in OXYS rats [30]. The function of Bdkrb2 genes (Ssc_10023113 and Ssc_10023117) in embryo vascular development were observed via confocal microscopy. Both overexpression of Ssc_10023113 and Ssc_10023117 resulted in vascular alterations, particularly the common cardinal veins (CCVs) and intersegmental vessel (Se). Although the two Bdkrb2 genes have similar function, the regulatory capacity of Ssc_10023117 was not so strong as...
Ssc_10023113 base on the deformation rates of embryos and vascular abnormalities.

Fig. 4 Tissue expression pattern of Bdkrb2 genes. A) Heatmap was constructed by comparing 20 tissues. The x-axis shows sampled tissues, with the prefix F_ for female and M_ for male samples, and the y-axis shows genes. Gene expression patterns can be roughly divided into gill-type, intestine-type and reproductive organ-type. B) The expressions of Bdkrb2 genes in ovarian wall. The x-axis shows genes and the y-axis shows TPM (Transcripts Per Million).

Fig. 5 Functional study of bdkrb2 genes in zebrafish. Overexpression of Ssc_10023113 and Ssc_10023117 affects the phenotype and vascular development of embryos. An amount of 1.5 ng/embryo of mRNA was injected into Tg (Fltl1a: EGFP) embryos at 1-4 cell stages. All embryos are shown with anterior to the left. A) Phenotype of embryos with overexpression of mRNA at 48 hours post-fertilization (hpf). Scale bar = 200 μm. a, control group, embryos without injection. f, control group, embryos with injection of equal volume of sterilized DEPC water. b-e, experimental group, embryos with injection of Ssc_10023113 mRNA. b, normal phenotype; c, mild phenotype; d, medium phenotype; d, serious phenotype. g-j, experimental group, embryos with injection of Ssc_10023117 mRNA. g, normal phenotype; h, mild phenotype; i, medium phenotype; j, serious phenotype. B) Deformation rates
of embryos in each category as shown in A). N is the number of total samples analyzed in each group. C) Vascular abnormalities of embryos with overexpression of mRNA at about 55 hpf. Scale bar = 100 μm. a’, control group, embryos with injection of equal volume of sterilized DEPC water. b’-d’, experimental group, embryos with injection of Ssc_10023113 mRNA. e’-g’, experimental group, embryos with injection of Ssc_10023117 mRNA. Yellow arrowheads indicate different types of vessels, whereas the red asterisks point out the abnormalities in the experimental group. H: heart, CCV: common cardinal veins, Sc: intersegmental vessel.

5. **Bdkrb2 genes play a role in the ovary wall in the reproductive cycle**

To investigate the function of *Bdkrb2* genes in the ovary in the reproductive cycle, samples of connective tissue rich in blood vessels covering the egg membrane, embryos and ovarian wall at different stage were collected for RNA-seq. The expression analysis provides more detailed information about the different regulatory pattern of angiogenesis and vasoconstriction in the three kinds of samples (see in Supplemental Fig6 and Supplemental Fig7). Angiogenesis and vasoconstriction related genes have continuous expression at pre-fertilization and gestation. All *Bdkrb2* genes are expressed in the ovarian wall, however, their expression periods were not similar (see in Fig5). Ssc_10023113, Ssc_10023116, Ssc_10023117, Ssc_10023118, Ssc_10023119 and Ssc_10023120 had an expression at the stage of pre-fertilization and pre-hatching, suggesting that these genes play a role in making preparations for fertilization and hatching. Furthermore, Ssc_10023113, Ssc_10023114 and Ssc_10023115 also highly expressed at the stages at the stage of hatching, suggesting that these genes play a role in hatching. In conclusion, all *Bdkrb2* genes make a contribution in the function of the ovary wall during the reproductive cycle.

![Fig.6 Expression pattern of Bdkrb2 genes in the ovary in the reproductive cycle](image)

**Fig.6** Expression pattern of *Bdkrb2* genes in the ovary in the reproductive cycle. A) Heatmap was constructed by comparing three kinds of samples at different stage of the reproductive cycle. The x-axis shows sampled tissues with the prefix C. for connective tissue rich in blood vessels covering the egg membrane and E. for embryos and O. for ovarian wall. Arabic numerals represent different stages. 1, pre-fertilization; 2, 1-cell; 3, 8-cells; 4, 16-cells; 5, gastrula stage; 6, 8-somites stage; 7, tailbud stage; 8, pre-hatching; 9, hatching.

**Discussion**

In this study, we screened eight *Bdkrb2* genes from the black rockfish genome. The number of *Bdkrb2* genes in vertebrate varies greatly, from one copy to eight copy. The BDKRB2 protein is
encoded by a single-copy gene in human, which contains 3 exons separated by 2 introns. The first and second exons are noncoding, while the third exon contains the full-length coding region [31]. The number of Bdkrb2 genes in the black rockfish is the most in the species we investigated. These genes were arranged in a tandem array on chromosome14, forming a gene cluster spanning about 50 kb. Three of which with typical structure forming seven transmembrane (TM) domains and the rest five only have six TM domains. Difference in number of TM domains resulted in 3D structure change. N-terminus of former kind of protein is extracellular and C-terminus is intracellular, however, both N-terminus and C-terminus of the latter kind of proteins are intracellular. BDKRB2 is a G protein receptor, which combines with ligand bradykinin via N-terminus and stimulate PI3K/Akt via intracellular domains to activate Calm, leading to efficient nitric oxide (NO) synthesis [15, 16]. The conformational changes can cause functional changes. The interaction with ligand may be affected in the latter kind of proteins. Here we provide a perspective that BDKRB2 with six TM domains can amplify intracellular signals when co-expressed with BDKRB2 with seven TM domains. Phylogenetic analysis showed that genes which encoded the former kind of proteins each belonged to a clade and the latter kind of genes were clustered with Ssc_10023113. The three clades show different evolutionary rate. The group of Ssc_10023113, Ssc_10023116, Ssc_10023117, Ssc_10023118, and Ssc_10023120 evolved faster than Ssc_10023114 and Ssc_10023115. Clustering and evolutionary distance suggested that Bdkrb2 genes in black rockfish may have functional differentiation. The tissue expression pattern provides evidence to support this insinuation. The result shows that eight Bdkrb2 genes can be roughly divided into three patterns, including gill-bias expression, intestine-bias expression and ovarian wall or genitalia-bias expression, illustrating that these genes perform the function in various combinations in different tissues. Preliminary functional experiment in zebrafish shows that Ssc_10023113 and Ssc_10023117 have a similar function in embryo development, however, the regulatory capacity of Ssc_10023117 was not as strong as Ssc_10023113. Taking protein structure into consideration, the protein encoded by Ssc_10023113 with seven TM domains and that encoded by Ssc_10023117 only with six TM domains. Lack of one TM domain resulted in function insufficiency. These proteins may not be able to interact with extracellular signals but able to interact with intracellular signals, because both N-terminus and C-terminus of the proteins are intracellular. We speculate that BDKRB2 with six TM domains can amplify intracellular signals when it co-expressed with BDKRB2 with seven TM domains. Moreover, Bdkrb2 genes play a role in vascular development. Overexpression of Ssc_10023113 and Ssc_10023117 mRNA resulted in similar vascular abnormalities. The development of intersegmental vessels (Se) and/ or common cardinal veins (CCVs) was affected. Bdkrb2 was one of genes related to cerebrovascular dysfunction with the development of Alzheimer’s disease-like pathology in OXYS rats. Its expression was found to be reduced at 20-day-old and 18-month-old, and was increased at 5-month-old in OXYS rats [30]. However, the underlying mechanisms need further research. Interestingly, Bdkrb2 genes have bias expression in ovarian wall, but not in oocytes of pre-fertilization adults. The RNA-sequencing data show that Bdkrb2 gene has a relatively high
expression in uterus and placenta, but a relatively low expression in ovary and testis in mammals (data from PRJNA280600, PRJNA66167, PRJNA238328). Both human and rat BDKRB2 proteins show an intense membrane labeling in the epithelial cells of endometrial glands and luminal [32]. A greater expression of BDKRB2 protein was observed in the early pregnancy samples and the location of signals in the uterus coincide with other vasoactive effectors such as NO, prostacyclin, growth factors and renin [33, 34]. The expression of Bdkrb2 was also detected in endometrial and prostate cancers [35]. These studies made Bdkrb2 related to the physiological and pathological functions of the uterus. Uterus is the organ where fetus is conceived and the placenta is developmentally crucial for reproductive success in placental mammals [36]. Black rockfish is viviparous. But unlike the placental mammals, black rockfish did not evolve a uterus to reproduce, whose offspring are gestated in ovary. The expression in ovarian wall but not oocytes suggest that ovarian wall evolved the function like mammalian uterus to meet the need of viviparity. The connective tissue rich in blood vessels covering the surface of the eggs may evolved the function like mammalian placenta.

Bdkrb2 genes also have bias expression in genitalia. Previous studies have shown the expression of KKS components in the male reproductive tract and the function related to smooth-muscle contraction, prostaglandin production, the motility and vitality of ejaculated spermatozoa and anion secretion [37-40]. Bdkrb2, an important component of KKS, showed segmental expression in rat efferent ducts and epididymis and played a role in glycerol and potentially water transport via AQP9, which was essential for concentration of spermatozoa and the establishment of luminal hypertonicity [41]. These data point to a regulatory role for Bdkrb2 in sperm maturation and storage. One peculiarity of viviparity in black rockfish is that long-term storage of sperm in the ovary. However, little has been known about the mechanism. Whether Bdkrb2 participates in the sperm preparation need further investigation.

We previously reported a model of black rockfish reproduction, and proposed that the rockfish ovarian wall has a similar function to the uterus of mammals [4]. Different WGCNA co-expression modules were identified in ovarian wall and oocytes. Genes in the module of ovarian wall are associated with placental development, cell adhesion, trophoblast invasion, calcium-sensing receptors, the NO-sGC-cGMP signalling pathway, and blood vessel function. Especially, the NO/cGMP signaling pathway, which is crucial to oocyte maturation, insemination, pregnancy and birth [9, 10]. The chemical messenger NO is an endothelium derived relaxing factor, which participates in the control of vasorelaxation in fetoplacental vessels [42]. Bdkrb2 can participate in regulating vasodilation, vasopermeability, matrix degradation cell proliferation, and myometrial contractility by controlling NO production via binding with bradykinin in the key sites for embryo attachment, implantation, placentation, maintenance of placental blood flow, and parturition [34]. Ovarian wall, connective tissue rich in blood vessels covering the egg membrane and embryos at different stages were collected for RNA sequencing to investigate the potential function of Bdkrb2 genes during breeding cycle in the black rockfish. Cluster analysis of gene expression profile was performed. Interestingly, Ssc_10023113 was clustered with Ssc_10023116, Ssc_10023117, Ssc_10023118, Ssc_10023119 and Ssc_10023120, consistent with the result of phylogenetic analysis. This clustering pattern also shows the functional differentiation of Bdkrb2 genes. The eight genes have time-dependent expression in the ovarian wall during the reproduction cycle.
Furthermore, all genes in the cluster mentioned above were relatively high expressed in the ovarian wall at the stage of pre-fertilization and pre-hatching, suggesting that these genes play a regulatory role in the preparation for fertilization and hatching. In a conclusion, more Bdkrb2 genes expressed in the ovarian wall to play a role in pregnancy and birth, providing a new view of viviparity adaption in black rockfish.

Conclusion

Our study suggests that the expansion of Bdkrb2 gene family is an adaptation for viviparity in black rockfish. Most Bdkrb2 genes have a bias expression in the ovarian wall at the stage of pre-fertilization and pre-hatching, indicative of functional significance of these genes. But due to changes of protein structure, the BDKRB2 with six TM domains operating together with the BDKRB2 with seven TM domains, collaborating in the regulation of vasodilation.

Methods

Fish and samples

Black rockfish (Sebastes schlegelii) were obtained from Zhucha Island (Qingdao, Shandong, China). Samples used for tissues RNA-seq were collected in November 2017. Six healthy 3-year-old fish (three males and three females) were randomly selected for sampling of heart, liver, spleen, kidney, brain, intestine, gill, muscle, ovarian wall, oocytes and gonad (testis or ovary). Samples of ovary tissues at different developmental stage (pre-fertilization, 1-cell, 8-cell, 16-cell, gastrula stage, 8-somites stage; tailbud stage, pre-hatching, hatching), including connective tissue rich in blood vessels covering the egg membrane, embryos and ovarian wall, were collected from March 2019 to May 2019 and used for RNA-sequencing.

Transcriptome data

The RNA-seq libraries of black rockfish were sequenced by the BGI-seq 500 platform. The transcriptomic data were mapped to the rockfish genome and the expression level of genes was calculated using Salmon [43] with default parameters. Genes of interest were subsequently visualized via the Heatmap package. Other mammal transcriptomic data was obtained from NCBI, including RNA sequencing of total RNA from 20 human tissues project (PRJNA280600), mouse ENCODE transcriptome project (PRJNA66167) and the project of rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages (PRJNA238328). Genes of interest were subsequently visualized via the histogram.

Sequence alignment and protein structure analysis

Sequence alignments and comparisons of homology between eight Bdkrb2 ORF sequences or amino acid sequences were performed using the MUSCLE algorithm in MEGAX [44]. The protein domains were predicted by SMART (available online: http://smart.embl-heidelberg.de [45] and the schematic diagram was constructed by the online tool IBS (available online: http://ibs.biocuckoo.org/online.php) [46]. 3D Structure prediction analysis was performed via Phyre2 online tool [47] and modified by PyMol software (https://pymol.org/2/).
Phylogenetic Analysis

The evolutionary history was inferred by using the Mrbayes software, mcmc=500000 generations [48]. All parameters are the default. This analysis involved 27 amino acid sequences, which were retrieved from NCBI (available online: http://www.ncbi.nlm.gov) and Ensemble (available online: www.ensembl.org). Homo sapiens (BDKRB2, NP_000614.1), Mus musculus (BDKRB2_isofrom X1, XP_011242293.1 and BDKRB2_isofrom X2, XP_006515505.1), Rattus norvegicus (BDKRB2_isofrom X1, NP_001257642.1 and BDKRB2_isofrom X2, NP_775123.2), Gallus gallus (BDKRB2_isofrom X1, XP_011242293.1 and BDKRB2_isofrom X2, XP_025006606.1), Podarcis muralis (BDKRB2, XP_028572589.1), Xenopus laevis (BDKRB2-like, XP_018085979.1 and XP_018087887.1), Callorhinchus milii (BDKRB2, XP_007910171.1), Danio rerio (Bdkrb2_like, XP_009291047.1), Larimichthys crocea (BDKRB2, ENSLCRP00005048508, ENSLCRP00005048527, ENSLCRP00005048529), Lepisosteus oculatus (BDKRB2, ENSLCP00000021802), Orzyzias latipes (BDKRB2, ENSORLP00000042622), Poecilia formosa (BDKRB2, ENSPREP00000007245), Xiphophorus maculatus (BDKRB2, ENSXMAP0000009989) and Sebastes schlegelii (Ssc_10023113, Ssc_100231114, Ssc_100231116, Ssc_100231116, Ssc_100231117, Ssc_100231118, Ssc_100231119 and Ssc_100231120) from local data.

mRNA Synthesis and Microinjection

Capped mRNA of different Bdkrb2 genes was synthesized with mMESSAGE mMACHINE@T7 (Ambion, Foster City, CA, USA). The primers were Ssc_10023113_mRNA_Fw/ Rv and Ssc_10023117_mRNA_Fw/Rv (see in supplemental Table1). Microinjection was performed on Harvard Apparatus PLI-100 (NatureGene, NV, USA) machine in one- to four-cell-stage embryos of transgenic strain (Fli1a: EGFP) with 1.5 ng mRNA for each embryo. Embryos without injection and with injection of sterilized DEPC water were the control group.

Abbreviations

Bdkrb2: Bradykinin receptor B2
NO: nitric oxide
VEGF: Vascular Endothelial Growth Factor
S-1P: Sphingosine-1-Phosphate
H$_2$O$_2$: Hydrogen Peroxide
KKS: kallikrein-kinin system
NOS: nitric oxide synthase
eNOS: endothelial nitric oxide synthase
ORF: open reading frame
TM: transmembrane
CCVs: common cardinal veins
Se: intersegmental vessel
TPM: Transcripts Per Million
hpf: hour post-fertilization

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Ethics approval and consent to participate
This study was approved by the Animal Care and Use committee of the Centre for Applied Aquatic Genomics at the Ocean University of China.

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Authors’ contributions
Jingjing Niu, Yan He and Jie Qi conceived and designed the research. Jingjing Niu, Weihao Song, Haiyang Yu, Jian Guan analyzed data. Jingjing Niu, Rui Li performed the experiment. Jingjing Niu wrote the manuscript. Jian Guan, Jie Qi and Yan He revised the manuscript.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.