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Molecular Characterization of the Duck SCF Gene and its Expression Changes in Different Color Plumage Bulbs

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Abstract: Stem Cell Factor (SCF) also called Steel Factor (SF), Mast cell Growth Factor (MGF) and KIT tyrosine Kinase receptor Ligand (KL) is a hematopoietic growth factor and ligand for the KIT tyrosine kinase receptor. In this study, the partial complementary DNA (cDNA) of SCF was cloned from the eye of duck by homology cloning and Rapid Amplification of Cdna Ends (RACE) approaches. Comparing with zebra finch, quail, chicken and mammalian SCF cDNA sequence, the phylogenetic tree displayed that duck SCF is highly conserved, it shares 63.8-94.3% similarity with the above species cDNA sequence. The rooted phylogenetic neighbor-Joining tree with bootstrap was done using SCF CDS sequences from duck and other species by DNAMAN software. The results showed that duck has close relationship with chicken, quail and zebro finch. The semi-quantitative RT-PCR analysis indicated that SCF was a universally expressed gene, it was detected in heart, liver, spleen, kidney, brain, back skin, muscle, eye, glandular stomach, belly skin. Q-PCR was employed to analyze the SCF gene expression in six type duck hair bulbs. The results showed that its expression had significant difference between W-W which is white hair bulb taken from white plumage with yellow beak and other types (BL-Bl which is black feather hair bulb taken from black plumage with black beak, W-L which is white feather hair bulb taken from white plumage with black beak ducks, W-WB which is white feather taken from white-black plumage with black beak, B-WB which is black feather taken from white-black plumage with black beak), $p<0.001$). The expression of SCF in BR (brown feather hair bulb taken from brown plumages with black beak ducks) has significant difference comparing with the above 5 hair bulb types ($p<0.05$). There is no significant difference between any of the following types which including BL-BL, W-L, W-WB and B-WB. The results indicated that SCF may be a critical gene on regulation gene expression for duck plumage diversity.

Key words: Cloning, SCF, duck, expression analysis, plumage diversity, sequence

INTRODUCTION

Stem Cell Factor (SCF) is a hematopoietic growth factor and ligand for the KIT tyrosine kinase receptor. This gene is also called Steel Factor (SF), Mast cell Growth Factor (MGF) and KIT tyrosine Kinase receptor Ligand (KL). SCF involved in cytokine-cytokine receptor interaction, hematopoietic cell lineage, cancer pathways and melanogenesis. Martin *et al*. (1990) isolated the human SCF cDNA and genomic clones using probes based on the sequence of rat and characterized this protein. In the mouse, SCF has two splice variants which called Kl1 and Kl2, Kl2 encodes an isoform lacking a 28 amino acid sequence preceding the transmembrane region of Kl1 (Huang *et al*., 1992). SCF can augment the proliferation of both myeloid and lymphoid hematopoietic progenitors in bone marrow cultures acting via PI3K (Martin *et al*., 1990). This gene is also a paracrine regulator of Leydig cell steroidogenesis (Rothschild *et al*., 2003). McGill reported that the expression of the melanocytic master transcriptional regulator MITF is regulated by SCF-KIT signaling and stimulates transcription of the key enzyme tyrosinase genes in melanocyte lineages. Compared with the normal rat liver tissue, SCF-mRNA from the Cholangiocarcinoma (CC) tissue was upregulated up to 20 fold whereas c-Kit-mRNA was upregulated up to 5 fold, Mansuroglu *et al*. (2009) believed that SCF-c-Kit system, via acting as a surviving factor for CC cells may contribute to tumor development.

Chicken stem cell factor was cloned by Jian-Huo *et al*. (1993). Wang *et al*. (2007) found that KL expression in undifferentiated and differentiated

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granulosa cells could be transiently down-regulated by HB-EGF. However, the duck SCF gene has not been cloned. In this study, the duck SCF gene is characterized and detected its expression in different color type plumage bulbs.

MATERIALS AND METHODS

Animals and tissue collection: The genetic background of the experimental ducks was explained by Gong *et al.* (2010) e.g., four white plumage with yellow beak ducks, four white plumage with black beak ducks, four black plumage with black beak ducks, four brown plumage with black beak ducks and four white-black plumage with black beak ducks were randomly selected from a population of F2 generation. Three hair bulbs from one duck was pooled as one sample. Four white hair bulb samples were taken from four white plumages with yellow beak ducks and marked as W-W. Four white hair bulb samples were taken from four white plumages with black beak ducks and marked as W-L Four black hair bulb samples were taken from four black plumages with black beak ducks and marked as BL-BL.

About 4 brown hair bulb samples were taken from four brown plumages with black beak ducks marked as BR. Four black hair bulb samples and four white hair bulb samples were taken from four white-black plumage with black beak ducks, marked as BL-WB and W-WB, respectively. Duck plumage pattern as (Fig. 1). Two ducks at 1 week old were anesthetized with ether and killed by the bleeding of jugular veins. Various tissues including heart, liver, spleen, kidney, brain, back skin, muscle, eye, glandular stomach and belly skin were surgically removed and put into marked 2 mL EP tuble in which 1 mL TRIzol reagent (Invitrogen, SanDiego, CA). One ceramic bead were prepared immediately then grinded 30 sec by easy grind immediately, then stored at -80°C until total RNA extraction.

RNA extraction and cDNA synthesis: The total RNA was isolated from the above collected tissues and 6 type hair bulbs by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The amount of total RNA was estimated by Spectrophotometer ND-1000 (Nano-Drop, USA). The first-strand cDNA was synthesized from 1 μg of DNase-treated (Biored CO., DNaseI) total RNA according to M-MLV reverse transcriptase kit (TOYOBO, Japan) at 42°C. The cDNA was used as the template for PCR reactions in gene cloning and expression profile analysis.

Cloning and sequencing of SCF cDNA fragment: Based on conserved regions in other SCF sequences including *Gallus gallus* (NM-205130.1|), *Mus musculus* (NM-013598.2|), human (gb|M59964.1|HUMSCF) and *Coturnix coturnix* (U43078.1|CCU43078), *Taeniopygia guttata* (XM-002196398.1) primers were designed using the Oligo 6.0-amplify duck SCF cDNA fragment from eye (Table 1). The PCR was performed in a final volume of 10 μL, containing 50-300 ng cDNA come from duck eye, 3 μM of each primer, 0.1 mM deoxynucleoside triphosphate, 20 mM Mg Cl_2, 10×buffer, 0.5U of DNA polymerase (TransGen Biotechnology Company, Beijing, P.R. China) on an thermal cycler (Applied Biosystems, Foster City, CA).

The PCR reaction procedure is as follows: 95°C for 4 min then 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. After a 5 min final extension at 72°C, the products were visualized on a 2.0% agarose gel using

Fig. 1: Duck plumage pattarn; A) black plumages with black beak duck; B) white-black plumage with black beak duck; C) brown plumages with black beak duck: D) white plumages with yellow beak duck; E) white plumages with black beak duck

Table 1: Primers used for RT-PCR, RACE and semi-quantitative RT-PCR

Primer name	Primer sequence (5'-3')	Tmp (°C)	Function
SCF-F_1	ATGAAGAAGGCACAAACTTGG	58	RT-PCR
SCF-R_{578}	TCCTAAGGGAGCTGGCAGCAA		
NUP	AAGCAGTGGTATCAACGCAGAGT		RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT		RACE
GSP1	GCACAAACTTGGATTATCAC	68	3'-RACE
GSP2	ACATCAGACATACCGGAAATATCAG		5'-RACE
NGSP1	GCCAGCTCCCTTAGGAATGAC	60	3'-RACE
NGSP2	GCAACCAACAGTGATTAGGCAAG		5'-RACE
SCF-F_{583}	GCCAGCTCCCTTAGGAATGAC	60	sqRT-PCR
SCF-R_{617}	TTGCTGCAACATACTTATCTC		andqRT-PCR
β-actin-F_{256}	GTATCCTGACCCTGAAGTACC	60	sqRT-PCR
β-actin-R_{454}	ACAGCCTGGATGGCTACATAC		andqRT-PCR

NGSP1 is the same as SCF-F_{583}

ethidium bromide staining. PCR products were carefully excised from the agarose gels then purified with a TransGen gel extraction kit (TransGen Biotechnology Company, Beijing, P.R. China). The purified PCR products were ligated and sub-cloned into the PEASY-T1 plasmid vector (TransGen Biotech) according to manufacturer's protocol. Clones were selected by blue-white screening, DNA sequencing was performed in Augct Company (Beijing, China) using an automated ABI3730 analyzer (Applied Biosystems, Foster City, CA,USA).

Rapid Amplification of 3' and 5' cDNA Ends (RACE): Two pairs of Gene-Sequence Primers (GSP) and Nested Gene-Sequence Primers (NGSP) were designed based on the above PCR product sequences which were subsequently used to design primers for 5'-RACE and 3'-RACE to obtain the entire SCF cDNA sequence. For 3'-RACE and 5'-RACE PCR, 10 μg of RNA isolated from the eye was used and the RACE reactions were performed by using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA) according to the manufacturer's protocols. About 10 pmol of 10×Universal Primer A Mix (UPM) and the GSP1 and GSP2 were used in the first 3'-RACE and 5'-RACE PCR, respectively. PCR cycling parameters were 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 min and 5 min at 72°C for the final extension. About 1 μL of PCR products from the first run were used as template in the second nest PCR run with NUP and NGSP as primers.

The temperature program included: denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 35 sec, annealing at 60°C for 35 sec and extension at 72°C for 1.5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels. 3'-RACE and 5'-RACE PCR products were gel-purified and sequenced as described above.

Cloning and sequencing of PCR products: The nucleotide sequences of SCF were analyzed using DNAMAN (version 6.0.40) software package and multiple alignment program. The sequences of different species were obtained with in NCBI. The phylograms were created by Neighbor-Joining (NJ) software with 1000 bootstrap trials after multiple alignments of sequence data by CLUSTALW (Thompson *et al*., 1994; Edgar, 2004; Tamura *et al*., 2007).

RT-PCR analysis of SCF expression in different tissues: To determine the distribution of duck SCF in various tissues, semi-quantitative RT-PCR was conducted for expression analysis. The SCF gene-specific primers (SCF-F583/SCF-R817) were designed based on the obtained cDNA sequence (Table 1). The conditions for PCR were denature at 94°C for 5 min followed by 30 cycles of 30 sec at 94°C, annealing at 60°C for 30 sec and extension at 72°C for 30 sec with a final extension at 72°C for 5 min. The control reactions using the gene-specific primers to duck β-actin (GenBank accession no: EF667345) were conducted with 30 cycles for PCR amplification from the same cDNA samples.

Table 2: The SCF mRNA GenBank accession numbers and Gene ID of different species

Species	Gene ID	CDS
Quail (*Coturnix coturnix*)	U43078	U43078.1|CCU43078
Chicken (*Gallus gallus*)	396028	NM_205130.1
Human	4254	gb|M59964.1|HUMSCF
Mouse (*Mus musculus*)	17311	NM_013598.2|
Pig (*Sus scrofa*)	397509	NM_214269.2
Horse (*Equus caballus*)	100034127	AF367704.1
Zebro finch (*Taeniopygia guttata*)	100225713	XM_002196398.1
Chimpanzee (*Pan troglodytes*)	452114	XM_509255.2
Rhesus monkey (*Macaca mulatta*)	574254	XM_001101381.2
Rat (*Rattus norvegicus*)	60427	NM_021843.3
Dog (*Canis familiaris*)	403507	NM_001012735.1
Cow (*Bos taurus*)	281885	NM_174375.2
Platypus (*Ornithorhynchus anatinus*)	100080577	XP_001511452.1
Opossum (*Monodelphis domestica*)	100021399	XM_001373535.1

All experiments were repeated 3 times Table 2. The PCR products were visualized on 2.0% agarose gels stained with ethidium bromide and visualized with ultraviolet light and band intensity was analyzed by using Quantity one software (Bio-Rad, Hercules, CA, USA).

Q-PCR analysis of SCF from six type hair bulbs: To check the SCF gene expression difference among six different hair bulb types, Q-PCR (quantitative PCR) was employed using β-actin gene expression as reference. The qPCR analysis was performed on Roche lightercycler® 480 using lightercycler® 480 SYBR Green master detection reagents (Roche Diagnostics 11367523). The primers of qRT-PCR were shown in Table 1. SCF-F583 located on exon6 according to chicken SCF precursor gene structure and SCF-R8175 was designed across exon8 and 9. All reactions were performed in triplicate within a PCR assay and under the same cycling conditions: denaturation at 95°C for 3 min followed by 40 of cycles of amplification (95°C for 20 sec, 60°C for 20 sec and 72°C for 20 sec) with single acquisition of fluorescence at the end of the extension step.

Melt curve analysis was performed over a range of 55-95°C in order to verify single product generation at the end of the assay. The data of quantification analysis was performed by using the Light Cycler analysis software.

RESULTS AND DISCUSSION

Duck SCF gene sequences and phylogenetic relationship with other species: By cDNA cloning and RACE analysis, we obtained bp of the SCF sequence. This sequence encode a aa protein. The alignment results showed that

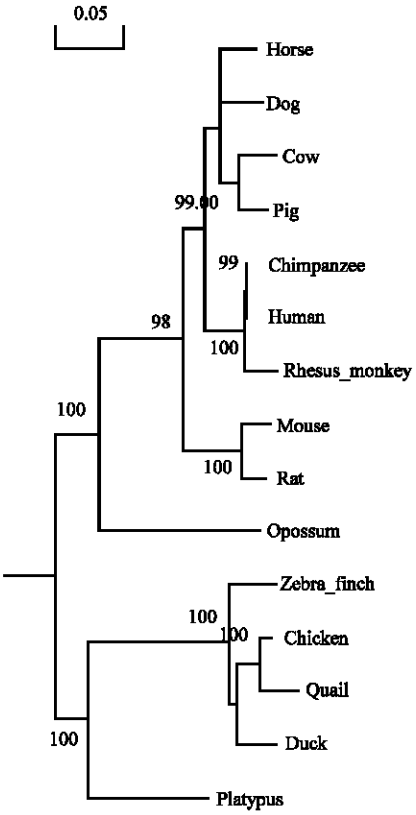

Fig. 2: Phylogenetic analysis of the SCF gene

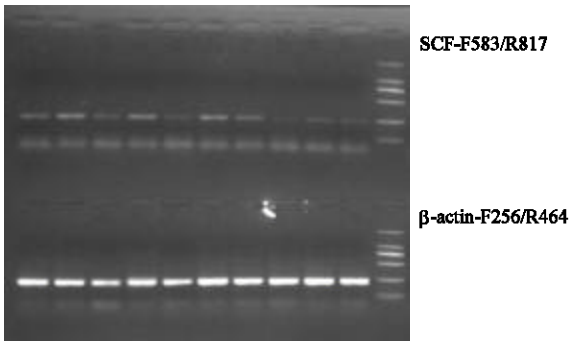

Fig. 3: Tissue expression of the duck SCF gene. 1: Eye; 2: Brain; 3: Back skin; 4: Heart; 5: Liver; 6: Spleen; 7: Kidney; 8: Glandular stomavh; 9: Leg muscle; 10: Belly skin. M: DL2000

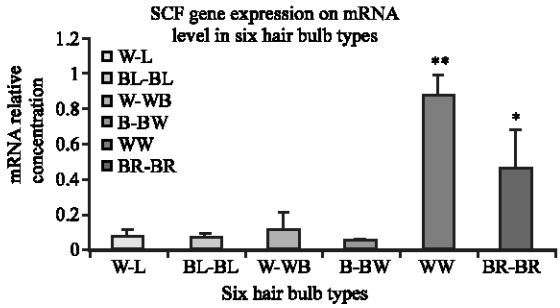

Fig. 4: Q-PCR analysis of the duck SCF gene in six hair bulb types

duck SCF shares a high identity with the nucleotide sequences of chicken (94.4%), quail (92.5%), zebra finch (92.5%) human (67.3%), pig (67.7%), horse (67.3%), opossum (70.0%), platypus (70.0%), rat (67.6%), dog (67.5%), rhesus monkey (63.8%) chimpanzee (67.5%) and mouse (67.6%). The deduced amino acid sequence of duck SCF shared significant sequence identities to SCF of other species including quail (93.7%), chicken (93.0%), Zebro finch (89%). The phylogenetic analysis showed that the duck SCF appears to be closely related to that of quail, zebra finch and chicken. The rooted phylogenetic NJ tree with 1000 bootstrap was shown in (Fig. 2).

Expression of SCF mRNAs in tissues: To determine the SCF gene expression levels in different tissues, simi-quantitative RT-PCR method was employed. The agarose gel electrophoresis of the PCR products for SCF-F583/R817 and β-actin-F256/R464 from individual samples showed that fragments of 255 and 229 bp were obtained, respectively (Fig. 3). The Simi-quantitative RT-PCR results showed that SCF gene is expressed in multiples tissues (Fig. 3).

Expression of SCF mRNAs in six hair bulb types: The quantitative RT-PCR results are shown in Fig. 4. The SCF gene expression in six hair bulb type demonstrate that SCF in white hair bulb from white plumage with yellow beak ducks (W-W) had the highest gene expression, next to brown hair bulb from brown plumage with black beak ducks (BR) while the other four types had lowest gene expression. The SCF relative expression had significant difference between W-W and other four types, e.g., BL-BL, W-L, W-WB and B-WB ($p<0.001$). BR also had significant difference comparing with the other 5 hair bulb types ($p<0.05$). There is no significant expression difference among BL-BL, W-L, W-WB and B-WB.

In this study, conserved regions of SCF gene in other species as well as RACE approach were used to design primers to obtain duck SCF sequence. The cloned duck sequences shared high homology with *Gallus gallus* (NM-205130.1|), *Mus musculus* (NM-013598.2|), human (gb|M59964.1|HUMSCF) and *Coturnix coturnix* (U43078.1|CCU43078) and *Taeniopygia guttata* (XM-002196398.1) indicating the function of this gene is conserved among different species. The Q-PCR result showed that SCF is expressed in eye, brain, back skin, heart, liver, spleen, kidney, glandular stomach, leg muscle and belly skin. This

result is in according with SCF has multiple function which involved in cytokine-cytokine receptor interaction, hematopoietic cell lineage, cancer pathways and melanogenesis. SCF can increase the proliferation of both myeloid and lymphoid hematopoietic progenitors in bone marrow cultures (Martin *et al*., 1990).

Vincent *et al*. (1998) reported that blocking interaction of Kl2 with Kit with soluble Kl protein inhibited the appearance of haploid cells and completion of meiosis. Rothschild *et al*. (2003) found that KITLG is a paracrine regulator of Leydig cell steroidogenesis. By incubation of CD34 (142230)-positive/CD38 (107270)-positive cord blood cells with IL9 (146931) and SCF, Matsuzawa *et al*. (2003) concluded that IL9 is a potent enhancer of SCF-dependent growth of human mast cell progenitors.

More studies on SCF focused on the relationship between SCF and pigmentation variation in human skin, hair and eye e.g., on hair and eye pigmentation, skin sensitivity to sun and freckling variants based on genome wide scan results. Sulem *et al*. (2007) found that a variant near KITLG (184745.0001.C/T dbSNP:rs12821256) was associated with hair color (611664) in Icelanders and Dutch. Miller *et al*. (2007) believed that the rs642742 SNP (184745.0002) located 326 kb upstream of the KITLG transcription start site, A/G allele can reflect difference between West Africans and Europeans skin pigmentation. Study on a 6 generation Chinese family with familial progressive hyperpigmentation (145250), Wang *et al*. (2009) identified a missense mutation in the SCF gene (N36S; 184745.0003, A to G transition at nucleotide 107 in exon 2 of the KITLG gene) which is a gain of function as responsible for this disease.

CONCLUSION

These results all indicated that the SCF gene may play important roles in pigmentation. However, the SCF gene is rarely studied in poultry species. This study is the first report of the SCF gene in the duck. Expression in six type duck hair bulbs indicated that SCF involved in duck plumage pigmentation.

More interesting, except the high expression levels in W-W and BR, the other four types including W-L (which taken from white plumage with black beak ducks), W-WB (which taken from white-black plumage with black beak) and W-W (hair bulb which from white plumage with yellow beak) are all un-pigmention feather but the SCF mRNA expression level had significant difference. This result demonstrated that mechanism of the white plumage is different and very complicated in different type of ducks.

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