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Nramp1 gene expression in different tissues of Meishan piglets from newborn to weaning

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ABSTRACT. *Natural resistance-associated macrophage protein gene 1* (*Nramp1*) plays an important role in the innate immune response of swine, and is believed to influence disease resistance. In this study, a real-time quantitative polymerase chain reaction technique was used to investigate *Nramp1* expression in 12 different tissues in newborn and 7-, 14-, 21-, 28-, and 35-day-old Meishan piglets. Results indicated that *Nramp1* was expressed to varying degrees in all sample tissues, although expression differed among growth stages. For example, *Nramp1* was highly expressed in the spleen, but minimally expressed in heart, liver, and muscle tissues among the various piglet age classes. Overall, *Nramp1* expression increased with age, reaching significant levels in 21- and 28-day-old animals. *Nramp1* was expressed in all 12 tissues tested; however, expression in spleen, lung, kidney, and thymus tissues was highest among newborns, which is consistent with this gene's role in innate immunity improvement. Before and after weaning, *Nramp1* was highly expressed in digestive (stomach) and intestinal (duodenum, jejunum, and ileum) tissues, further indicating a genetic

role in both immune regulation to compensate for weaning stress and enhanced development of intestinal immunity.

Key words: Pig; *Nramp1*; Differential expression; Disease-resistant breeding

INTRODUCTION

Natural resistance-associated macrophage protein gene 1 (Nramp1) is a member of the *Nramp* family, originally discovered on chromosome 1 in mice. *Nramp1* encodes a phosphoglycoprotein that functions as an ion channel during molecular transport (Cellier et al., 1995; Vidal et al., 1996). It is primarily expressed by macrophages in endothelial cell organs, including peripheral blood leukocytes, and spleen and lung tissues, which affects not only autoimmunity, but also resistance to Salmonella and various other intracellular pathogenic microorganisms (Blackwell, 1996; Lam-Yuk-Tseung et al., 2006; McDermid and Prentice, 2006; Tanaka et al., 2007; Kadkhodazadeh et al., 2016). In addition, another study found that the *Nramp1* gene could influence Bacillus Calmette-Guérin (BCG) tuberculosis vaccine efficacy, indicating that *Nramp1* plays an important immune regulation function with regard to pathogenic response (Badawy et al., 2013). Similarly, He et al. (2013) found that A24101991G of *Nramp1* was significantly associated with salmonellosis resistance.

In pigs, the *Nramp1* gene was initially cloned by Tuggle et al. (1997) and is located on chromosome 15 q23-26 (Sun et al., 1998). The 15 kb *Nramp1* gene is comprised of 15 exons and 14 introns. Many studies have shown that polymorphism of pig *Nramp1* is correlated with both immune function (Wu et al., 2008) and diarrhea in piglets (Gu, 2006; Liu et al., 2010; Zhao et al., 2013). Among indigenous Chinese pig breeds (specifically Tibetan and Huai pigs) and in hybrid boar, *Nramp1* gene expression exists in all tissues (Ying and Zhang, 2007; Chen and Liu, 2009; Ding et al., 2014), whereas some tissue specificity was reported in foreign pigs, in which *Nramp1* was not expressed in ileum, colon, or brain tissues (Zhang et al., 2000). Despite a correlation between polymorphism of *Nramp1* and diarrhea in piglets, and some profile variation in tissue expression data, it has not been determined whether expression of *Nramp1* is related to general disease resistance in piglets (nonspecific immunity). Immune regulatory functions of *Nramp1* in intestinal tissues (specific immunity) are also not well understood.

Piglet growth from birth to weaning is significantly affected by food changes (i.e., transition from breast milk to feed), as is immunity to disease. Because the piglets no longer receive maternal antibodies from breast milk, immune responses are easily stimulated by the external environment, and diarrhea is more likely to occur. Therefore, this study measured *Nramp1* expression in various tissues from Meishan piglets at different developmental stages from newborn to weaning (ages ranging from newborn to 7-, 14-, 21-, 28- and 35-day-old), and investigated possible mechanisms for *Nramp1*-associated gene regulation on weaning- and external pathogen-related stress. A real-time fluorescent quantitative polymerase chain reaction (qPCR) technique was used to detect *Nramp1* mRNA expression levels in various tissues. The results provide a theoretical basis for further study of *Nramp1* function and regulatory mechanisms.

MATERIAL AND METHODS

Experimental materials and reagents

Experimental pigs were obtained from Meishan Pig Conservation Breeding Co., Ltd. in Kunshan, Jiangsu Province, China. Six piglets from each litter were selected as experimental animals, one of which was slaughtered at each developmental stage (e.g., newborn, 7-, 14-, 21-, 28-, and 35-day-old). At the same time, five piglets with similar characteristics (e.g., size and weight) were also selected from half-sib families for slaughter experiments. Thus, five piglets were tested at each developmental stage. Twelve (12) samples were obtained from heart, lung, liver, spleen, stomach, kidney, thymus, lymph node, muscle, duodenum, jejunum, and ileum tissues, and preserved in liquid nitrogen until needed. TRIZOL reagent was purchased from Invitrogen (USA), reverse transcription (HiScript Q Select RT SuperMix for qPCR) and quantitative fluorescence (AceQ qPCR SYBR Green Master Mix) kits were both purchased from Vazyme Biotech Co., Ltd. (Nanjing, China).

Primer design for qPCR

Using Primer Express 5.0 software, an *Nramp1* primer was designed according to *Nramp1* gene sequences found in the GenBank database (accession No. NC_010457.4), and included qPCR-crossed exons to avoid contamination of genomic DNA. The *GAPDH* gene was used as an internal control, and primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Primer information is shown in Table 1.

Table 1. Primers used for real-time PCR.

Gene	Primer sequence (5'→3')	Product length	GenBank accession No.
Nramp1	F: GTGAGGACAGCCAAGTTGAG	197 bp	NC_010457.4
	R: TCTCCTCCTGCTCCAATCCAG		
GAPDH	F: CATCATCCCTGCTTCTACTGG	187 bp	NC_010447.4
	R: TCTCCTCCTGCTCCAATCCAG		

Total RNA extraction and qPCR amplification

The TRIZOL method was used to extract total RNA from all tissue samples in strict accordance with TRIZOL reagent manual protocols. Total RNA was visualized using 1% formaldehyde denaturing agarose gel electrophoresis, and RNA concentration and urity were measured using a NanoDrop ND-1000 nucleic acid/protein concentration meter (GE, USA). RNA was frozen and stored at -70°C until needed. cDNA was synthesized using RNA as a template in 10 µL reactions containing 2 µL 5 x qRT SuperMix II and 500 ng total RNA, with the volume made up of RNase-free ddH$_2$O. Cycling parameters were 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min. Reaction mixtures were stored at 4°C.

Real-time fluorescent quantitative PCR experiments contained 2.0 µL cDNA, 0.4 µL upstream and downstream primers (10 µM), 10 µL 2X SYBR Premix ExTaq II, and 0.4 µL 50X ROX Reference Dye II, with the volume made up of 20 µL RNase-free ddH$_2$O. All experiments were performed in triplicate. Cycling parameters were 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s, for 40 total cycles. To analyze the specificity of amplification

products, samples were collected at the end of the PCR amplification process for melting curve determination, and were subjected to thermal cycling at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s.

Statistical analysis

Relative quantitative results were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Significant differences in *Nramp1* expression among various tissues at different developmental stages were determined using the general linear model (GLM) multivariate statistical method in SPSS17.0 software. Data are reported as means ± standard deviation.

RESULTS

Determination of total RNA integrity and purity

The results of 1% formaldehyde denaturing agarose gel electrophoresis following RNA extraction (**Figure S1**) revealed three RNA bands (28S, 18S, and 5S), with no obvious degradation or DNA contamination. The RNA A260/A280 ratio of RNA and protein concentration measured by NanoDrop ND-1000 was 1.8-1.9, which indicated high RNA integrity and purity, and thus, suitability for use in subsequent experiments.

qPCR amplification and melting curves

An amplification-reaction kinetics curve was plotted against cycle number, and the amount of fluorescence automatically generated by the system was subtracted from the measured fluorescence change. Amplification product specificity was judged based on the melting curve, which indicated that the *Nramp1* gene had been amplified successfully, with only one specific qPCR product and no primer dimers or nonspecific products (**Figure S2A** and **S2B**).

Analysis of *Nramp1* expression in different tissues and developmental stages

The average expression level of *Nramp1* in muscle tissue taken from newborn Meishan piglets was used as a reference for comparison with expression levels in other tissues and at other developmental stages. Results indicated that *Nramp1* mRNA expression increased steadily with age from newborn to 28-day-old piglets, then clearly decreased at 35 days old (Figure 1). *Nramp1* was expressed at relatively low levels in heart, liver, and muscle tissue, whereas the gene was expressed at relatively high levels in spleen, lung, kidney, thymus, and lymphatic tissues. This was particularly evident in spleen tissue taken from newborn, 7-, and 14-day-old piglets (Figure 2). However, expression increased significantly in stomach tissue, and clearly increased in intestinal tissue, at 21 days old. By 28 days old, *Nramp1* expression in stomach tissues had reached its maximum and had increased in kidney and intestinal tissues. Expression peaked in kidney tissues at 35 days old, although expression in other tissues had begun to decrease at this point.
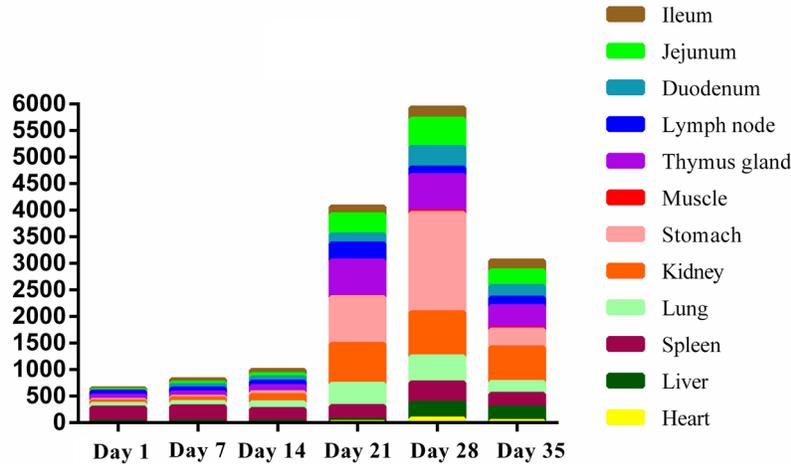
Figure 1. Overall expression of *Nramp1* gene in 12 different tissues in Meishan piglets at various developmental stages.
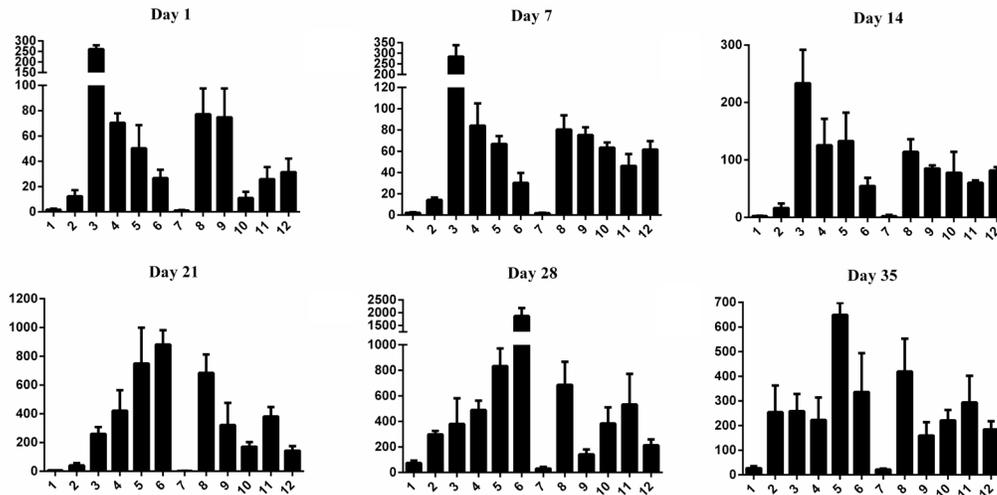

Figure 2. Expression profiles of *Nramp1* gene in 12 different tissues in Meishan piglets at various developmental stages. Items 1-12 represent heart, liver, spleen, lung, kidney, stomach, muscle, thymus, lymph, duodenum, jejunum, and ileum, respectively.

Expression trends and difference analysis of *Nramp1* gene in various tissues of Meishan piglets with increasing age

The average expression rate of *Nramp1* in muscle tissue taken from newborn Meishan piglets was used as a reference for comparison with expression levels found in nine other tissues (spleen, lung, kidney, stomach, thymus, lymph, duodenum, jejunum, and ileum) at

various developmental stages. Expression of *Nramp1* in different tissues varied considerably with age (Figure 3): *Nramp1* expression was consistently high in spleen tissue, and reached significant levels (P < 0.05) at 28 days old, but was down-regulated at 35 days old. Similarly, expression was also up-regulated with age in lung, kidney, stomach, duodenum, jejunum, and ileum tissues, reaching a highly significant peak (P < 0.01) at 21 days old, then down-regulated at 35 days old. Expression of *Nramp1* in the thymus was up-regulated with age, reaching a highly significant peak (P < 0.01) at 21 days old, and then decreasing at 35 days old, although levels at the latter developmental stage were still significantly higher than those at 14 days old. Expression of *Nramp1* in lymphatic tissue gradually increased in newborn to 21-day-old piglets, peaking at 21 days old (P < 0.01), but was then down-regulated at 28 days old, and up-regulated again at 35 days old.
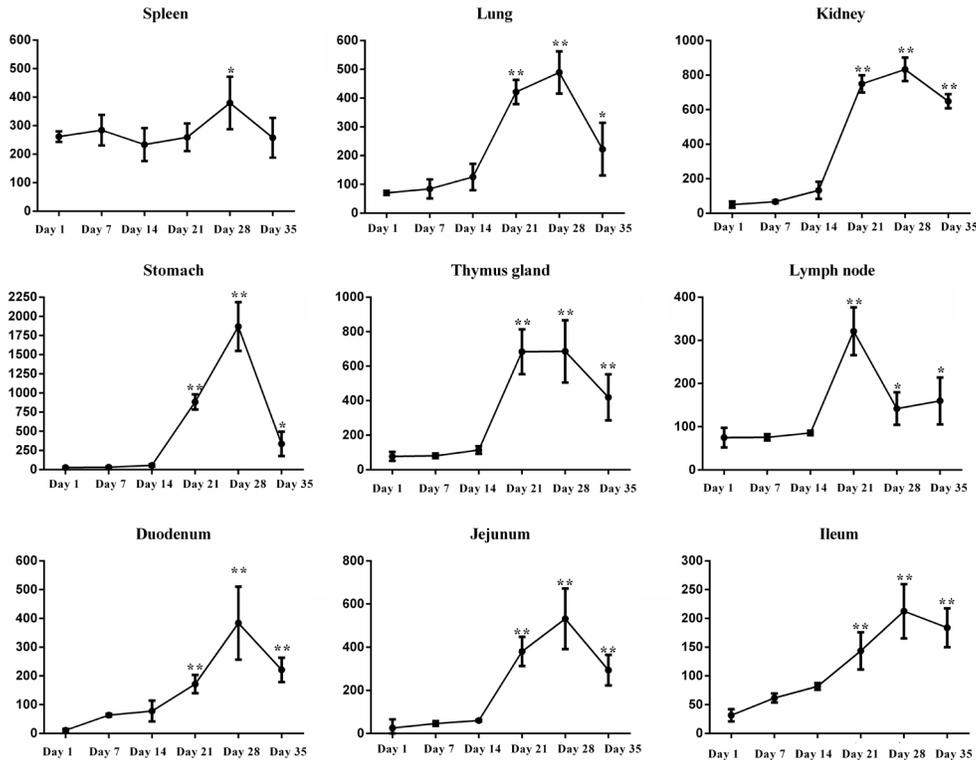

Figure 3. Trends and difference analysis of *Nramp1* expression in various tissues in Meishan piglets with increasing age, from newborn to weaning.

DISCUSSION

Nramp1 possesses conserved sequence elements that clearly place it in the *Nramp* transporter family. It serves an apparent role in host defense against microbial infection (Blackwell, 1996; Govoni and Gros, 1998; He et al., 2013), and is considered an important

immune response-related gene (Wu et al., 2008; Chen et al., 2012; Badawy et al., 2013). Previous studies have indicated that the *Nramp1* protein serves a role in pathogenic infection resistance via a mechanism involving the transport of metal ions such as Mn^{2+} and Fe^{2+}. Such ions are essential to the function of bacterial immune defense enzymes, and bacteria lose viability without them (Supek et al., 1996). In addition, *Nramp1* can transport divalent metal ions that activate macrophages in autoimmune and a variety of other infectious diseases, thereby generating reactive oxygen species that kill microorganisms and host cells (Cellier et al., 2007). Moreover, *Nramp1* plays an important role in nitric oxide generation and inflammatory response (Fritsche et al., 2008).

Based on existing research, differential expressions of *Nramp1* in 12 tissues taken from Meishan piglets ranging in age from newborn to weaning were investigated in this study to establish a basic data profile for *Nramp1* tissue expression and regulation among piglet age classes. Results indicated that *Nramp1* was expressed in all tissues tested regardless of individual tissue specificity, although expression at different developmental stages did vary significantly. This finding was consistent with *Nramp1* tissue expression among hybrid boar and indigenous Tibetan and Huai breeds, but differed from that in foreign pig breeds (Zhang et al., 2000; Ying and Zhang, 2007; Chen and Liu, 2009; Ding et al., 2014). *Nramp1* was expressed at relatively high levels in the spleen, which is the largest immune response-related organ in the body that develops and produces macrophages and lymphocytes, and is key to both humoral and cellular immunity in Chinese pig breeds. It is logical, therefore, that high expression of *Nramp1* in spleen tissue is likely consistent with the body's capacity for immune response.

Overall, *Nramp1* expression was relatively low in heart, liver, and muscle tissues, relatively high in spleen, lymph, thymus, kidney, and lung tissues, and was consistently highest in spleen tissues during the early stages of Meishan piglet development (newborn to 14 days old). During this time, the digestive tract is not fully developed as food intake consists of milk secreted by the sow. As a result, the immune system is also incomplete, and resistance to external stimulation/pathogens is mainly produced by maternal antibodies in milk and acquired via congenital immunity. High expression of *Nramp1* in immune tissues thus indicates a potentially important regulatory role in congenital immunity in piglets.

At 21 days old, *Nramp1* expression was up-regulated in both stomach and intestinal tissues. Interestingly, previous studies have shown that a sow's milk yield peaks approximately 21 days after parturition, with both yield and nutrient content diminishing after this time (Lan, 2003). Thus, piglets begin to take in exogenous feed when they are about 21 days old, at which point the stomach and intestinal tissues of their digestive organs and digestive tract suffer increased stress, and are more susceptible to invasion from external stimulation/pathogens found in food and the surrounding environment. We speculated that the high expression of *Nramp1* in these tissues is therefore consistent with a piglet's own immune response capability.

At 28 days old, piglets enter the early weaning stages, at which point, weaning stress becomes the primary cause of diarrhea. Because *Nramp1* expression significantly increased in multiple tissues at this stage, it suggested that piglets might improve their immunity to weaning stress by increasing the expression of *Nramp1*. Furthermore, *Nramp1* expression was consistently elevated in the spleen throughout all stages of growth and development, implicating *Nramp1* itself as a major component in macrophage-associated resistance in endothelial organs. This result indicates that *Nramp1* presents a clearly defined expression trend in piglet age classes from newborn to weaning.

In the majority of other sampled tissues, *Nramp1* expression increased gradually with age, reaching significantly high levels (P < 0.01) at 21 days old, continuing to increase at 28 days old, and then declining at 35 days old. As stated previously, piglets generally begin to consume exogenous feed at around 21 days old, and are weaned at around 28 days old, at which point they are most vulnerable to invasion by external pathogens. Before and after weaning, the body's immune response declines due to changes in feeding and the loss of maternal antibodies present in milk, thereby increasing vulnerability to intestinal bacteria and viral diarrhea in particular. High levels of *Nramp1* expression in immune-associated and intestinal tissues at 21 and 28 days old suggests that high levels of *Nramp1* expression are associated with both non-specific and specific immunity in piglets.

Study sampling limitations included the selection of five half-sib piglets rather than full-sibs for expression differences analysis. Piglets selected represented the same growth stages, although differences in number and quality of litters were noted. In addition, only mRNA expression level (without protein expression level) analysis was performed in this study, and was not rigorous. All experimental animals used were piglets of normal phenotype without symptoms of pathological diarrhea.

Results demonstrated a close relationship between *Nramp1* expression and immune response in piglets. Specific relationships between *Nramp1* expression and diarrhea in piglets require further study focusing on expression differences between normal and diarrhea-prone Meishan piglets, combined with susceptibility analysis for *Nramp1* knock-down and over-expression in individuals. The results of this study provide a platform for future research into the regulatory role of, and molecular mechanisms underpinning, the *Nramp1* gene, which may prove *Nramp1* to be an effective molecular genetic marker.

CONCLUSION

To establish a basic data profile for *Nramp1* tissue expression and expression regulation among piglet age classes, real-time quantitative PCR was used in this study to investigate *Nramp1* expression in 12 different tissues in newborn, 7-, 14-, 21-, 28-, and 35-day-old Meishan piglets. Results indicated that *Nramp1* was expressed in all tissues to varying degrees, and was highly expressed in the spleen, but minimally expressed in heart, liver, and muscle tissues at different growth stages. *Nramp1* expression increased with age and reached significant levels in 21- and 28-day-old animals. In newborns, *Nramp1* was expressed at the highest levels in spleen, lung, kidney, and thymus tissues. Before and after weaning, *Nramp1* was highly expressed in both digestive and intestinal tissues, indicating a role in immune regulation during weaning stress and in intestinal immunity in particular. The results of this study provide a platform for future research into the regulatory role of, and molecular mechanisms underpinning, *Nramp1*.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Supplementary material

Figure S1. 1% agarose gel electrophoresis separation of total RNA.

Figure S2. Amplification curve (**A**) and melting curve (**B**) of qPCR analysis of *Nramp1* expression in different tissues.