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# The sodium channel gene family is specifically expressed in hen uterus and associated with eggshell quality traits

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## Abstract

**Background:** Eggshell quality is important for the poultry industry. During eggshell formation a mass of inorganic minerals is deposited. The Sodium Channel (SCNN1) gene family plays an essential role in cation transportation. The objective of this study was to investigate the pattern of expression of members of the SCNN1 gene family, their variation and their effects on eggshell quality.

**Result:** The highest expression of SCNN1a, SCNN1b, and SCNN1g genes were in the active uterus during eggshell mineralization, while SCNN1d showed its highest expression level in the quiescent uterus (no egg present). Nineteen candidate SNPs from the four genes were genotyped in a population of 338 White Leghorn layers. Association analysis between SNPs (haplotypes/diplotypes) and eggshell traits was performed. Among seven significant SNPs, five SNPs were associated with eggshell strength, eggshell thickness, eggshell percentage or/and egg weight, while the other two SNPs within SCNN1d were only associated with eggshell percentage. These SNPs had a 0.25-6.99% contribution to phenotypic variance, depending on the trait. In haplotype analysis, SCNN1b and SCNN1d were associated with egg weight. The SCNN1b and SCNN1g were significantly associated with eggshell weight while only SCNN1g explained 2.04% of phenotypic variance. All the alleles of the members of SCNN1 gene family were associated with eggshell percentage and eggshell thickness, and others members had an association with eggshell strength except for SCNN1a. The contribution of different haplotypes of the SCNN1 gene family to eggshell phenotypic variance ranged from 0.09% to 5.74%.

**Conclusions:** Our study indicated that the SCNN1 gene family showed tissue expression specificity and was significantly associated with eggshell traits in chicken. This study provides evidence that genetic variation in members of the sodium channel can influence eggshell quality.

**Keywords:** Sodium channel gene family, Eggshell quality, Cation transportation, Phenotypic variance, Uterus

## Background

Chicken eggs have a high nutritive value as a human food source and provide a less expensive animal protein for consumers than other foodstuffs such as meat and milk [1]. Improving eggshell quality is critically important for the poultry industry. A fragile eggshell brings economic loss at each stage of the production process. It has been estimated that in the process of egg production more than ten percent of total eggs could not be collected or are lost [2]. The eggshell has been shaped through evolution to resist physical and pathogen challenges from the external environment, to satisfy the metabolic and nutritional needs of the developing embryo by regulating gas and water exchange, and to serve as a calcium store [3,4].

The eggshell is a complex and highly structured calcitic bioceramic. As the forming egg traverses the oviduct, the eggshell membranes are assembled in the isthmus and the eggshell is deposited in the uterus [3]. About 94% of eggshell mineral is calcium carbonate, with other inorganic minerals such as magnesium carbonate, calcium phosphate and magnesium phosphate [5]. Ion transportation plays a very important role in the

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process of eggshell formation. The ion channel super-family includes voltage-gated $K^+$ channels, voltage-gated $Ca^{2+}$ channels, $Na^+$ channels and non voltage-gated $Na^+$ channels, etc. Amiloride-sensitive $Na^+$ channels are a diverse group of ion channels which are essential for controlling the regulation of $Na^+$ transport into cells and across epithelia [6]. In the quail uterus, net $Na^+$ flux to the serosal side is observed in vitro [7]. $Na^+$ was actively transported across the uteruine epithelium into the plasma while net $Ca^{2+}$ secretion rose progressively when perfused $Na^+$ concentrations were increased. These observations suggested a positive influence of $Na^+$ absorption on net $Ca^{2+}$ secretion in the avian uterus [8]. Furthermore, new evidence suggests that the concentration and transfer of $Na^+$ can directly influence the transportation of calcium and bicarbonate ions in chicken uterus [9].

The amiloride-sensitive $Na^+$ channels are made up of four subunits α, β, γ, and δ (encoded by the SCNN1a, SCNN1b, SCNN1g and SCNN1d genes, respectively) [10]. These four genes are all members of the Sodium Channel Gene Family (SCNN1 gene family) [11,12]. The three distinct but similar subunits α, β, and γ can form a non-voltage gated sodium channel, for example, the epithelial sodium channel (ENaC). The amiloride-sensitive ENaC is a membrane constituent of many salt-reabsorbing epithelia and its activity limits the salt-reabsorption rate [13]. The pore-forming α subunit spans the cell membrane; with the accessory β and γ subunits whose extracellular domains interact with the loops of the α subunits, they together compose a heterotrimeric functional channel [14]. The δ subunit has similar features as the α subunit.

In order to deposit eggshell, the uterus mucosa must transfer $Ca^{2+}$ from the plasma to the uterine lumen. Previous studies illustrated that the ion concentrations (mainly including $Ca^{2+}$, $Na^+$, $K^+$, $HCO_3^-$ and $Cl^-$) of the uterine fluid change during the different stages of calcification [9]. One mechanism of $Ca^{2+}$ transportation across the uterine mucosa is $Na^+/Ca^{2+}$ exchange, which increases the $Ca^{2+}$ efflux indirectly by creating an $Na^+$ gradient providing energy [14]. However, it has been reported that net $Ca^{2+}$ secretion might not be critically dependent upon $Na^+$ absorption [8]. As the ENaC is a non-voltage gated channel and a regulator of fluid volume, it transports $Na^+$ ions from the lumen into the cell in order to establish a voltage difference and maintain osmolarity of either side of the cell's membrane [15]. Then it indirectly helps the $Ca^{2+}$ transportation and secretion. The disruption of sodium reabsorption by specific inhibitors in perfused uterus or in vitro causes reduced $Ca^{2+}$ secretion [16,17], revealing a strong relationship between $Na^+$ and $Ca^{2+}$ transfer and validating the putative presence of $Na^+/Ca^{2+}$ exchangers in uterine cells. Recent study showed that expression of members of the SCNN1 gene family increased significantly in the

uterus compared with magnum and duodenum during the active phase of calcification. Moreover, SCNN1G was expressed at a higher level in the presence of eggshell calcification than in its absence of eggshell calcification [9].

Based on this assembly of evidence to support a role for $Na^+$ in $Ca^{2+}$ transport during eggshell formation, we hypothesized that the SCNN1 gene family could affect eggshell quality traits. In the current study, our goals were to investigate the tissue expression patterns of the SCNN1 gene family, to identify polymorphisms in each gene and to study their association with chicken eggshell quality traits.

## Results

### Expression pattern of SCNN1 gene family

The expression status of the SCNN1 gene family was analyzed by real time quantitative PCR (qPCR). As shown in Figure 1, results revealed that three family members (SCNN1a, SCNN1b and SCNN1g) had a higher expression level in active uterus than in other chicken tissues. The highest relative expression of the SCNN1g gene was detected in the active uterus during eggshell deposition (Figure 1c) ($p < 0.05$ for all comparisons). The highest relative expression of SCNN1a and SCNN1b were in active uterus, but they were not significantly different from the quiescent uterus (egg in magnum but not uterus) and isthmus. On the other hand, the highest relative expression of SCNN1d was observed in quiescent uterus (Figure 1d). If the SCNN1 gene family is involved in eggshell formation, it should be expressed in the uterus at a higher level in the laying stage uterus than the developing immature uterus (pullet). The uterine expression level of all SCNN1 gene family members was significantly increased in 49-week adult hens compared to 16-week pullets (Figure 2). We compared the expression level of the four different SCNN1 genes between the active and quiescent uterus during a daily laying cycle. The relative expression levels of SCNN1b, SCNN1d and SCNN1g were far less than that of SCNN1a ($p < 0.05$) in both active and quiescent uterus (Additional file 1: Figure S1). SCNN1b is the second most highly expressed gene among the four members in both active and quiescent uterus (Additional file 1: Figure S1). At the same time we compared the gene expression in active uterus and kidney tissues from the 49-week adult hens. The results showed that the relative expression of all members of this gene family in kidney were significantly lower than in uterus ($p < 0.05$) (Figure 1).

Our qPCR results illustrated that all members of the SCNN1 gene family had a relatively abundant expression in both uterus and isthmus. Analysis of their expression supported our hypothesis that these genes had a potential role in eggshell formation. Therefore we performed
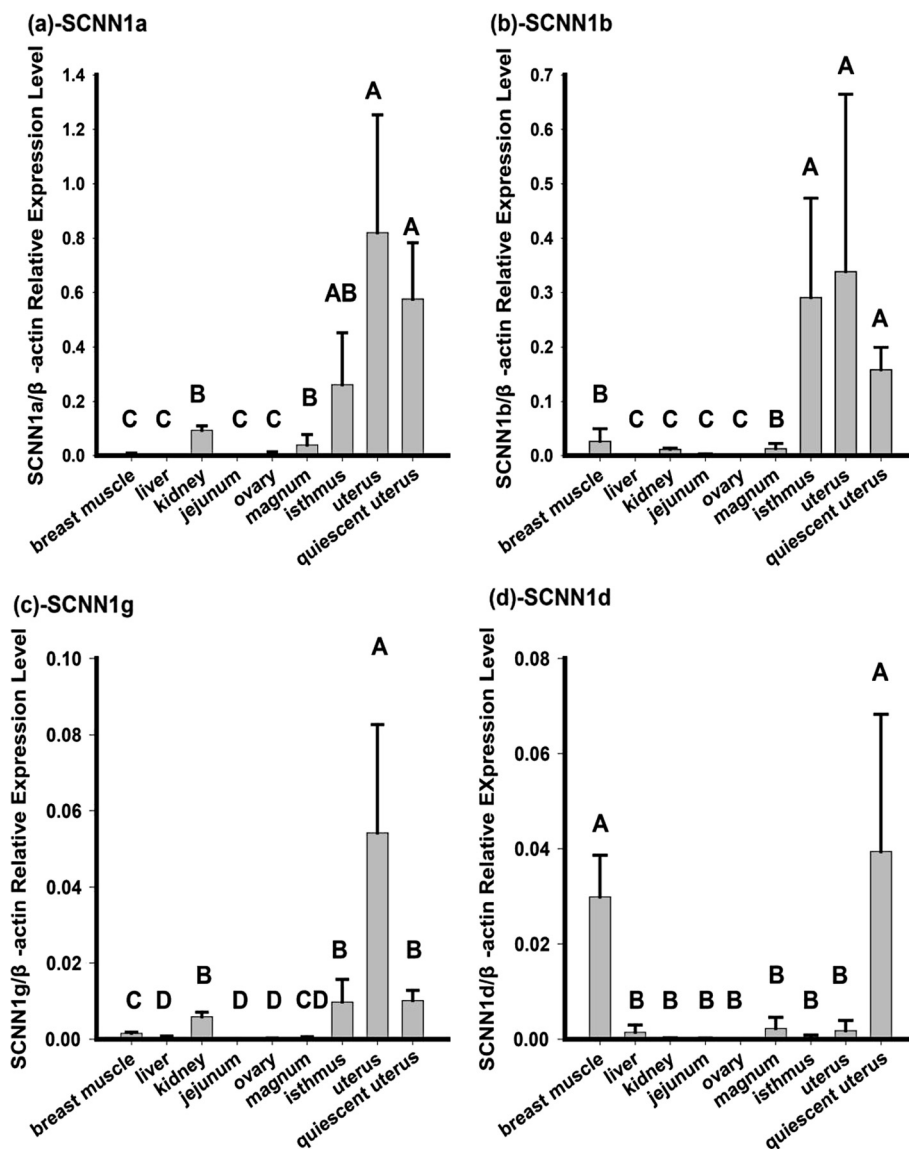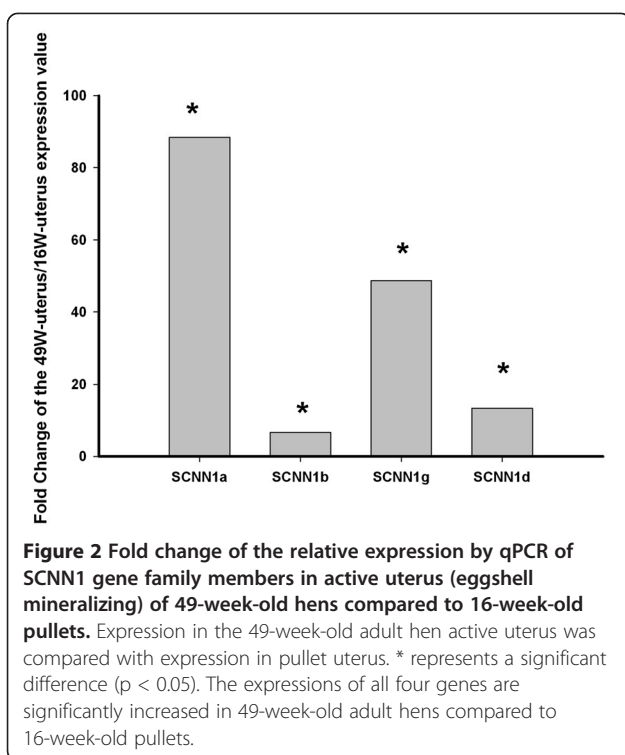
**Figure 1 Comparison of relative expression of SCNN1 gene family members vs β-actin measured by qPCR in different tissues.** The kidney was collected from four normal 49-Week-old White Leghorn layers while all others tissues, breast muscle, liver, jejunum, ovary, magnum, isthmus, active uterus (during eggshell deposition) and quiescent uterus (no egg present at time of tissue collection) were collected from four normal 55-week-old White Leghorn layers. The y-axis indicates the relative expression level of each SCNN1 family member. Different letters (A,B,C, D) represent significant difference ($P < 0.05$). Panel **(a)**, Panel **(b)**, Panel **(c)** and Panel **(d)** represents the expression of the SCNN1a, SCNN1b, SCNN1g and SCNN1d, respectively.

an association analysis between SNPs/haplotypes/diplotypes and egg weight and eggshell quality traits.

### SNP detection and association with egg weight and eggshell quality traits

Basic statistical analyses of eggshell quality traits are presented in Table 1. As there were some missing data, the sample size varied depending on the of each trait was not same as each other. Phenotypes of the samples were in the normal range.

Of the 19 SNPs genotyped, SNP rs15731913 was not detected, SNP rs15731904 had no polymorphism (allele G frequency = 1), rs14282986 and rs14075352 were only detected in a few individuals in the studied population. Single-marker association analysis revealed that in the overall cohort, seven SNPs were significantly associated ($p < 0.05$) with at least one of the eggshell quality traits (Table 2). The CPV of these significantly associated SNPs ranged from 0.25% (rs13532838 to eggshell thickness (EST)) to 6.99% (rs14282978 to eggshell percentage

**Figure 2 Fold change of the relative expression by qPCR of SCNN1 gene family members in active uterus (eggshell mineralizing) of 49-week-old hens compared to 16-week-old pullets.** Expression in the 49-week-old adult hen active uterus was compared with expression in pullet uterus. * represents a significant difference (p < 0.05). The expressions of all four genes are significantly increased in 49-week-old adult hens compared to 16-week-old pullets.

(ESP)). As shown in Table 2, rs13532836 (SCNN1b), rs14282978 (SCNN1d), rs15181931 (SCNN1d), and rs15181934 (SCNN1d) were significantly associated with ESP. Of the four SNPs, only rs13532836 and rs15181931 were significantly associated with egg weight (EW) (the contributions to phenotypic variance (CPV) were 1.04% and 0.83% respectively).

Interestingly, among the seven SNPs which were significantly associated with egg weight and eggshell quality traits, we found that the SNPs associated with eggshell strength (ESS) or EST were all located in SCNN1b or SCNN1g genes. SNP rs13532838 of SCNN1b revealed an association with ESS and EST (P = 0.011 and 0.033, respectively). SNP rs14075350 of SCNN1b had an

**Table 1 Descriptive statistics for chicken egg weight and eggshell traits**

| Trait[1] | Sample number | Mean | SD[2] | Minimum | Maximum |
|---|---|---|---|---|---|
| EW (g) | 319 | 55.68 | 3.81 | 44.30 | 68.05 |
| ESW (g) | 294 | 7.47 | 0.76 | 4.60 | 9.89 |
| ESP (%) | 294 | 13.96 | 1.27 | 9.83 | 18.54 |
| ESS (Kg/cm$^3$) | 314 | 3.07 | 0.62 | 1.28 | 4.65 |
| EST (mm) | 313 | 0.32 | 0.02 | 0.25 | 0.37 |

[1]EW = Egg Weight; ESW = Eggshell Weight; ESP = Eggshell Percentage; ESS = Eggshell Strength.
EST = Eggshell Thickness; % indicates these traits relative to EW.
[2]SD = standard deviation.

association with EST (P = 0.045), and SNP rs15009191 of SCNN1g was significantly associated with EST (P = 0.008). We also observed an overdominance effect of the heterozygote of SCNN1d (rs15181934) on eggshell percentage.

Multiple-markers in each gene association analysis were performed to promote our understanding of each gene's effect on egg weight and eggshell quality traits. Additional file 2: Table S1 shows the major haplotypes (frequency > 1%) and genotypes (frequency > 0.5%) in all four genes. Because each gene contained different numbers of SNPs which were in LD (Additional file 3: Figure S2), the haplotype numbers of each gene varied. In each gene, there was a main haplotype (frequency > 30%). The results indicated that there were significant associations between haplotype and eggshell quality traits. As there were so many and complicated comparison results, we only report the comparisons between the most significantly different diplotypes (Table 3). Almost all haplotypes in our study were not in Hardy-Weinberg equilibrium. Possible reasons are discussed later. The SCNN1b (p < 0.05) and SCNN1d (p < 0.01) genes had an association with EW and SCNN1d had a 0.62% CPV on EW. The SCNN1b (p < 0.05) and SCNN1g (p < 0.01) genes had an association with ESW, ESP, ESS and EST traits and both of them had a main CPV (1.64%, 5.43%, respectively) on trait ESS. On the EST trait, compared with the CPV of SCNN1b (0.78%), the gene SCNN1g had a larger CPV (0.82%). The SCNN1a gene (p < 0.01) was significantly associated with ESP and EST traits. The SCNN1d (p < 0.05) gene showed a significant association with ESP, ESS, and EST traits while it only had a major CPV (5.74%) on ESP trait.

## Discussion and conclusions

Some efforts were performed to pursue the markers which are related with eggshell qualities. Polymorphisms of the eggshell organic matrix genes were considered to be related with eggshell breaking strength, eggshell thickness, dynamic stiffness [18]. A genome-wide SNP scan found some novel loci affected the eggshell quality [19]. These investigations are helpful to identify loci which are potentially useful in the breeding. However, current studies are very limited in terms of the population and studied genes. This study focused on the SCNN1 gene and their effects on the eggshell quality. In general, it is observed that the egg production rate is reduced with increasing hen age, and the incidence of thin-shelled and cracked eggs is markedly increased [20]. However, the plasma levels of ionized $Ca^{2+}$ do not significantly change in hens between 33 and 122 weeks of age. Thus, the decreased eggshell thickness may involve changes in shell gland function rather than availability of $Ca^{2+}$ for secretion [21]. A declining ability of the epithelium to transport $Ca^{2+}$ might be one of the major

**Table 2 Means for eggshell-quality traits among genotypes and the SNP contribution to phenotypic variance (CPV) of the single-nucleotide polymorphism**

| Gene | SNP | Trait[1] | Genotype(number)/Mean(SD)[2] | | | CPV[3] (%) |
|---|---|---|---|---|---|---|
| SCNN1b | rs13532836 | | CC(214) | TC(69) | TT(53) | |
| | | ESP | 13.95(1.30)[ab] | 13.81(1.35)[b] | 14.05(1.18)[a] | NA |
| | | EW | 55.58(3.88)[ab] | 56.53(4.03)[b] | 55.19(3.69)[a] | 1.04 |
| SCNN1b | rs13532838 | | CC(81) | TC(164) | TT(90) | |
| | | ESP | 13.84(1.45)[b] | 14.08(1.30)[a] | 13.89(1.04)[ab] | 0. 28 |
| | | ESS | 2.96(0.60)[b] | 3.07(0.59)[ab] | 3.16(0.68)[a] | 1.14 |
| | | EST | 0.31(0.02)[b] | 0.32(0.02)[ab] | 0.32(0.02)[a] | 0.25 |
| SCNN1b | rs14075350 | EST | CC(151) | CT(158) | TT(28) | 0.60 |
| | | | 0.32(0.02)[a] | 0.31(0.02)[b] | 0.32(0.02)[ab] | |
| SCNN1g | rs15009191 | | CC(232) | CT(91) | TT(13) | |
| | | ESP | 13.93(1.20)[ab] | 14.09(1.47)[a] | 13.65(1.16)[b] | NA |
| | | EST | 0.32(0.02)[a] | 0.31(0.02)[b] | 0.31(0.02)[b] | 3.24 |
| SCNN1d | rs15181931 | | CC(179) | CA(126) | AA(31) | |
| | | ESP | 14.16(1.34)[a] | 13.81(1.22)[b] | 13.52(1.00)[c] | 3.96 |
| | | EW | 55.36(3.91)[a] | 55.78(3.74)[ab] | 56.80(3.42)[b] | 0.83 |
| SCNN1d | rs14282978 | | CC(1) | CT(33) | TT(302) | |
| | | ESP | 13.99(0.00)[ab] | 13.44(1.08)[b] | 14.02(1.28)[a] | 6.99 |
| SCNN1d | rs15181934 | | CC(135) | TC(19) | TT(183) | |
| | | ESP | 13.83 (1.16)[b] | 14.16(1.34)[c] | 13.33(1.15)[a] | 6.35 |

[1]EW = Egg Weight; ESP = Eggshell Percentage; ESS = Eggshell Strength.
EST = Eggshell Thickness after correction; % indicates these traits relative to Egg Weight.
[2]Different superscript (a, b, c) in the same row means phenotypes are significantly different between different genotypes ($P < 0.05$).
[3]CPV = SNP contribution to phenotypic variance (%).

reasons for decreasing eggshell quality in older hens. Could non-$Ca^{2+}$ transporting genes related to ion transport affect the final eggshell quality? This pilot study on sodium channel related genes provides evidence to address this question.

Previous studies showed that SCNN1a, SCNN1b and SCNN1g are highly expressed in the uterus during the eggshell formation [9,22] while SCNN1g expression increases quickly during the eggshell formation stage. Genes highly and specifically expressed in a given tissue likely contribute to its development and function [15]. Our qPCR results indicated that the genes encoding the ENaC subunits, SCNN1a, SCNN1b, SCNN1g and SCNN1d, are all highly expressed in uterus (Figure 1). A strong relationship between uterine $Na^+$ and $Ca^{2+}$ transfers has been previously established [16,17], and the $Na^+/Ca^{2+}$ exchangers are likely present in uterine cells [9]. $Na^+$ was actively transported across the uterus epithelium into the plasma while net $Ca^{2+}$ secretion rose progressively when perfused $Na^+$ concentrations were increased [8]. The $Na^+/K^+$ ATPase responsible for $Na^+$ re-absorption in the plasma membrane has been characterized and is upregulated during the period of shell calcification [14]. In this study we found that expression of

all members of the SCNN1 gene family also increased significantly during eggshell formation in the uterus (Figure 2). The rates of $Na^+$ and anion transport are altered with age and in response to molting, which could potentially contribute to the decrease in shell thickness often associated with eggs from older birds [21]. These observations suggested a positive influence of $Na^+$ absorption on net $Ca^{2+}$ secretion [8]. Expression results and $Na^+$ changes during the laying period strongly suggests that the SCNN1 gene family is involved in eggshell formation via influencing $Ca^{2+}$ transport.

This study also explored association of different genotypes of the members of the SCNN1 gene family with eggshell qualities. We searched the AnimalQTL database [23] to find the potential QTLs which are closely related with SCNN1 gene regions. However, we didn't find the SCNN1 gene fall in any known QTL regions and also are not closely related QTLs in the current researches. Our association analysis results between SNPs/haplotypes and eggshell traits demonstrated that the SCNN1a, SCNN1b and SCNN1g mainly had effects on eggshell thickness and eggshell strength while the SCNN1d gene mainly had an effect on eggshell percentage. One SNP (SCNN1d, rs15181934) has overdominance effects on

**Table 3 Means for eggshell-quality traits among genotypes and the contribution to phenotypic variance (CPV) of the diplotypes**

| Trait[1] | Gene | Diplotypes(number) | Mean(SD)[2] | P-value | CPV[3] (%) |
|---|---|---|---|---|---|
| EW | SCNN1b | H2H6(15) | 56.48(3.06)[a] | 0.0200 | NA |
| | | H8H8(21) | 53.88(2.88)[b] | | |
| | SCNN1d | H2H5(8) | 58.96(3.39)[a] | 0.0024 | 0.62 |
| | | H4H4(174) | 55.36(3.86)[b] | | |
| ESW | SCNN1b | H7H4(6) | 7.84(0.39)[a] | 0.0148 | NA |
| | | H6H6(5) | 6.85(0.48)[b] | | |
| | SCNN1g | H5H7(14) | 7.84(1.03)[a] | <0.0001 | 2.04 |
| | | H11H1(5) | 6.19(1.15)[b] | | |
| ESP | SCNN1a | H1H2(8) | 14.53 (1.64)[a] | 0.0025 | NA |
| | | H2H4(30) | 13.68(1.11)[b] | | |
| | SCNN1b | H3H4(6) | 13.67(0.89)[a] | 0.0006 | 0.09 |
| | | H1H6(8) | 13.54(0.87)[b] | | |
| | SCNN1d | H4H4(174) | 14.17(1.34)[a] | <0.0001 | 5.74 |
| | | H1H5(6) | 12.76 (0.93)[b] | | |
| | SCNN1g | H2H2(49) | 13.71 (0.94)[a] | <0.0001 | NA |
| | | H11H1(5) | 13.36(0.34)[b] | | |
| ESS | SCNN1b | H3H4(6) | 3.18(0.65)[a] | 0.0021 | 1.64 |
| | | H6H6(5) | 2.60(0.30)[b] | | |
| | SCNN1d | H3H5(99) | 3.11(0.05)[a] | 0.0278 | NA |
| | | H5H5(23) | 2.85(0.11)[b] | | |
| | SCNN1g | H1H9(9) | 3.64(0.73)[a] | <0.0001 | 5.43 |
| | | H2H6(12) | 2.61(0.50)[b] | | |
| EST | SCNN1a | H1H2(8) | 0.33 (0.02)[a] | 0.0085 | NA |
| | | H4H4(165) | 0.32 (0.02)[b] | | |
| | SCNN1b | H3H4(6) | 0.32(0.03)[a] | 0.0002 | 0.78 |
| | | H5H1(12) | 0.30(0.03)[b] | | |
| | SCNN1d | H1H5 (6) | 0.30 (0.03)[a] | 0.0069 | NA |
| | | H4H4(174) | 0.32(0.02)[b] | | |
| | SCNN1g | H1H9(9) | 0.33(0.02)[a] | 0.0021 | 0.82 |
| | | H5H5(13) | 0.31 (0.02)[b] | | |

[1]EW = Egg Weight; ESW = Eggshell Weight; ESP = Eggshell Percentage after correction; ESS = Eggshell Strength; EST = Eggshell Thickness after correction; % indicates these traits relative to Egg Weight.
[2]Different superscript (a, b) means significantly different between different diplotypes of the same gene for certain trait.
[3]CPV = SNP contribution to phenotypic variance (%).

eggshell percentage. This effect might be useful for commercial breeding, especially for designing line crosses. When we constructed diplotypes based on the haplotypes, we found many different diplotypes. Our association analysis based on these diplotypes might be affected by the relatively small number of observations for some diplotypes.

However, almost all the SNPs were not in Hardy-Weinberg equilibrium in the population we studied. As our breeding population has suffered artificial selection, this is similar to most modern poultry breeding populations. Moreover, breeding populations generally are nonrandomly mated, with a relatively small population (around 800 birds per generation). All these reasons could cause some genes to not be in Hardy-Weinberg equilibrium [24]. Could the lack of HWE be caused by genotyping errors? We added replications for all SNPs of 4 randomly selected individuals to validate the accuracy of the genotyping methods used in this study. The genotyping method used in this study has been shown to be accurate in many other studies of this field [25-27]. Furthermore, we also used the direct sequencing method to

verify the genotype accuracy for two SNPs (rs15181934, n=128; rs14075350, n=64) in randomly selected individuals. Error rate ranged from 7.8% (rs14075350)-11.2% (rs15181934) for two randomly selected SNPs. The error rate used in this study is comparable with other studies which used the same method. We manually checked the distribution of the genotyping error for two SNPs, and found the genotype errors generally are consistent with the genotype distribution in the whole samples. This means the genotyping errors didn't show any bias trends for different genotypes. These randomly happened errors didn't change the final conclusions. It is likely that the SCNN1 gene family has undergone selection related to breeding programs to improve eggshell quality traits.

In summary, members of the SCNN1 gene family are highly expressed in the adult hen active uterus compared to the immature pullet uterus. SCNN1a, SCNN1b and SCNN1g were more highly expressed in the active hen uterus than any other tissue in this study. We identified 7 SNPs that could influence eggshell quality and/or egg weight in the White Leghorn population. The results of our expression and association study provide evidence to suggest that this Sodium Channel can affect eggshell quality, especially eggshell strength and eggshell thickness.

## Method
### Animal sampling and data collection
Oviposition time was recorded daily in one week for 20 random selected 55-week-old White Leghorn layers from a relatively large population (around 500 chickens). In order to observe gene expression changes during uterus development, we also randomly selected four 16-week-old pullets (sexually immature, non-egg laying) and four 49-week-old White Leghorn layers (mature, egg producing) to obtain uterus samples. Kidney tissues were collected from four 49-week-old hens. Liver, breast muscle, jejunum, ovary, magnum, isthmus and uterus (containing an egg in the process of eggshell mineralization-active uterus) were collected from four 55-week-old hens 18–20 hours post ovulation, to represent overall gene expression levels. Another four 55-week-old hens were processed 2–4 hours post ovulation to obtain four uterine samples with egg in magnum and not in the uterus (quiescent uterus). These hens were from one hatch and were reared in the same environment at the China National Center for Poultry Performance Testing. Tissue samples for expression analysis were snap-frozen in liquid nitrogen and then stored at −80°C until RNA extraction was performed. The entire sampling process from chicken sacrifice to tissue snap freezing was finished within less than 10 minutes. Total RNA from tissues was extracted with E.Z.N.A™ Total RNA Kit (OMEGA Bio-tek Inc., USA), and dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Three-hundred and thirty-eight White Leghorn layers including 40 sire families, from one hatch and reared in the same environment, were utilized for association analysis. Blood samples were collected from the wing vein, quickly homogenized with the anticoagulant agent acid citrate dextrose (ACD) and then stored at −20°C. The chicken genomic DNA was extracted using a phenol/chloroform method. Birds were individually caged. At the age of 40 weeks, eggs were collected in 3 consecutive days from each hen for the measurement of egg quality. Egg weight (EW), eggshell weight (ESW), eggshell strength (ESS) and eggshell thickness (EST) were measured within 12 h after collecting eggs, as described in our previous publications [28,29], except for eggshell weight measurement. Eggshell weight refers to the wet eggshell which still contained the eggshell membranes with adhering water and albumen. Dividing EW by ESW, we get the eggshell percentage (ESP). As the ESP and EST data did not satisfy normal distribution, they were calibrated by the box-cox method when conducting T-Test [30,31].

The animal experiments were approved by the Animal Welfare Committee of China Agricultural University.

### Cloning and expression of SCNN1 gene family
cDNA was obtained by the reverse transcription polymerase chain reaction (RT-PCR) that was performed in a total volume of 50 μL, using 1 μg of total RNA, 2 μL oligo T18 primer (10 μM) (synthesized by Sangon Co. Ltd. Beijing, China), 1 μL murine leukemia virus (MLV) reverse transcriptase, 10 μL of 5 × Buffer (ZePing Bioscience & Technologies Co. Ltd., Beijing, China), 1 μL RNAase inhibitor (TaKaRa Co. Ltd., Beijing, China), 24 nmol dNTP mixture (DingGuo ChangSheng Biotech Co. Ltd., Beijing, China), and sterile deionized water. A negative control was performed with sterile water as the template in each reaction. The optimum reverse transcription thermal cycling parameters were as follows: 10 min at 25°C, 1 hour at 37°C, 5 min at 95°C in a Mastercycler gradient (Eppendorf Limited, Hamburg, Germany). The quality of cDNA was assured through the agarose gel figure and the housekeeping gene β-actin in order to avoid the genome DNA pollution. We did PCR to amplify our target gene, ligated the purified PCR products using pMD19T vector (Takara Biotechnology Co., Ltd) at 16°C for 6 hours. Then we transformed the ligated product into E.coli to get our cloning gene using the plate paint isolation methods and blue-white selection. All selected bacterial colonies are identified by PCR using the bacteria liquid as template and sequencing (Sangon Co. Ltd. Beijing, China).

All primers (Table 4) were designed using Primer Express 3.0 software (Applied Biosystem AB Inc., USA) based on the chicken full-length mRNA sequences

**Table 4 Primer sequences used in qPCR for SCNN1 gene family members and internal control gene β-actin**

| Primer name | Sequence (5′→3′) | Tm(°C) | Product size (bp) | Position |
|---|---|---|---|---|
| SCNN1a-F | TCATGTTCAGCGCCATCCT | 60 | 175 | Exon3+4 628-802 |
| SCNN1a-R | TTCCCGCACTGCATCCA | 60 | | |
| SCNN1b-F | GAAGTTCCCAGCAGTCACAGTCT | 60 | 168 | Exon2+3 277-444 |
| SCNN1b-R | GTGGCGTTGCTGTTGTTCAG | 60 | | |
| SCNN1d-F | CACCATCCACGGCACCAT | 60 | 183 | Exon1 90-272 |
| SCNN1d-R | AACATCTTGGGCTCCGAATG | 60 | | |
| SCNN1g-F | TGGGACAAAGGACAGAAAATC | 60 | 141 | Exon10+12 1450-1590 |
| SCNN1g-R | GCCGAAGTTGGACAGAAGGA | 60 | | |
| β-actin-F | TATGTGCAAGGCCGGTTTC | 60 | 110 | Exon1+2 113-222 |
| β-actin-R | TGTCTTTCTGGCCCATACCAA | 60 | | |

predicted by EnsEMBL(v.62) and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). Each pair primer had only one amplification product. All amplification products were sequenced to confirm validity of the primers. The amplification product of every gene by our designed primers is unique in this SCNN1 gene family. PCR was performed in a total volume of 20 μL, including 1 μL of first-strand cDNA, 0.5 μL dNTP mixture(10 mM), 2 μL of 10×Taq Buffer, 0.5 μL of Taq enzyme (ZeXing Biotech Co. Ltd., Beijing, China), 0.5 μL of each primer (10 μM), and 15 μL of sterile deionized water. The optimum thermal cycling parameters were as follows: 5 min at 95℃, followed by 32 cycles of 30 s at 95℃, 30 s at 60℃, and 30 s at 72℃, and a final extension of 5 min at 72℃ in a Mastercycler gradient.

The real-time quantitative PCR (qPCR) was run using the ABI 7300 system (Applied Biosystems). First-strand cDNA from RT-PCR (above) was used as template. The housekeeping gene β-actin (GenBank Reference Sequence: NC_006101.3) of chicken was used as an internal control. Each reaction mixture consisted of a total volume of 15μL with 7.5 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.2 μL of each primer (10 μM), 1.5 μL of cDNA, and 5.6 μL of ultrapure ribonuclease-free water. The qPCR procedure was at 95℃ for 10 min; 40 cycles of 95℃ for 15 s and 60℃ for 1 min; 95℃ for 15 s; 60℃ for 30 s; and 95℃ for 15 s. Each individual sample and no-template controls were run in triplicates.

The quantitative values of each target gene were obtained from the threshold cycle (Ct). The expression values were calculated by the formula $2^{-\Delta\Delta Ct}$. They were normalized using the expression values for the β-actin gene to obtain the relative expression of target genes. The relative gene expression was analyzed by the double standard curves method [28,29,32]. All the expression values were transformed using log2 when conducting

T-Test. Statistical analysis of differential expression between tissues was performed using the T-Test implemented in the SAS System release 8.0 (SAS Institute Inc., USA).

### Single nucleotide polymorphisms detection

We used the NCBI SNP bank (NCBI, http://www.ncbi.nlm.nih.gov/), Ensembl Data mining tool BioMart (http://www.ensembl.org/) and the UCSC Genome Bioinformatics (http://genome.ucsc.edu/ ) to conduct online searching for potential SNPs in the SCNN1 gene family DNA sequences. All SNPs had an rs# number which information could be accessed on dbSNP. All the SNP positions were reported based on the reference chicken genome (Genome assembly: WASHUC2). A high-throughput genotyping method, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), was used to distinguish these SNPs genotypes. The genotypes were analyzed by MALDI-TOF MS based on the Sequenom's MassARRAY iPLEX Platform (Sequenom, San Diego, CA). In these chip analyses, we randomly designed 4 repeats to ensure the reliability of the technology. SNPs with a genotype call rate < 85% and minor allele frequency (MAF) < 1% across all individuals were discarded. Firstly, we designed a preliminary experiment to test the polymorphism and compatibility of all SNPs in the four genes for small samples (n=50). Finally based on the preliminary experiment results (SNP frequency, position and mutation type), we identified 19 SNPs in the four genes that were used in our study (Table 5).

### Statistical analysis

The Hardy-Weinberg equilibrium test and frequencies of SNPs and genotypes were analyzed using the FREQ procedure of SAS 8.0 (SAS Institute Inc., Cary, NC). For the SNPs in one gene, genotype data was used in the

**Table 5 Details of the SCNN1 gene family members and SNPs selected**

| Gene | Gene location | No. of SNPs[1] | SNP rs#. | SNP location (Chr: bp) | Mutations type | Major allele (Fre)[2] |
|---|---|---|---|---|---|---|
| SCNN1a | chr1: 80,034,908-80,045,394 | 2 | rs14845039 | 1:80035441 | Synonymous coding (C/T) | T(0.71) |
| | | | rs13886292 | 1:80039016 | Intronic (G/C) | G(0.82) |
| SCNN1b | chr14: 7,002,221-7,011,192 | 6 | rs13532836 | 14:7005977 | Intronic (A/G) | C(0.74) |
| | | | rs13532838 | 14:7007309 | Synonymous coding (A/G) | T(0.51) |
| | | | rs14075350 | 14:7011699 | 5'UTR (C/T) | C(0.68) |
| | | | rs13532842 | 14:7011842 | Intronic (A/G) | A(0.64) |
| | | | rs15731904 | 14:7003218 | Intronic (A/G) | G(1.00) |
| | | | rs15731913 | 14:7006347 | Intronic (C/G) | NA |
| SCNN1d | chr21: 2,435,983- 2,440,820 | 4 | rs14282978 | 21:2436106 | Synonymous coding (C/T) | T(0.95) |
| | | | rs15181931 | 21:2438805 | Intronic (T/G) | C(0.72) |
| | | | rs15181934 | 21:2442772 | 5'UTR (G/A) | T(0.57) |
| | | | rs14282986 | 21:2437824 | Intronic (A/G) | T(0.70) |
| SCNN1g | chr14: 7,019,365- 7,028,441 | 7 | rs10730783 | 14:7018929 | 3'UTR (A/G) | G(0.82) |
| | | | rs15009191 | 14:7019233 | 3'UTR (A/G) | C(0.83) |
| | | | rs15009198 | 14:7022273 | Intronic (A/G) | C(0.77) |
| | | | rs15009204 | 14:7023020 | Intronic (A/C) | T(0.57) |
| | | | rs15009207 | 14:7027589 | Intronic (A/G) | T(0.56) |
| | | | rs15009209 | 14:7027788 | Intronic (A/G) | T(0.57) |
| | | | rs14075352 | 14:7019820 | Intronic (C/G) | C(0.90) |

[1]No. of SNPs = the number of SNPs selected in each gene. All SNP positions were reported based on the reference chicken genome (Genome assembly: WASHUC2).
[2]Major allele and its frequency; NA means that the allele was not detected.

haplotype analysis using Simwalk2 (2.91) [33,34]. The following model was designed for the association analysis between the SNP/haplotype/diplotype and eggshell quality traits using the VARCOMP procedure: $Y_{ij} = \mu + S_i + G_j + e_{ij}$, where $Y_{ij}$ was the observed value of eggshell quality traits of the $i^{th}$ individual with $j^{th}$ genotype; μ was the population's mean; $S_i$ was the fixed effects of the $i^{th}$ sire family, $G_j$ was the random effects for the $j^{th}$ genotype, and $e_{ij}$ represented the residuals. The contribution of the SNP/haplotype/diplotype to the phenotype variation (CPV) was estimated using the equation: $CPV = V_{SNP/haplotype/diplotype} \div V_{phenotype}$; where $V_{SNP/haplotype/diplotype}$ and $V_{Phenotype}$ were the SNP/diplotype and phenotypic variance, respectively. The $V_{Phenotype}$ was calculated using the quotation: $V_{phenotype} = V_{SNP/haplotype/diplotype} + V_{environment}$ variances.

We used the VARCOMP procedure implemented in the SAS system and the residual maximum likelihood (REML) method to estimate the variance component for each variable used in the model.

The HaploView program [35] was used in the linkage disequilibrium (LD) analysis between SNPs in same gene. The LD block was defined according to the confidence interval method [36]. The genealogy and LD results were used in statistical analysis of haplotype.

## Additional files

**Additional file 1: Figure S1.** Comparison of relative expression of SCNN1 gene family members vs β-actin in uterine tissues by qPCR. Gene expression compared between active uterus (during eggshell deposition) and quiescent uterus (no egg present), in tissues collected from four normal 55-week-old White Leghorn layers. The y-axis indicates the relative expression level of SCNN1 family members compared with β-actin. Vertical bars represent the mean ± SD (n = 4). # represent the a, b, d or g. The expression of SCNN1a, SCNN1b and SCNN1b were reduced to some degree in the quiescent uterus, that of SCNN1d increased about 4-fold. The relative expression levels of SCNN1b, SCNN1d and SCNN1g were far less than that of SCNN1a (P<0.05).

**Additional file 2: Table S1.** Major haplotypes (frequency >1%) and diplotypes (frequency >0.5%) for SCNN1 family members.

**Additional file 3: Figure S2.** Haploview plot illustrates the linkage disequilibrium of SCNN1 gene family members for the main informative SNPs. The downward-pointing Triangle black box represents LD block. The number in the square represents the value of D'. The darker the square color shows and the larger the D' value, the higher the level of two sites linkage disequilibrium displays. All SNPs $r^2 \geq 0.933$ and their minor allele frequencies were > 0.05. In the SNP name, the first letter a, b, d and g are the abbreviation of gene SCNN1a, SCNN1b, SCNN1d and SCNN1g respectively and rs## represents the last two number of the SNP ID.

materials/analysis tools: GYX, GQY, ZCH, NY. Wrote the paper: YFF, ZHC, NY. All authors read and approved the final manuscript.

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