Molecular characterization and tissue expression profile of porcine *Ephrin-B2*

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ABSTRACT. Ephrin-B2 (EFNB2) is a signaling molecule that plays an important role in cell adhesion, proliferation, and migration in humans. However, little is known about this molecule in pigs. In order to investigate whether *EFNB2* is associated with the skeletal muscle in pigs, we cloned the full-length cDNA of *EFNB2* (GenBank accession No. KF500033) from the longissimus dorsi muscle of Yorkshire pigs by rapid amplification of cDNA ends. The results indicated that its full-length cDNA comprises 1991 bp, with an open reading frame of 1002 bp, a 5' end of 88 bp, and a 3' end of 901 bp. We analyzed the homology of porcine *EFNB2* with sequences from other species, and the phylogenetic tree showed that pig *EFNB2* was most closely related to that from sheep, followed by domestic cats and wolf, with mackerel being the most distantly related. Porcine EFNB2 is a water-soluble protein with a theoretical molecular weight of 36,928.1 Da, an isoelectric point of 8.98, and a hydrophilic transmembrane-spanning region. It contains 19 glycosylation sites and eight phosphorylation sites. The tertiary structure of the EFNB2 protein showed a forniciform helix structure. The porcine *EFNB2* gene was expressed in ten different tissues from 25-day-old Shaziling and Yorkshire piglets, with the highest expression observed in the longissimus dorsi. These results lay the foundation for further study on the *EFNB2* gene in pigs.

Key words: Pig; *EFNB2*; Cloning; Expression profiles

INTRODUCTION

The ephrin family is one of the largest families of receptor protein tyrosine kinases, and, in humans, is made up of ephrin-B2 (EFNB2), ephrin-A2 (EFNA2), ephrin-A5 (EFNA5), and ephrin-B4 (EPHB4). These proteins are related to the formation of blood vessels, axons, human gastric cancer, and tumors (Kataoka et al., 2002; Wu et al., 2004; Das et al., 2010). The interaction between ephrin and its receptor plays a pivotal role in the formation of blood vessel endothelial cells (Noren et al., 2006) and promotes the angiogenesis (Yu et al., 2003). At the same time, it regulates the migration of neuroblasts and cellular proliferation (Katsuta et al., 2013; Thomas et al., 2013). Furthermore, it mediates the production of alveoli neurofibrilla by targets in the remote distance (Xie et al., 2011; Bennett et al., 2013), constructed a p*EFNB2*-shRNA vector, and showed that it could decrease the activity of HeLa cells and induce apoptosis through repression of *EFNB2* gene expression.

EFNB2 is located on *Homo sapiens* chromosome 13 (*HSA*13), *Mus musculus* chromosome 8, and *Rattus norvegicus* chromosome 16. The full-length human *EFNB2* gene spans 45,240 bp with a 3290-bp 5'-untranslated region (UTR) and a 25-bp 3'-UTR. Human *EFNB2* is composed of five exons and four introns; however, little information is known about the porcine *EFNB2* gene. We have mapped this gene to *Sus scrofa* chromosome 11 using the somatic cell hybrid panel and the INRA-Minnesota 7000-rad porcine x Chinese hamster whole-genome radiation hybrid panel (Ma et al., 2005). In this study, we cloned the swine *EFBN2* gene using the rapid amplification of cDNA ends (RACE) technique and analyzed the similarity of its cDNA with that from other species. In addition, expression of the *EFNB2* gene in 10 tissues from one 25-day-old Shaziling and Yorkshire piglets was investigated by quantitative PCR. The results of this study aim to provide insight into the role of the *EFNB2* gene in the formation of meat in pigs.

MATERIAL AND METHODS

Sample collection

Samples were collected from three castrated full-sibling 25-day-old males from each of the indigenous Chinese Shaziling pigs and the European Yorkshire pigs, including the longissimus dorsi, crureus, lung, liver, pancreas, intestine, cecum, heart, spleen, and kidney. Samples were then snap-frozen with liquid nitrogen, stored at -80°C, and used for quantitative real-time PCR.

RNA extraction, reverse transcription, cloning, and sequencing

RNA was extracted from different tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol. Total RNA was treated with DNase I before the generation of first-strand cDNA, which was synthesized from RNA by reverse transcription PCR. Total RNA was reverse transcribed by M-MLV reverse transcriptase (Promega, Madison, WI, USA), oligo dT primer, and ribonuclease inhibitor in a total volume of 50 μL. The PCR conditions were as follows: 5 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at 55°C, 60 s at 72°C (Zhang et al., 2010). Primers were designed based on the conserved sequences using the software Primer premier 5.0 as shown in Table 1.

Table 1. *EFNB2* gene cloning and expression of primer sequences.

Application of primers	Primer sequences (5'-3')	Annealing temperature (°C)	Size (bp)
5'-RACE	F1: TCTTCGGCGCGAGTCTTCCA	60	1327
	R1: AGCAGTGGTATCAACGCAGAGTACACGCGGG		
3'-RACE	F2: GTGTCTGCAACGGACTGATG	55	726
	R2: AAGCAGTGGTATCAACGCAGAGTAC		
QPCR	F3: GCCCAGTGACATTATCATCCC	59	155
	R3: CCGAGGCTCAGACCTTGTAGTA		
GAPDH	F4: ATTTGGCTACAGCAACAGGGT	59	172
	R4: AAGTCAGGAGATGCTCGGTG		

RACE

In order to obtain the cDNA sequence of *EFNB2*, two pairs of primers were designed to amplify the 5' and 3' ends of the *EFNB2* gene (Table 1). The PCR products were separated on a 1.5% agarose gel. Following gel purification, the RT-PCR products were cloned into the pMD19-T vector (Tiagen, Dalian, China) and sequenced. The full-length cDNA sequence was obtained using the DNAStar 7.1 software (http://www.gpxz.com).

Phylogenetic analysis

Artificial calibration graphs of peak quality were spliced and the *EFNB2* gene sequences of 13 species were downloaded from GenBank to analyze homology. A phylogenetic tree was constructed with the full-length cDNA of previously published *EFNB2* gene sequences using the neighbor-joining method with MEGA version 5.0 (Tamura et al., 2011).

Bioinformatic analyses

The amino acid phosphorylation sites were deduced using net Phos (http://www.cbs.dtu.dk/services/NetPhos/) and glycosylation sites were identified by NetOGlyc (http://www.cbs.dtu.dk/services/) and Protscale (http://web.expasy.org/protscale/). The hydrophilicity and amino acid domains were analyzed using smart (http://smart.embl-heidelberg.de/) and the tertiary structure using SWISS-MODEL (http://swiss-model.expasy.org/).

Real-time quantitative PCR analysis

To evaluate the expression of porcine *EFNB2* in different tissues, quantitative PCR was carried out using the SYBR Green I method in a total of 25 µL, which contained 2 µL cDNA, 12.5 µL 2X SYBR premix ex Taq II, 0.5 µL primers, 10.0 µL ddH$_2$O. PCR was performed as follows: 2 min of pre-incubation at 95°C followed by 40 cycles for 50 s at 95°C, 30 s at 59°C, and 40 s at 72°C. Three technical replicates were performed with each sample, and the arithmetic mean Ct value was obtained. The relative expression of the target gene and standardization to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were calculated using $2^{-\Delta\Delta Ct}$ method, and Yorkshire pig small intestine was used as the control group.

RESULTS

Sequence analysis and comparison of amino acid sequence homology between animal EFNB2

Using 5'- and 3'-RACE sequence assembly, the full-length 1991-bp cDNA sequence of the porcine *EFNB2* gene was obtained (GenBank accession No. KF500033), including a 1002-bp open reading frame (ORF), a 88-bp 5'-UTR, and a 901-bp 3'-UTR, encoding 333 amino acids. The deduced amino acid sequence is shown in Figure 1.

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CTCCTTGTCTGCCCGGAAGATTGGGGCTTTCGGCCCGCACCCCGTTCCTCCGACGCCAGG
AGTGCGCGGAGCTGGGAGCGGCTTCGCCATGGCCGCGAGGAGGGACTCCGTGTGGAAGTA
                        M   A   A   R   R   D   S   V   W   K   Y
CTGCTGGGGAGTTTTGATGGTTTTATGCAGAACTGCGATTTCCAGATCGATAGTTTTAGA
  C   W   G   V   L   M   V   L   C   R   T   A   I   S   R   S   I   V   L   E
GCCCATCTATTGGAATTCCTCGAACTCTAAATTTCTACCTGGACAAGGACTGGTACTATA
  P   I   Y   W   N   S   S   N   S   K   F   L   P   G   Q   G   L   V   L   Y
CCCACAGATAGGAGACAAATTGGATATTATTGCCCCAAAGTGGACTCTAAAACTGTTGG
  P   Q   I   G   D   K   L   D   I   I   C   P   K   V   D   S   K   T   V   G
CCAGTATGAATATTATAAAGTTTATATGGTGGATAAAGACCAAGCAGACAGATGCACTAT
  Q   Y   E   Y   Y   K   V   Y   M   V   D   K   D   Q   A   D   R   C   T   I
TAAGAAGGAAAATACCCCTCTCCTCAACTGTGCCAGACCAGATCAAGATGTAAAATTCAC
  K   K   E   N   T   P   L   L   N   C   A   R   P   D   Q   D   V   K   F   T
TATCAAGTTCCAAGAATTCAGCCCTAACCTCTGGGGTCTAGAATTTCAGAAGAACAGAGA
  I   K   F   Q   E   F   S   P   N   L   W   G   L   E   F   Q   K   N   R   D
TTATTACATTATATCTACATCAAATGGGTCTTTGGAGGGCCTGGATAACCAGGAGGGAGG
  Y   Y   I   I   S   T   S   N   G   S   L   E   G   L   D   N   Q   E   G   G
GGTGTGCCAGACAAGAGCCATGAAGATCCTCATGAAAGTTGGACAAGATGCAAGTTCCGC
  V   C   Q   T   R   A   M   K   I   L   M   K   V   G   Q   D   A   S   S   A
TGGGTCAACCAGGCATAATGAGCCAACAAGACGTCCAGAACTAGAAGCTGGTACAAATGG
  G   S   T   R   H   N   E   P   T   R   R   P   E   L   E   A   G   T   N   G
AAGAAGTTCAACAACAAGCCCCTTTGTCAAACCAAATCCAGGTTCCAGCACAGACGGCAA
  R   S   S   T   T   S   P   F   V   K   P   N   P   G   S   S   T   D   G   N
CAGCGCCGGACATTCTGGGAACAATATCCTCGGTTCCGAAGTGGCCTTATTCGCAGGGAT
  S   A   G   H   S   G   N   N   I   L   G   S   E   V   A   L   F   A   G   I
TGCTTCAGGATGCATCATCTTCATCGTCATCATCATCACGCTGGTGGTCCTGCTGCTCAA
  A   S   G   C   I   I   F   I   V   I   I   I   T   L   V   V   L   L   L   K
GTACCGGCGGAGACACCGCAAGCACTCGCCGCAGCACACGGCCACGCTGTCGCTCAGCAC
  Y   R   R   R   H   R   K   H   S   P   Q   H   T   A   T   L   S   L   S   T
GCTGGCCACGCCCAAGCGCGGCGGCAACAACAACGGCTCGGAGCCCAGTGACATTATCAT
  L   A   T   P   K   R   G   G   N   N   N   G   S   E   P   S   D   I   I   I
CCCGCTGAGGACTGCAGACAGTGTCTTCTGCCCCCACTACGAGAAGGTCAGCGGGGACTA
  P   L   R   T   A   D   S   V   F   C   P   H   Y   E   K   V   S   G   D   Y
CGGGCACCCGGTGTACATAGTCCAGGAGATGCCCCCGCAGAGCCCGGCGAACATTTACTA
  G   H   P   V   Y   I   V   Q   E   M   P   P   Q   S   P   A   N   I   Y   Y
CAAGGTCTGAGCCTCGGCGGTGCCTTCGCTTTCCCGGAGGAAACTTACTGTCCCGCTGCC
  K   V
GCCTACGGAGGGTCGAATGCCCCCTGTGTCTGCAACGGACTGATGCACAGCGGGGCGGGG
CGGGGCGGGGCGGGGGTACCCTCCTTCTCCGAAGAGCCCCGTGGAATCGGACAGCTTACT
AGTCTGTAGCGTTTCGGCCGCGGTGACGACGGATGCGCCCCGGCAGGTGGAAGACTCGCG
CCGAAGAAGCCGGTCGGGACTGCGGCGCGCGCCCGGCCCCTCTCCTCCTCTGGGTACCAG
GGCGCTCAGGCCTCCGCAGGAGCCATGGGAAGACAGAGCGGTAGGTGGGCAGGAGCGAGA
GCCGTGAGCATCCTGGGCGCGGGGGACCCCCGGGGACCCTCCCCGCCCCCTGCCCCCGGG
TCGCTTTGGCCCGGAGATGCCGTCCTTTGCCTCGGGTGTGGCTCCCCTCCCCCGCAAGCC
GCACCCCGGACTTGCCTTGTCGCACGGACTCTGGCTGCGTGAGGCTTTGGGAGAGCGCCA
GCTTTTAGTCCTCACCGTTGCGCACACTCCTGTTATCCAGGGCTCCGCAGGCCTTCACAT
AGATGTCCTCTCACGCCACACGCTGTCTGCATCACTCATGGAACATTACCGTTTGGCGTC
TCCGAGTCCATCCGTTTGCAGCAACCGCTCCAGTCGAACGGGTCCCACACTGAGAGCGGT
CGCCTTGGCTAAGCAGGAGCCTTCCAGCCAGACGCTGGGCTGTCCGCCGCGGAAGAGGAG
GAGTTCACCGAAGGAGGGGCCGGGGAGAGGGCTGTCTGCAAATGCAGCTGGGTGCTGCTG
CCTCCGAAACGGCGTTGGGAAGGGCAACCCCAATGAGATAGCACAGCAAAAAAAAAAAAA
AAAAAAAAAA
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Figure 1. Complete cDNA sequence of the *EFNB2* gene in pigs and its deduced amino acid.

Phylogenetic analysis of porcine *EFNB2*

In order to clarify the genetic relationship of swine *EFNB2* with that from other species, the nucleotide sequences of different genes were downloaded from GenBank, and included those from *H. sapiens* (human) (NM_004093.3), *M. musculus* (mouse) (NM_010111.5), *Rattus norvegicus* (rat) (NM_1107328.2), *Pan troglodytes* (chimpanzee) (XM_001134800.3), *Nomascus leucogenys* (gibbon) (XM_003270196.1), *Papio anubis* (baboon) (XM_ 3914058.1), *Felis catus* (cat) (NM_001114344.1), *Callithrix jacchus* (white-tufted-ear marmoset) (XM_ 002 742528.3), *Macaca mulatta* (macaque) (XM_002691986.1), *Canis lupus familiaris* (dog) (NM_001114296.1), *Ovis aries* (sheep) (XM_004012234.1), *Danio rerio* (zebrafish) (NM_131023.1), and *Xenopus laevis* (African clawed frog) (NM_001122597.1). Comparisons of homology with the porcine *EFNB2* cDNA sequence are shown in Figure 2. *S. scrofa* and *O. aries* were grouped into one cluster with the closest distance. *F. catus* and *C. lupus familiaris* were clustered into one group, which suggested that swine *EFNB2* had a closer genetic relationship with mammalian sequences in comparison with fish and amphibian sequences. The results were consistent with the molecular evolution of species.
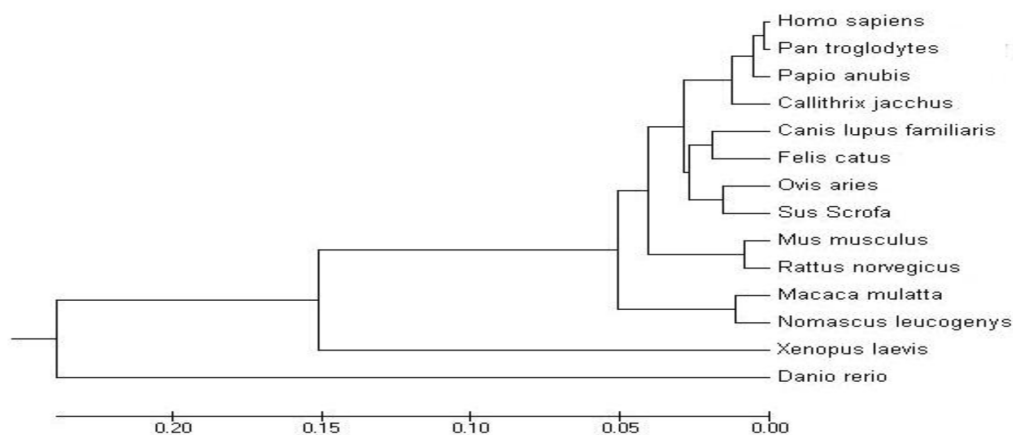

Figure 2. Phylogenetic tree of the *EFNB2* gene.

Analysis of porcine EFNB2 protein structure

Physicochemical analysis showed that the molecular weight of the deduced porcine EFNB2 protein was 36,928.1 Da with a theoretically isoelectric point (pI) of 8.98. It was estimated to consist of 20 amino acids, with Ser accounting for 9.3%, and Trp accounting for 1.2% of the total amino acid residues. Analysis of potential phosphorylation and glycosylation sites showed that 11 Ser, one Tyr, and four Thr residues may be protein kinase phosphorylation sites. The EFNB2 protein contained eight potential N-glycosylation sites. EFNB2 was predicted to be a water-soluble protein. Amino acid residues 228-250 formed a transmembrane region and amino acids 264-274 formed a low complexity sequence. The tertiary structure of EFNB2 was predicted using SWISS- MODEL EFNB2, which showed EFNB2 to contain a curved spiral structure (Figure 3).

Figure 3. Domain area of EFNB2 tertiary protein structure. The tertiary structure was predicted as a bent helical structure. Red represents α-helices, yellow β-sheets, deep blue turns, light blue coils, and green other.

Porcine *EFNB2* gene expression in two breeds of pig

Using the relative quantitative method, the expression level was defined as 100 in the small intestine of Yorkshire pigs. Relative expression levels of *EFNB2* mRNA were analyzed in different tissues. As shown in Figure 4, expression of *EFNB2* mRNA in Shaziling pig was highest in the longissimus dorsi muscle, followed by the cecum, pancreas, liver, crureus muscle, heart, spleen, small intestine, lung, and kidney. In the Yorkshire pig, the highest expression was found in the longissimus dorsi muscle, followed by the pancreas, liver, heart, spleen, crureus muscle, cecum, small intestine, lung, and kidney. The expression of *EFNB2* mRNA in the longissimus dorsi muscle was the highest in both Yorkshire and the Shaziling pig. The *EFNB2* gene may therefore be involved with intracellular signal transduction in the skeletal muscle of pigs.
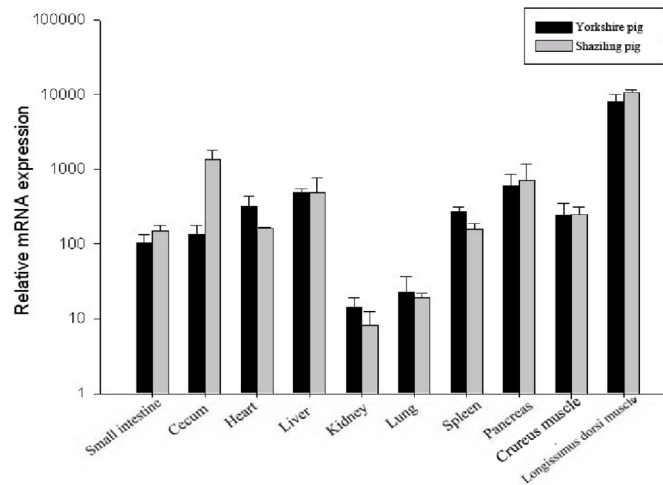

Figure 4. Tissue distribution of *EFNB2* mRNA in different tissues and two pig breeds.

DISCUSSION

In this study, the *EFNB2* gene was cloned from a Yorkshire pig by RACE. A 1991-bp full-length cDNA included a 1002-bp ORF, an 88-bp 5'-UTR, and a 901-bp 3'-UTR, encoding 333 amino acids with a theoretical molecular weight of 36,928.1 Da.

Phylogenetic analysis of the *EFNB2* gene showed that sequences from pigs and sheep clustered into a class, cats and wolves formed a class, human and chimpanzee formed a class, baboon and monkey clustered into the second class; and mice and rats clustered into the third category. The phylogenetic relationship between pigs and sheep was the closest, and the furthest relationship was between pig and chub mackerel. The *EFNB2* gene has been highly conserved throughout evolution and may therefore have played an important role in the biological processes of growth and development. Through hydrophilic analysis, we found that *EFNB2* encodes a water soluble protein, which is involved in the regulation of cellular proliferation and angiogenesis (Noren et al., 2006), indicating that EFNB2 also contributes to replication, translation, and cellular proliferation. Therefore, the findings of this study are consistent with previous research findings on the function of this gene. Protein phosphorylation and glycosylation are common and important post-translational protein modifications, and are common in the regulation of biological processes (Wang and Bai, 2012).

Bioinformatic prediction showed that he EFNB2 protein contains 11 serine phosphorylation sites, four tyrosine phosphorylation sites, four threonine phosphorylation sites, and eight glycosylation sites, which may play important roles in signal transduction, differentiation, and cell growth.

The amount of muscle fibers is determined before the swine fetus is 90-days-old, but the metabolic characteristics are determined after birth, at 30-days-old (Picard et al., 2010). Results obtained from real-time PCR analysis revealed that the *EFNB2* gene is expressed in 10 tissues obtained from 25-day-old Yorkshire and Shaziling pigs. There was high expression in the longissimus dorsi, which indicated that the *EFNB2* gene plays an important role in the regulation of muscle growth and development. There was a significant difference in the expression in the cecum between the Yorkshire and Shaziling pigs, which may be associated with inconsistency in the growth rate of Chinese native and European pig breeds.

In conclusion, the full-length cDNA of the porcine *EFNB2* gene was cloned. The mRNA expression profile of *EFNB2* in ten different tissues of 25-day-old Yorkshire pigs and Shaziling pig was determined. The results of this study provide an insight into the function of the *EFNB2* gene in pigs.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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