The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes

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ABSTRACT The yolk sac membrane plays a major role in the transport of nutrients from the yolk contents to the chick embryo. We examined whether the yolk sac membrane expresses genes for nutrient digestion, enzymes, and nutrient transporters. We evaluated relative mRNA abundance of the digestive enzymes aminopeptidase N (APN) and sucrase-isomaltase (SI); the nutrient transporters oligopeptide transporter Pept1, cationic amino acid transporter CAT1, and sodium glucose transporter SGLT1; and the micronutrient transporters type IIb sodium phosphate cotransporter NPT2b, calcium transporter TRPV6, and zinc transporter ZnT-1 from embryonic d 11 (11E) to 21E (day of hatch) by real-time reverse-transcription PCR. The yolk sac membrane expressed all the examined genes, which exhibited several patterns of expression. Relative abundance of APN mRNA increased in the yolk sac membrane from 11E to 17E and decreased from 17E to 20E. Expression of PepT1 increased from 11E to 15E and decreased from 15E to 20E. In contrast, CAT1 expression decreased from 11E to 13E and increased from 15E to 17E. Expression of SGLT1 increased between 15E and 20E and decreased substantially between 20E and 21E. Expression of NPT2b increased during incubation and exhibited the highest relative expression of all the examined genes, particularly on 20E to 21E. Expression of TRPV6 decreased from 11E to 13E and increased substantially from 15E to 19E. No significant difference was found between the sampled days for ZnT-1 or SI expression, with the latter exhibiting the lowest relative expression of all the genes studied. These results present the first documentation of nutrient transporter and digestive enzyme gene-expression patterns in the yolk sac membrane, and provide a basis for future research on the capacity of the yolk sac membrane for nutrient digestion and transport.

Key words: yolk sac membrane, chick embryo, nutrient transporter, digestive enzyme, gene expression

INTRODUCTION

The yolk sac membrane (YSM) is a complex extracellular structure that is responsible for the transfer of nutrients needed for energy and tissue growth from the contents of the yolk sac to the chick embryo (Noble and Cocchi, 1990). The YSM develops from the hindgut of the embryo and begins to envelop the yolk in the initial phase of development. By d 5 of incubation (5E), the yolk is totally surrounded by the YSM, which has developed elaborate folds and a microvillus structure (Speake et al., 1998).

The uptake of yolk lipids by the YSM has been well documented (Lambson, 1970; Noble and Cocchi, 1990; Speake et al., 1998, 2003). Yolk lipid uptake is slow during the first 2 wk of incubation and very rapid during the third and final week of incubation. This uptake is facilitated by endocytosis of low-density lipoproteins through the inner endodermal columnar cells of the YSM to the surrounding blood vessels and to the embryonic circulation. Other than endocytosis, the molecular mechanisms by which proteins, carbohydrates (COH), and minerals are absorbed through the endodermal cells of the YSM during embryonic development are unknown.

In the last period of incubation, nutrients that are swallowed with the amniotic fluid reach the intestine of the embryo (Romanoff, 1960; Oegema and Jourdian, 1974; Sugimoto et al., 1999). During this time, the embryonic intestine is undergoing morphological, cellular, and molecular changes—accelerated proliferation and differentiation of enterocytes, increased absorptive capacity and uptake of nutrients, and elevated expression of genes involved in digestion and absorption processes in the epithelial enterocytes (Uni et al., 2000; Geyra et al., 2001; Uni, 2006). The mRNA abundance of the digestive enzymes sucrase-isomaltase (SI) and amino-
peptidase N (APN) is high in the intestine of the chick embryo on 19E (Uni et al., 2003). Those of the peptide transporter (PepT1) and the sodium glucose transporter (SGLT1) increase substantially from 18E to 21E (Chen et al., 2005; Gilbert et al., 2007), and genes coding for different transporters of amino acids show high expression levels on 18E (Gilbert et al., 2007). Similar results have been found in turkey embryos (de Oliveira et al., 2009).

Although the expression of nutrient digestion and transport genes has been studied in the intestine of the developing chick embryo, no study has elucidated the molecular and cellular mechanisms in the YSM (other than endocytosis of lipid droplets) that are involved in nutrient uptake by the endothelial cells. The objectives of the current study were therefore to examine whether the YSM expresses nutrient transporters and digestive enzymes, and to examine their expression pattern during incubation. Real-time reverse-transcription PCR was used to investigate 2 digestive enzymes (APN and SI), 3 nutrient transporters (PepT1, the cationic amino acid transporter CAT1, and SGLT1), and 3 micronutrient transporters (the type IIb sodium phosphate cotransporter NPT2b, the calcium transporter TRPV6, and the zinc transporter ZnT-1) for their gene expression pattern during the second half of incubation (11E to 21E) in the YSM of broiler embryos.

**MATERIALS AND METHODS**

**Egg and YSM Sampling**

Fertile eggs (n = 100, mean weight = 70.2 g, SD = 4.1 g), collected on the same day from 50-wk-old broiler breeder Cobb hens, were obtained from a commercial breeder farm (Brown, Hod Hasharon, Israel). Eggs were incubated in a Petersime hatchery (Petersime, Zulte, Belgium) at the Faculty of Agriculture of the Hebrew University in Rehovot under standard conditions (37.5°C; 60% RH, with automatic egg turning). After 10 d of incubation (on 10E), all eggs were candled, and unfertilized eggs and those with dead embryos were removed.

Eight eggs, representing the weight distribution of the eggs at set, were selected at 11E, 13E, 15E, 17E, 19E, 20E, and 21E (day of hatch; no later than 30 min after hatch). Embryos and chicks at hatch were killed by cervical dislocation, the yolk sac was removed, and the YSM was separated from the yolk contents, rinsed in a 0.9% autoclaved saline solution, and placed in microcentrifuge tubes at −80°C for mRNA analysis.

**Total RNA Isolation**

Total RNA was isolated from YSM tissue using TRI-Reagent RNA/DNA/Protein Isolation Reagent 5 (1 mL/50 to 100 mg of tissue) according to the manufacturer’s protocol (Sigma-Aldrich, St. Louis, MO): homogenization of 50 to 100 mg of tissue with 1 mL of TRI-Reagent, phase separation using chloroform, RNA precipitation using isopropanol, RNA wash using 75% ethanol, and RNA solubilization with nuclelease-free water. Concentration of RNA was determined spectrophotometrically with a NanoDrop ND-1000 instrument (Thermo Scientific, Wilmington, DE).

**Real-Time PCR**

Total RNA was reverse-transcribed to cDNA in a 20-μL volume containing 1 μg of extracted RNA. Reverse transcription was carried out using an EZ-First Strand cDNA Synthesis Kit for reverse-transcription PCR according to the manufacturer’s protocol (Biological Industries, Beit Haemek, Israel). The reaction was performed at 70°C for 10 min, followed by 60 min at 42°C and 15 min at 70°C. The SGLT1, SI, CAT1, TRPV6, NPT2b, and ZnT-1 gene-specific primers were designed, using Primer Express software (Applied Biosystems, Foster City, CA), for SYBR Green detection according to the published cDNA sequences for each of the studied genes (Table 1). The APN and PepT1 gene-specific primers were constructed according to the method in a previous study (Gilbert et al., 2007). Real-time PCR was performed on a Stratagene MX 3000P instrument (Stratagene, Amsterdam, the Netherlands). The 20-μL PCR mixture consisted of 10 μL of Platinum SYBR Green qPCR SuperMix (Invitrogen Carlsbad, CA), 5 μL water, and 1 μL of each primer, which was added to 3 μL of the cDNA diluted 1:25. All PCR were performed in triplicate in A6gene PCR plates (Thermo Fisher Scientific Inc., Waltham, MA) closed with Absolute QPCR seals (Thermo Fisher Scientific Inc.) under the following conditions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min. In addition, to ensure amplification of a single product, a dissociation curve was determined under the following conditions: 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Specificity of the product was also confirmed by running samples on a 1.5% agarose gel, excising for purification using a Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech, Bade City, Taiwan), and sequencing (Weizmann Institute of Science, Rehovot, Israel).

Calculations of threshold cycles, amplification efficiencies, and Ro values (the starting fluorescence value, which is proportional to the relative starting template concentration) were performed using the Data Analysis for Real-Time PCR Excel workbook (Peirson et al., 2003). The raw fluorescence data for each of the examined genes were exported to the Data Analysis for Real-Time PCR Excel workbook, in which the efficiency (E) of each individual reaction was determined using linear regression analysis of the fluorescence data from the exponential phase of each amplification. One-way ANOVA detected no significant difference in amplification efficiencies among sampling days; therefore, the average E-value (arithmetical mean of E-values of all samples) for each of the genes was used for the Ro cal-
Table 1. Primers used for quantitative real-time PCR analysis of yolk sac membrane (YSM) gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Primer (5′)</th>
<th>Primer (3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APN</td>
<td>Y17105</td>
<td>AATAAGGGCTCCTGAGAAAC</td>
<td>AGCGGATACGCCCGTGTT</td>
</tr>
<tr>
<td>PepT1</td>
<td>NM_204365</td>
<td>CCCCCTGAGGAGATCAGTGTTGGCA</td>
<td>CAAAAGAGCAGCAAGACGA</td>
</tr>
<tr>
<td>CAT1</td>
<td>AJ810850</td>
<td>CCTACGAGTTCTTGGCTCTCAT</td>
<td>TTGTATTGACTTTTCGCCAAAA</td>
</tr>
<tr>
<td>SI</td>
<td>BU124113</td>
<td>TCAGATTCTCAGGATGTCCAA</td>
<td>AACAGAGCCGGTAACCCAGTA</td>
</tr>
<tr>
<td>SGLT1</td>
<td>AJ236903</td>
<td>TGGCCAGGGCTTATTTGGG</td>
<td>GGGCATGTCTTTCCAGAGAG</td>
</tr>
<tr>
<td>NPT2b</td>
<td>NM_204474</td>
<td>AACACTGCTTCCCATTTG</td>
<td>GTTGTGGTGTTGACAAATTT</td>
</tr>
<tr>
<td>TRPV6</td>
<td>XM_416530</td>
<td>ATCATCTTCCAGACAGAG</td>
<td>GTGGCCATGATGCGAAAAGC</td>
</tr>
<tr>
<td>ZnT-1</td>
<td>NM_204475</td>
<td>AAAGAGCCTGGGTTTGGATT</td>
<td>ATGGACAGGAGCAGGAGAG</td>
</tr>
<tr>
<td>SGLT1</td>
<td>AJ236903</td>
<td>TCAAATTCCCTACGATGTCCAA</td>
<td>ACAAAGAGCGGTAACCCAGTA</td>
</tr>
<tr>
<td>TRPV6</td>
<td>XM_416530</td>
<td>ATCATCTTCCAGACAGAG</td>
<td>GTGGCCATGATGCGAAAAGC</td>
</tr>
<tr>
<td>ZnT-1</td>
<td>NM_204475</td>
<td>AAAGAGCCTGGGTTTGGATT</td>
<td>ATGGACAGGAGCAGGAGAG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>X00182</td>
<td>ATGAATCCGGACCCTCCATT</td>
<td>AGCCATGGCAATTCCTTGCTT</td>
</tr>
</tbody>
</table>

1APN = aminopeptidase N; PepT1 = oligopeptide transporter; CAT1 = cationic amino acid transporter; SI = sucrase-isomaltase; SGLT1 = sodium glucose transporter; NPT2b = type IIb sodium phosphate cotransporter; TRPV6 = calcium transporter; ZnT-1 = zinc transporter.

calculation in each sample. The Ro values of β-actin were not significantly different (P > 0.05) among the different treatments (embryonic days); therefore, mRNA abundance was normalized for RNA loading to β-actin as the internal control [(Ro target gene/Ro β-actin) × 100].

**Statistical Analysis**

All data were subjected to one-way ANOVA. Prior to ANOVA, means ± SD for each data set were calculated. Heterogeneity of variance (expressed as an increase in SD with the mean) was observed between sampling days for APN, CAT1, SGLT1, SI, NPT2b, TRPV6, and ZnT-1; therefore, logarithmic (ln) transformation was carried out for the data on these genes. Differences among means were tested by contrasts using Student’s t-test. For log-transformed data, values are presented as back-transformed means ± CI (back-transformation resulted in asymmetric CI; CI also reflected the relationship between variance and mean). All statistical analyses were carried out using JMP 8 software (SAS Institute Inc., Cary, NC).

**RESULTS AND DISCUSSION**

We investigated the mRNA abundance of several genes that are normally expressed in the small intestine and that are responsible for nutrient uptake and digestion in the YSM tissue during the last 10 d of incubation. The YSM-expressed genes APN, SI, PepT1, CAT1, SGLT1, NPT2b, TRPV6, and ZnT-1 exhibited several patterns of expression during embryonic development (Figures 1, 2, and 3).

The fertile egg yolk, before setting in the incubator, contains approximately 15% protein (Romanoff, 1967; Shenstone, 1968, Yadgary et al., 2010). The uptake of protein from the yolk sac is difficult to evaluate because protein and water pass from the egg albumen compartment and from the amniotic sac to the yolk sac during incubation (Ar, 1991; Sugimoto et al., 1999, Yadgary et al., 2010). It is therefore of interest to investigate mechanisms of yolk protein breakdown and peptide or amino acid uptake by the YSM. Accordingly, we examined gene expression of the digestive enzyme APN, the di- and tripeptide transporter PepT1, and the cationic amino acid transporter CAT1 in the YSM.

The abundance of APN mRNA increased in the YSM from 11E to 17E and decreased significantly from one day to the next between 17E and 20E (Figure 1A). The APN is a membrane-bound zinc metalloproteinase that cleaves amino acids from the N terminus of oligopeptides (Hooper, 1994) and is expressed in various tissues and organs, among them the lungs, kidneys, liver, stomach, and small intestine, with high levels of activity in the latter (Jardinaud et al., 2004). This is in agreement with the report of Sihn et al. (2006), who found moderate levels of APN enzyme activity and gene expression (by a semiquantitative method) in the YSM during the first 9 d of incubation. However, the current study is the first to examine APN expression in the YSM during the last week of incubation, when yolk utilization is most rapid (Speake et al., 1998).

Expression of the PepT1 gene (SLC15A1), which encodes a transporter of di- and tripeptides (Fei et al., 1994; Daniel and Kottra, 2004), increased in the YSM between 11E and 15E and decreased between 15E and 20E. No change was observed between 20E and 21E (Figure 1B). The YSM gene-expression levels of CAT1 (SLC7A1), a high-affinity, low-capacity basolateral transporter of cationic amino acids (Verrey et al., 2004; Bröer, 2008), exhibited sporadic changes during embryonic development, with a significant decrease from 11E to 13E and a significant increase from 15E to 17E (Figure 1C).

The expression patterns of APN and PepT1 genes may be indicative of a change in the capacity of the YSM to digest and transport yolk peptides during the second half of incubation. The expression pattern of the CAT1 transporter differed from those of APN and PepT1. This difference may be associated with the locations of the transporters in the absorptive cells: CAT1 at the basolateral membrane, and APN and PepT1 at the brush-border membrane. Different expression patterns for basolateral vs. brush-border membrane amino acid transporters have also been observed in the small intestine of broilers (Gilbert et al., 2007).
Carbohydrates can be found in the yolk contents (0.5 to 2%), and their levels increase from midincubation to 19E and decrease from 19E to 21E (Thommes and Just, 1964; Yadgary et al., 2010). Carbohydrate digestion and absorption by various brush-border enzymes and transporters have been documented in epithelial absorptive cells (Ferraris, 2001), but they have never been examined in the YSM. Therefore, we examined gene expression of the apical surface enzyme SI and the glucose transporter SGLT1 in the YSM.

Whereas SI, an enzyme located at the brush-border membrane of enterocyte cells that is responsible for the final steps in starch and glycogen digestion (Naim et al., 1988; Fransen et al., 1991; Ferraris, 2001), exhibited low relative mRNA abundance in the YSM (with no significant difference among sampling days) (Figure 2A), relative abundance of SGLT1 mRNA increased between 15E and 20E and decreased substantially from 20E to 21E (Figure 2B). The SGLT1 protein (SLC5A1) is a high-affinity Na⁺-dependent transporter that is primarily found in the intestine (Ferraris, 2001), where it absorbs glucose or galactose from the gut lumen (Wright and Turk, 2004). The expression pattern of SGLT1 found here in the YSM coincided with the change in COH amount in the yolk sac during the same period (Thommes and Just, 1964; Yadgary et al., 2010). It can be speculated that SGLT1 transports glucose into the endodermal cells of the YSM for the process (demonstrated by Willier, 1968) of glycogen synthesis and storage.

Minerals such as calcium, phosphorus, and zinc are essential for normal growth and development of the avian embryo (Savage, 1968; Richards, 1997; Underwood and Suttle, 2001; Dibner et al., 2007). Although transporters of minerals have been studied in several tissues, including the intestine of the mature chicken, no work has been done on mineral transporters during chick embryonic development in the YSM or in the intestine in the pre- to posthatch period. We therefore examined the gene expression of 3 mineral transporters in the YSM: NPT2b, TRPV6, and ZnT-1.

The NPT2b gene, which encodes the major sodium phosphate cotransporter and is primarily expressed at the brush-border membrane of enterocytes (Hilfiker et al., 1998; Han et al., 2009), exhibited elevated expression levels in the YSM between 11E and 21E, with high relative expression on 17E and on 20E to 21E (Figure 3A).

Gene expression levels of TRPV6, a calcium-channel protein that is coexpressed in several tissues (Müller et al., 2000; Peng et al., 2000; Hoenderop et al., 2001; Khanal et al., 2008), decreased in the YSM between 11E and 13E, and increased substantially between 15E and 19E. The higher levels of TRPV6 gene expression on 19E were followed by lower levels of expression on the last 2 d of incubation (Figure 3B).

The ZnT-1, a zinc exporter located at the basolateral membrane of the cell that exhibits a broad pattern of expression (Palmiter and Findley, 1995; Tako et al.,
was also expressed in the YSM. Its expression decreased between 11E and 15E and increased 2-fold between 15E and 19E, although no significant difference was found among the sampled days.

In all of the genes examined in the current trial, YSM gene expression levels decreased between 20E and 21E, or were very low compared with previous days (Figures 1, 2, and 3). Although previous studies recorded an accelerated consumption of the yolk sac toward hatch (Yadgary et al., 2010) and posthatch (Noy and Sklan, 1999), the observed decrease in YSM mRNA levels may be explained by the transfer of yolk nutrients directly from the yolk sac into the intestine (Esteban et al., 1991; Sulaiman et al., 1996) and not just through the YSM (Lambson, 1970; Noble and Cocchi, 1990); Noy and Sklan (2001) indicated that from the day of hatch to d 4 posthatch, the major route of yolk utilization is via the yolk stalk into the small intestine. It is possible that the shift from the route through the YSM to the

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route through the intestine occurs as early as 20E to 21E; thus, nutrient transport and digestive mechanisms are downregulated in the YSM during this period.
This is the first study showing the molecular mechanisms, other than endocytosis of lipid droplets, that are involved in the transport of nutrients through the YSM. Our results demonstrate that the YSM expresses genes that are usually expressed by intestinal cells and are related to CHO, disaccharide, protein, amino acid, calcium, zinc, and phosphate digestion and transport from the yolk contents to the blood circulation of the embryo.

Because the YSM is an extension of the hindgut of the embryo (Noble and Cocchi, 1990), it is reasonable to assume that the 2 tissues share similar gene expression. Thus, it is quite likely that additional transporters of amino acids, COH, and minerals, which were not examined in this work, are expressed in the YSM. A comprehensive study of the different nutrient transporter genes and their proteins in the YSM compared with the intestine will contribute to a better understanding of the capacity of the yolk sac for nutrient absorption, and may lead to new perspectives on the role of the yolk sac in the supply of nutrients, other than fat, to the embryo for its optimal development, and on the shift in nutrient uptake through the YSM to that by the intestine.

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