Animal Reproduction Science 118 (2010) 297–309

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Effect of fetal size on fetal placental hyaluronan and hyaluronoglucosaminidases throughout gestation in the pig☆

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ARTICLE INFO

Article history:
Received 6 February 2009
Received in revised form 12 June 2009
Accepted 29 June 2009
Available online 5 July 2009

Keywords:
Pig – hyaluronan
Allantochorion
Glycosaminoglycan
Hyaluronidase

ABSTRACT

The trophoblast-endometrial epithelial cell bilayer of porcine placenta undergoes microscopic folding during gestation, and the folded bilayer is embedded in fetal placental stroma. We hypothesized that hyaluronan was a component of fetal placental stroma, and that hyaluronoglucosaminidases played a role in bilayer folding. Gilts were unilaterally hysterectomized-ovariectomized (UHO) at 160 days of age, mated at estrus and killed on days 25, 45, 65, 85 or 105 of gestation. Fetal placental tissues were collected to evaluate hyaluronan and hyaluronoglucosaminidase content. Fetal placental hyaluronan concentration increased ($P < 0.01$) between day 25 and 45 of gestation, remained high throughout gestation, and was greater ($P < 0.05$) in the fetal placenta of the smallest compared to the largest fetuses on day 105 of gestation. Hyaluronan was localized to fetal placental stroma. Three cDNAs for hyaluronoglucosaminidase 1 (two 1379 and one 1552 bp) and one cDNA (1421 bp) for hyaluronoglucosaminidase 2 were cloned from day-85 fetal placental RNA. Gene expression analysis indicated that the 1379 bp form of hyaluronoglucosaminidase 1 mRNA did not differ, the 1552 bp form increased, and the 1421 bp form of hyaluronoglucosaminidase 2 decreased during pregnancy. Amount of all three mRNAs was greater ($P < 0.05$) in fetal placenta of the smallest compared to the largest fetuses. Zymography indicated 70 and 55 kd protein isoforms of hyaluronoglucosaminidase in fetal placental tissue. Both forms increased with advancing gestation and were greater in fetal placenta of the smallest compared to the largest fetuses ($P < 0.05$). These results are consistent with a role for hyaluronan and hyaluronoglucosaminidases in the development of the microscopic folds of the pig placenta during gestation.

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1. Introduction

The pig placenta is a diffuse, epitheliochorial type of placenta, and is made up of areolar and interareolar areas. Areolae are specialized fetal placental structures that develop adjacent to the openings of the endometrial glands, and have been shown to take up gland products by fluid phase pinocytosis (Raub et al., 1985). The interareolar areas are primarily composed of a folded trophoblast/endometrial epithelial bilayer, which is embedded in a loose fetal placental stroma (Friess et al., 1980; Vallet and Freking, 2007). Maternal and fetal capillaries form on either side of the folded epithelial bilayer (Leiser and Dantzer, 1988), thus the folded bilayer represents the surface area of exchange for small molecules between the sow and the fetus during gestation. Comparisons of fetal weight to fetal placental weight ratios between the largest and smallest fetuses throughout gestation suggest that placental efficiency increases as gestation advances and is greater for fetal placenta of small fetuses throughout gestation (Vallet and Freking, 2007). Likewise, allometric slopes

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0378-4320/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.anireprosci.2009.06.019

generated by regression analysis of the natural log of fetal weight compared to the natural log of fetal placental weight indicated that fetal weight sparing mechanisms exist that preserve fetal weight when fetal placental weights were reduced (Vallet and Freking, 2007). This suggests that placental compensatory mechanisms exist to improve fetal nutrient transport in placenta of small fetuses. Depths of the microscopic folds increase as gestation advances, and are greater in placenta of small fetuses compared to large fetuses (Vallet and Freking, 2007). Thus, the increase in depth of folds during gestation and in placenta of small fetuses likely increases the surface area of exchange, and contributes to the efficiency of nutrient transfer to the fetus. Limited information is available regarding the control of development of the placental microscopic folds during gestation in swine.

Because the folded bilayer is embedded in fetal placental stroma, development of the folds takes place through biochemical interaction between cells of the epithelial bilayer and the stroma. We hypothesized that to generate the folded pattern, stromal tissues must be degraded during pattern formation. Thus, epithelial bilayer folding depends on secretion of the appropriate enzymes to degrade stromal tissue components. Stromal tissues are composed of a variety of extracellular matrix components, which is dependent on the stromal tissue type. Glycosaminoglycans (GAG) represent a major portion of extracellular matrix components, and previous results indicated that hyaluronan is the most abundant GAG found in porcine fetal placental tissue (Steele and Froseth, 1980). Thus degradation of fetal placental stroma likely involves degradation of hyaluronan by hyaluronoglucosaminidases. The objectives of the current experiment were to further characterize fetal placental stromal tissue hyaluronan content and explore hyaluronoglucosaminidase gene expression and activity in fetal placental tissue during gestation.

2. Materials and methods

All animal experimentation was approved by the USMARC Institutional Animal Care and Use Committee. Gilts used for this experiment were subsets of gilts described in Freking et al. (2007). Briefly, white crossbred gilts from the USMARC ovulation rate, uterine capacity and randomly selected control lines were unilaterally hysterectomized-ovariectomized (UHO) at approximately 160 days of age and allowed to recover. Gilts were UHO to induce a crowded intrauterine environment, which reduces the size of the smallest fetus in the litter and provides a better comparison between largest and smallest fetuses. At 250 days of age, gilts were mated to boars from their respective lines. Gilts were killed for tissue collection on day 25, 45, 65, 85 or 105 of pregnancy in four separate replicates. At time of tissue collection, the remaining uterine horn was recovered, opened along the antimesometrial side, and each fetus was weighed. Gilts from the third and fourth replicates described in Freking et al. (2007) were used in this experiment. In both replicates, the largest and smallest living fetuses in each litter were identified. In the third replicate, a 5 g sample of placental tissue was collected from the largest and smallest fetus of each gilt. Tissues were frozen in liquid nitrogen and stored at −80 °C. These tissues were used for hyaluronan and hyaluronoglucosaminidase measurements. In the fourth replicate, uterine wall samples (fetal placenta and adjacent uterus) were collected within the placentas associated with the largest and smallest fetuses in the litter from control line gilts on days 45, 65, 85, and 105 of gestation. These tissues were fixed in buffered formalin and used for hyaluronan-specific staining. In both replicates, tissues for this experiment were collected within 20 min of the time animals were killed. To standardize sample collection, fetal placental and uterine wall samples collected from day 45 of gestation onward were collected from an area of the placenta midway between the paraplacental zone and the junction of the amnion with the allantochorion.

2.1. Hyaluronan assay

Samples (0.5 g) of frozen fetal placental tissue from all three selected lines were homogenized in 10 ml homogenization buffer (9.6 M urea, 2% Triton X-100, 50 mM dithiothreitol, 5 mM K2CO3) using a Polytron homogenizer. Hyaluronan content was measured using a commercial assay (Corgenix, Broomfield, CO) according to directions contained in the kit.

2.2. Hyaluronan-specific histochemical staining

Formalin fixed uterine wall samples were incubated overnight in 75% ethanol, and the tissue was dehydrated by incubation in an increasing graded series of ethanol concentrations up to absolute alcohol. Samples were then incubated in three changes of xylene followed by three changes of Paraplast embedding compound (McCormick Scientific, St. Louis, MO) and embedded in Paraplast. Embedded samples were sectioned (6 μm) and mounted onto Superfrost-plus coated slides (Fisher Scientific, Pittsburg, PA). Slides were processed through xylene and a decreasing graded series of ethanol concentrations to rehydrate tissues. Slides were then incubated in 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 1% Triton X-100 for 1 h. Slides were then incubated with biotinylated hyaluronan binding protein (Sigma–Aldrich Chemical Co., St Louis, MO) in buffer alone (specific binding) or with exogenous hyaluronan (nonspecific binding control) for 1 h at room temperature. Slides were washed five times in buffer and then incubated with avidin-peroxidase in buffer. Slides were then washed five times in buffer, once in 0.9% NaCl, and then incubated with 3,3′-diaminobenzidine for color development. Slides were then processed through a graded series of increasing ethanol concentrations followed by xylene, allowed to dry, and a cover slip was applied with Permount (Fisher Scientific).

2.3. Hyaluronoglucosaminidase cDNA cloning

Previously reported porcine hyaluronoglucosaminidase 1 and 2 full-length cDNA sequences (GenBank accession numbers NM214441 and NM214440, respectively) were used to design primer pairs that would amplify the coding regions of both cDNAs (Table 1). Total RNA recovered

Table 1
Primer sequences used for reverse transcription polymerase chain reaction amplification of fetal placental hyaluronoglucosaminidase (HYAL)1 and 2 mRNAs from total RNA isolated from a day-85 fetal placental tissue sample are listed.

Primer name	Sequence
HYAL1 forward	5′-TCAAAGCCTGCTCTCTCAGCTC-3′
HYAL1 reverse	5′-GCATGTGCCAGTCACCG-3′
HYAL2 forward	5′-ACACTGGCTCTGGTGTTGG-3′
HYAL2 reverse	5′-AGCCAGGTGAGAGACCCTTAC-3′

from a day-85 fetal placental tissue sample using an RNeasy kit (Qiagen, Valencia, CA) was reverse transcribed using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and each cDNA specific reverse primer. The cDNAs were then amplified by polymerase chain reaction (PCR) using the following PCR parameters: 95 °C for 30 s followed by 35 cycles of 95 °C for 15 s, 58 °C for 1 min, and 72 °C for 2 min which was then followed by 10 min incubation at 72 °C. The amplified products were purified using GENECLEAN II (Qbiogene, Carlsbad, CA). Isolated cDNAs were then ligated into PCRII vector and transfected into bacteria using a TA cloning kit (Invitrogen, Carlsbad, CA). Colonies were screened using PCR with the same hyaluronoglucosaminidase 1 and 2 specific primer pairs used for amplification of cDNAs, and clones displaying amplified products were sequenced using dye terminator sequencing (Amersham, Piscataway, NJ).

2.4. Hyaluronoglucosaminidase mRNA measurement using real time reverse transcription PCR

Only fetal placental tissues from the randomly selected control line gilts from replicate three were used to evaluate mRNA concentration using real time reverse transcription (rt) PCR. Primers (Table 2) were designed for real time rtPCR based on sequences obtained for hyaluronoglucosaminidase 1 and 2 cDNA clones resulting from day 85 fetal placental total cellular RNA. For hyaluronoglucosaminidase 1, a common reverse primer was used in both assays, and the assays were rendered specific for each cDNA by designing the forward primer to either bridge putative intron 5 (1379 bp form) or be within putative intron 5 (1552 bp form). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Table 2, Blomberg et al., 2006) were used to measure GAPDH mRNA concentrations for use as a correction factor for variations in RNA isolation and sample aliquoting. Total cellular RNA was isolated from fetal placental tissues using RNeasy kits and samples were DNase I treated on the column using DNase I provided by the manufacturer. Total RNA was quantified using a ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE). Total cellular RNA samples (1 μg) were reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each cDNA sample was assayed in duplicate (25 ng tcRNA equivalents) along with 0.25 μM forward and reverse primers and 12.5 μl of Taq, SYBR green, and ROX supermix (Bio-Rad) in a 25 μl reaction. Amplification was performed using a PTC-200 thermocycler fitted with a chromo 4 fluorescence detector (Bio-Rad). The PCR conditions included denaturation (95 °C, 2 min) followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 70 °C for 45 s. Melting curve analysis and gel electrophoresis were used to confirm the amplification of a single product of the predicted size.

Table 2
Primer sequences used for real time reverse transcription polymerase chain reaction analysis of fetal placental hyaluronoglucosaminidase (HYAL) 1 and 2 mRNAs are listed.

Primer name	Sequence
1379 bp specific HYAL1 forward	5′-GAGAACACAAGAACCAAGGAATC-3′
1552 bp specific HYAL1 forward	5′-CAGTGCCCTAGGTGGACC-3′
Common HYAL1 reverse	5′-CACCCGATCCTTGAGTGAG-3′
HYAL2 forward	5′-CGGTATAGGTCTCCCAGTTCTG-3′
HYAL2 reverse	5′-CAGGCGCAGTATGAATTTGAG-3′
GAPDH forward [7]	5′-GGCGATGCTGGTGCTACGT-3′
GAPDH reverse	5′-CATGGTTCACGCCCATCAC-3′

2.5. Hyaluronoglucosaminidase zymography

Similar to the real time rtPCR measurements, zymography was only performed on samples from the randomly selected control line gilts from replicate three. Zymography was performed as described by Miura et al. (1995) with a few modifications. Briefly, 7.5% SDS acrylamide gels were prepared with 200 μg/ml human umbilical chord hyaluronan (Sigma–Aldrich Chemical Co.) incorporated into the gel. Fetal placental tissue (0.5 g) was homogenized in 10 ml SDS gel loading buffer (Buhi et al., 1989) without mercaptoethanol, homogenates were centrifuged at 3000 × *g*, and the supernatant was frozen at −20 °C. An aliquot of each fetal placental homogenate (200 μl) was then subjected to electrophoresis on the hyaluronan impregnated gel. Gels were incubated sequentially in 2.5% Triton X-100 for 2 h, followed by two changes of 0.1 M sodium formate, 0.15 M NaCl buffer pH (3.5) for 2 h each, followed by an overnight incubation in 0.1 M sodium formate, 0.15 M NaCl (preliminary incubations of hyaluronan containing gels were performed at pH 3.5, 5.0, 6.0, and 7.0). Subsequently, gels were incubated twice in 20 mM Tris–HCl, pH 8.0 for 2 h, and then in the same buffer plus 0.1 mg/ml Pronase at 37 °C overnight. The gel was subsequently incubated in 20% ethanol, 10% acetic acid for 1 h followed by staining with Stainsall (Sigma–Aldrich Chemical Co.; Stainsall is a superior stain for hyaluronan compared with Alcian Blue (Miura et al., 1995; Scott et al., 1964; Volpi and Maccari, 2002; results not shown) in the same solution. Gels were imaged, the images were inverted, and densitometry was performed on the inverted images using 2D-Advanced software (Nonlinear Dynamics, Durham, NC).

2.6. Statistical analysis

Fetal and fetal placental weights and hyaluronan concentrations in fetal placental tissue samples were analyzed using PROC MIXED (SAS Institute, Cary, NC) with a model that included fixed effects of selection line, day of gestation, size of the fetus, and all interactions. Gilt (day*line) was included as a random effect. Orthogonal contrasts were used to separate individual interaction contrasts to explore the day of gestation by size of fetus interaction more fully. Hyaluronoglucosaminidase and GAPDH real time rtPCR

threshold cycles (CT) were converted to relative units by assuming that the amount of product at the CT was 1 unit and using the formula 2^{-CT} to calculate the units in the starting sample. Use of 2 in this equation assumes 100% PCR efficiency but experimentation using values from 1.8 to 2 (80% to 100% efficiency) made little difference to the relative relationships among sample values. This resulted in the generation of very small numbers, which were then rescaled separately for each mRNA to larger values by multiplication by a constant. Analysis of target mRNA data was hampered by the fact that GAPDH and the other "housekeeping" mRNAs displayed similar day of gestation and fetal size effects, making their use as correction factors questionable. The need for correction of mRNA data is based on the lability, recovery rate, and accurate measurement of total RNA isolated from tissues. All of these factors have significantly improved in the past two decades. Furthermore, differences between samples in lability, recovery rate, and measurement of the resulting RNA would be expected to be distributed randomly, and thus only increase the error variance of the experiment. Thus, the need for correction is not well supported on statistical grounds and in many cases the way correction is practiced has numerous drawbacks (Ivell, 1998). Here, systematic significant differences in "housekeeper" mRNAs precluded their use as correction factors. To solve this problem, we fitted effects of day of gestation, fetal size and the day by fetal size interactions to the GAPDH mRNA measurements, and then used the residuals (actual value minus mean value for that day by size combination) to correct target mRNA values. Use of the residuals removed systematic experimental-design-based differences in the "housekeeper" mRNA while retaining the error correction function of the "housekeeper" mRNA data. In the current experiment, use of the residuals as a covariate did not change relationships between the uncorrected means of the target mRNA in the experiment, it only reduced the error variance (not shown). In our view, this is clearly preferential to the use of

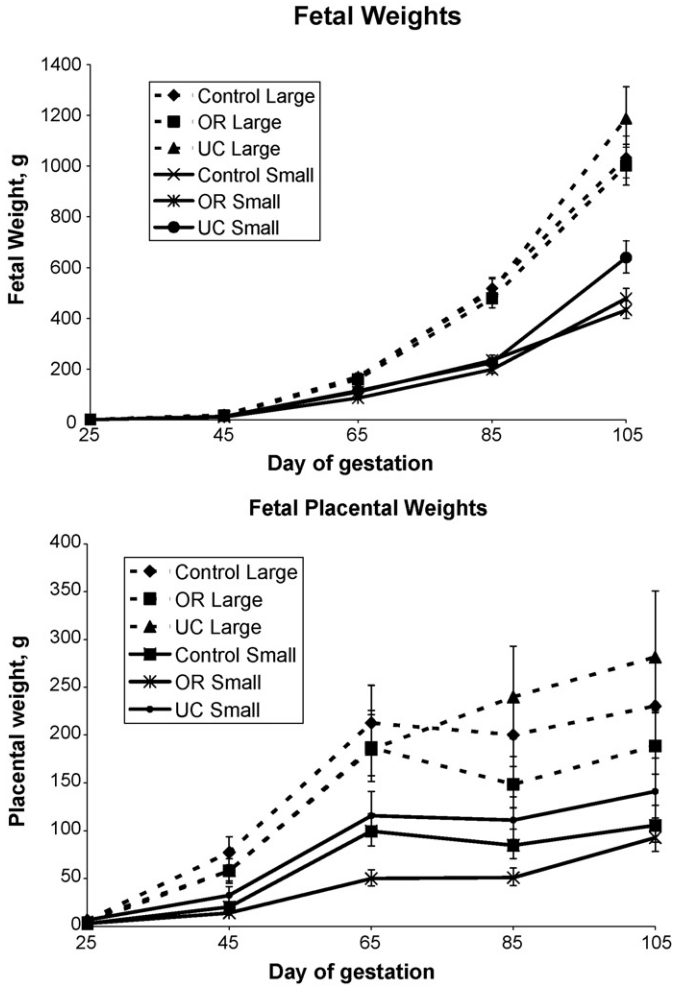

Fig. 1. Least squares means for fetal (top graph) and fetal placental (bottom graph) weights from replicate three of Freking et al. (2007) are illustrated. Effects of day, line size of fetus, and the size of fetus by day interaction ($P < 0.01$) were observed for fetal weights. Number of observations for the control, ovulation rate (OR) and uterine capacity (UC) selected lines were 6, 6, and 6 for day 25; 7, 6, and 4 for day 45; 9, 9, and 6 for day 65; 8, 8, and 6 for day 85; 8, 9, and 5 for day 105. Effects of day, line, size of fetus, the size of fetus by day interaction, and the size of fetus by line interactions ($P < 0.01$) were observed for fetal placental weights. These results are very similar to those reported for replicate four (Vallet and Freking, 2007).

a correction factor which itself varies systematically with treatment effects for unknown reason. Thus, hyaluronoglucosaminidase mRNA relative units were analyzed using PROC MIXED and a model that included GAPDH residuals as a covariate, and day of gestation, size of fetus, and their interaction as fixed effects (line was not included because only control line gilts were measured). Gilt (day) was included as a random effect. Finally, densitometry results from hyaluronoglucosaminidase zymography were analyzed using PROC MIXED and a model similar to that described for hyaluronoglucosaminidase mRNA data.

3. Results

3.1. Fetal and fetal placental weights

Fetal and fetal placental weights (including numbers of observations) from replicate three are presented in Fig. 1. Fetal and fetal placental weights from replicate four (used for hyaluronan staining) were reported by Vallet and Freking (2007). Using data from the third replicate, effects of day, line, size of fetus, and the size of fetus by day interaction (all $P < 0.01$) were observed for fetal weights. Day, line, size of fetus, size of fetus by day interaction, and size of fetus by line interactions (all $P < 0.01$) were observed for fetal placental weights. In general, patterns of change in fetal and fetal placental weights for the third and fourth replicates used in this experiment were very similar.

3.2. Hyaluronan assay

Pig fetal placentae collected on day 25 of gestation appeared to consist primarily of a clear gel, and attachment of the fetal placenta to the endometrium was not very robust. By day 45, fetal placental tissue and its attachment to the endometrium were more substantial. The results of hyaluronan measurement in fetal placentae of the largest and smallest fetuses throughout gestation (including numbers of observations) are illustrated in Fig. 2. Hyaluronan content of the pig fetal placenta increased dramatically between day 25 and 45 and remained relatively constant from day 45 to day 105. Separation of the day by size interaction using orthogonal contrasts indicated an interaction ($P < 0.05$) between day 105 and the rest of gestation with fetal size, indicating that hyaluronan content increased more in the fetal placentae of the smallest fetuses from day 85 to 105 compared to the largest fetuses.

3.3. Hyaluronan-specific histochemical staining

Representative photomicrographs of hyaluronan-specific staining of placental tissue on day 105 of gestation are presented in Fig. 3. A comparison of slides treated with biotinylated hyaluronan binding protein alone to those treated with biotinylated hyaluronan binding protein preabsorbed with exogenous hyaluronan indicated that fetal placental stroma surrounding the microscopic folds of the placenta contained hyaluronan. Similar results were obtained from day 45 to 105 of gestation (not shown). There were no apparent differences between placentae from the smallest and largest fetuses.

3.4. Hyaluronoglucosaminidase cDNA cloning

Cloning of hyaluronoglucosaminidase cDNAs from fetal placental tissue total cellular RNA resulted in three cDNA clones (clones 1 and 2–1379 bp, GenBank accession number EU925133, and clone 3–1552 bp, GenBank accession number EU925134) similar to porcine hyaluronoglucosaminidase 1 cDNA (GenBank accession number NM214441) and a single clone (1421 bp, GenBank accession number EU925135) that was nearly identical

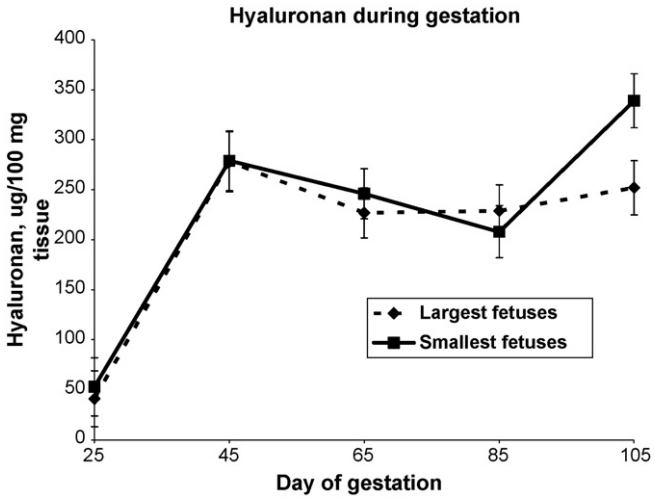

Fig. 2. Least squares means for fetal placental hyaluronan concentration during gestation in the pig are illustrated for fetal placentae associated with the smallest and largest fetuses in litters from randomly selected control, ovulation rate, and uterine capacity lines from this experiment. No effect of line was detected, thus data were combined across line for presentation. Number of observations were 12 (day 25), 17 (day 45), 24 (day 65), 22 (day 85), and 22 (day 105). Orthogonal contrasts indicated an interaction between day 105 and the rest of gestation with size of the fetus was present ($P < 0.05$). In addition, hyaluronan increased ($P < 0.01$) dramatically between day 25 and 45 of gestation, which was coincident with a substantial increase in the integrity and attachment strength of fetal placentas.

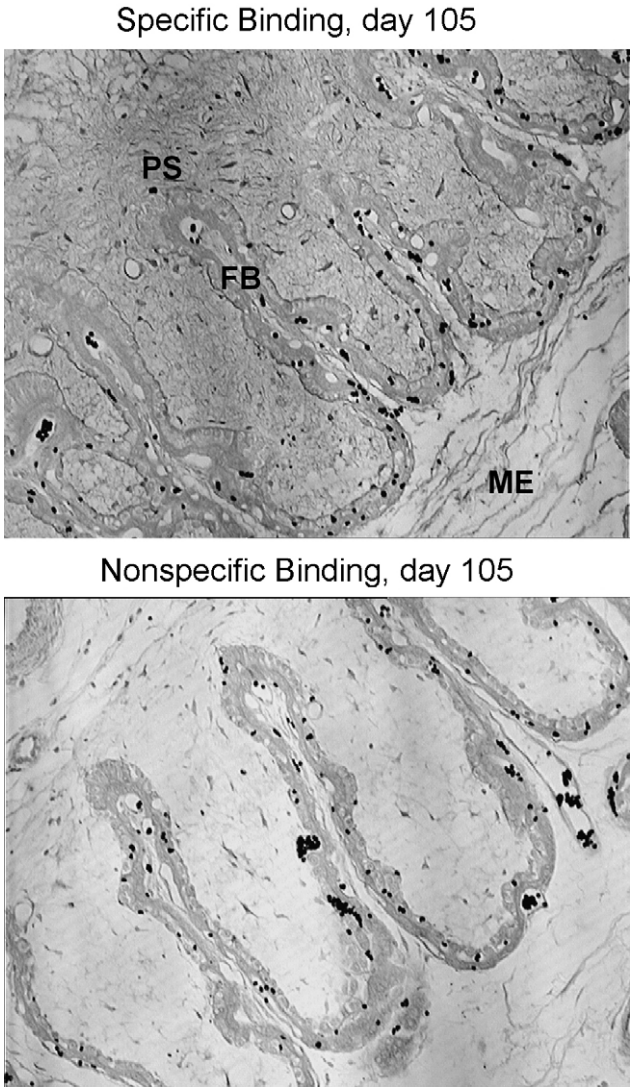

Fig. 3. Representative photomicrographs of uterine wall sections from day 105 of gestation that were histochemically stained for hyaluronan are illustrated. The top image shows staining using biotin labelled hyaluronan binding protein and thus indicates specific staining, the bottom image shows staining in the presence of exogenous human umbilical chord hyaluronan and therefore illustrates nonspecific staining. Fetal placental stromal areas were specifically stained from day 45 to 105 of gestation. The fetal placental stroma (PS), folded bilayer (FB), and maternal endometrium (ME) are indicated.

to porcine hyaluronoglucosaminidase 2 cDNA (GenBank accession number NM214440). Results of alignment of the three hyaluronoglucosaminidase 1 clones with other known porcine hyaluronoglucosaminidase 1 cDNAs (GenBank accession numbers NM214441, liver cDNA AK232421, intestine cDNA AK231433) is summarized in Fig. 4. Differences were observed at nucleotide positions 47, 135, 143, 200, 808, 863 (single nucleotide polymorphisms), 962 and 1052 (alternative exons; numbering based on 1379 bp sequence). The 1379 bp cDNAs coded for a protein of 435 amino acids. The 173 bp alternative exon included in clone 3 introduced a stop codon and codes for a putative truncated hyaluronoglucosaminidase protein of 338 amino acids. The single nucleotide polymorphism at 143 corresponds to either a proline or leucine at amino acid 28, the single nucleotide polymorphism at 200 corresponds to either an arginine or histidine at amino acid 47, the nucleotide polymorphism at 808 corresponds to either an alanine or serine at amino acid 250 and the nucleotide polymorphism at 863 codes for a glutamate or a glycine at amino acid 268. The nucleotide polymorphism at 47 was outside the coding region and at 135 was silent. The hyaluronoglucosaminidase 2 cDNA clone begins nine codons from the putative translation start site (after alignment with the previously reported sequence, GenBank accession number NM214440) and codes for 467 amino acids. Thus, the complete protein is predicted to be 476 amino acids in length. The alignment indicated two sequence differences, one at 556 (position relative to the 1421 bp sequence), which was silent, and several nucleotides beginning at 1387. The nucleotide sequence beginning at nucleotide1387 codes for a leucine, threonine, tryptophan starting at amino acid 472 compared to a tyrosine, proline, glycine in the previously identified sequence. (Fig. 5)

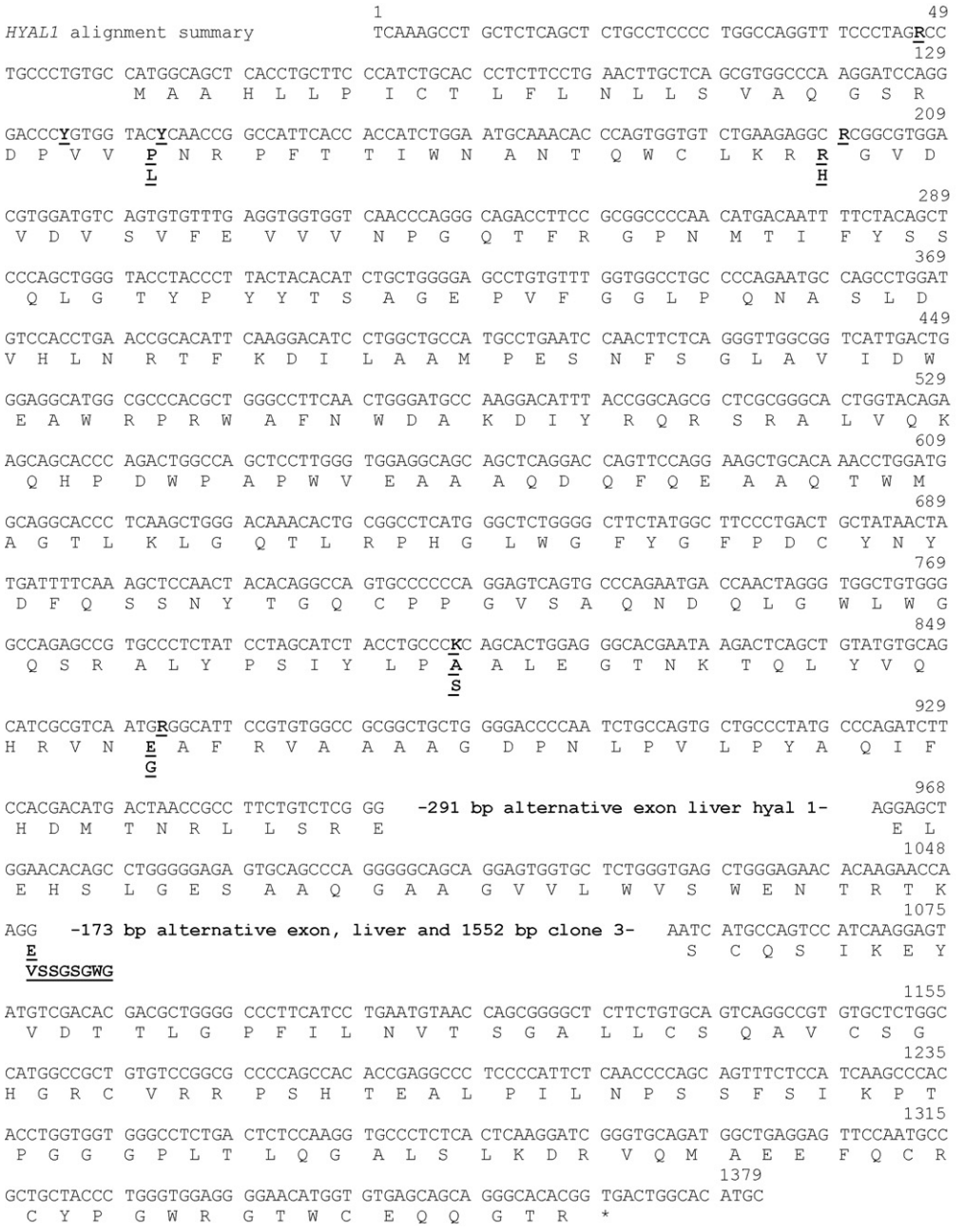

Fig. 4. A summary of the cDNA and amino acid sequence alignments of the three hyaluronoglucosaminidase (HYAL) 1 clones obtained in this experiment with three previously reported hyaluronoglucosaminidase 1 cDNAs is illustrated. Sequence differences observed are underlined and emboldened. The inclusion of the 173 bp alternative exon in the 1552 bp HYAL 1 clone obtained introduced several alternative amino acids (emboldened and underlined) and a stop codon, truncating the C-terminal end of the resulting hyaluronoglucosaminidase protein.

3.5. Hyaluronoglucosaminidase mRNA measurement using real time rtPCR

Real time rtPCR values for GAPDH mRNA are illustrated in Fig. 6. Measurements for mRNA corresponding to the 1379 bp and 1552 bp transcript variants of hyaluronoglucosaminidase 1 and to hyaluronoglucosaminidase 2 are illustrated in Fig. 7. Results indicated that GAPDH gene expression decreased ($P < 0.01$) with day of gestation and was greater ($P < 0.05$) in placentae of the smallest fetus compared to the largest fetus, with no interaction. Two alternative control mRNAs (ribosomal protein large P2 and (-actin) were also measured and both showed a similar pattern to that of GAPDH (results not shown). This result rendered correction of hyaluronoglucosaminidase mRNA data using endogenous control mRNA data of ques-

tionable value, necessitating use of GAPDH residuals as a correction factor. GAPDH residual corrected gene expression of the 1379 bp form of hyaluronoglucosaminidase 1 indicated no effect of day and an effect ($P < 0.05$) of size of fetus with no interaction. Least squares means indicated that gene expression was greater in fetal placentae of the smallest compared to the largest fetuses. Analysis of GAPDH residual corrected gene expression of the 1552 bp form of hyaluronoglucosaminidase 1 resulted in effects of both day of gestation and size of fetus ($P < 0.05$ and $P < 0.01$, respectively), with no overall day by size of fetus interaction. However, separation of the overall day by size of fetus interaction into individual orthogonal contrasts indicated an interaction was present ($P < 0.05$) between early (day 25 and 45) and late (65, 85, and 105) gestation and size of fetus, indicating that expression differences were greater in fetal placentae of the smallest fetuses compared to the largest fetuses during late gestation compared to early gestation. Finally, GAPDH residual corrected hyaluronoglucosaminidase 2 mRNA data indicated effects of both day of gestation and size of the fetus (both $P < 0.01$), with no significant interaction. Results indicated that hyaluronoglucosaminidase 2 gene expression decreased as gestation advanced and was greater in fetal placentae of the smallest fetus compared to the largest fetus.

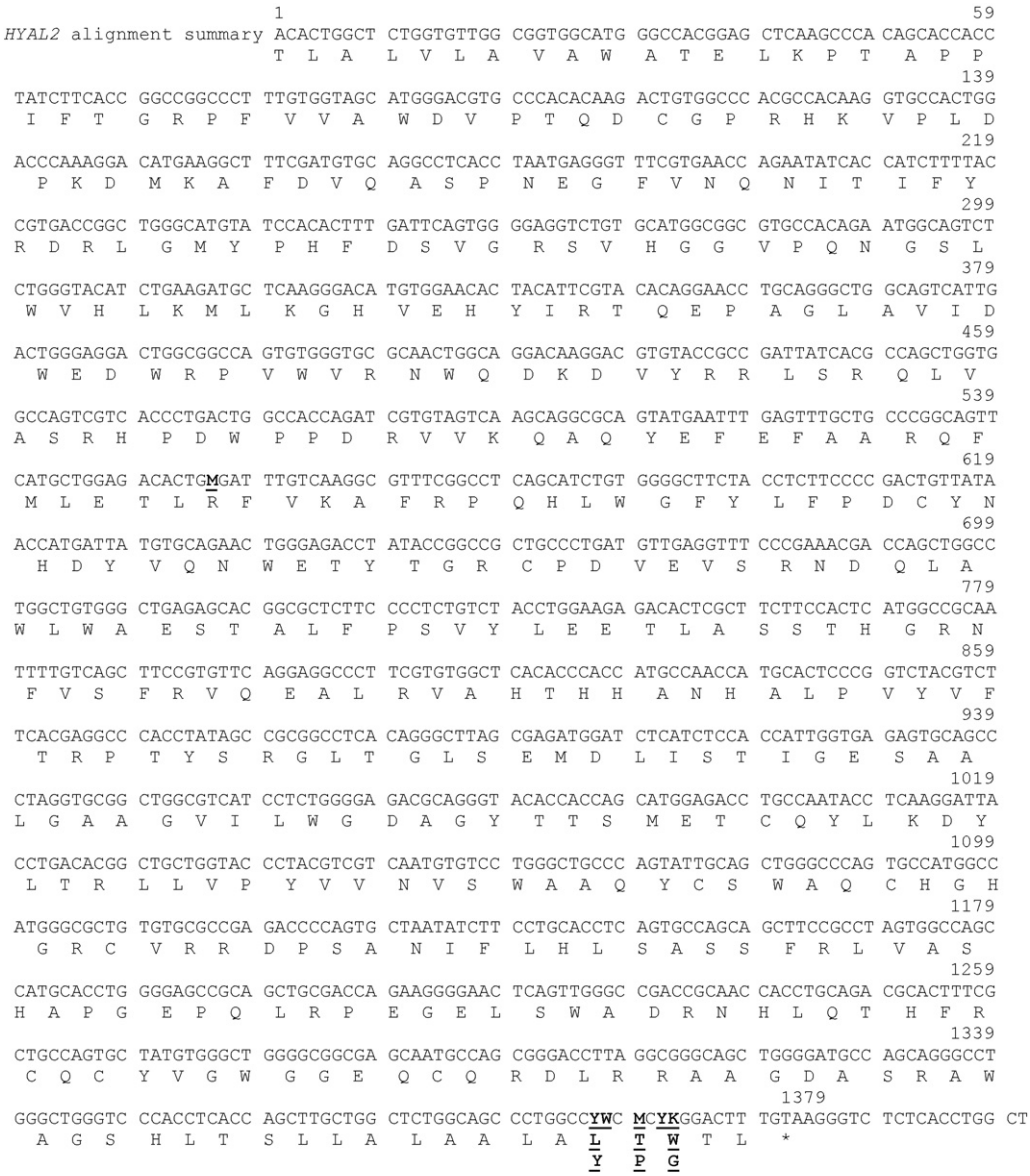

Fig. 5. A summary of the cDNA and amino acid sequence alignments of the hyaluronoglucosaminidase (HYAL) 2 clone obtained in this experiment with a previously reported hyaluronoglucosaminidase 2 cDNA is illustrated. Sequence differences observed are underlined and emboldened.

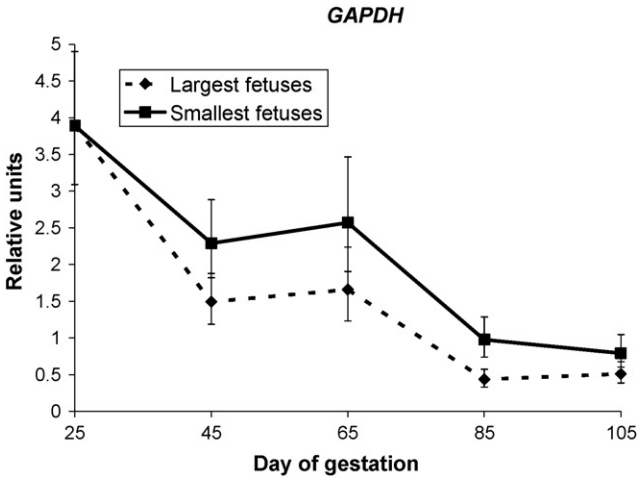

Fig. 6. Least squares means for GAPDH relative units during gestation in the pig are illustrated for fetal placenta associated with the smallest and largest fetuses in litters of control line gilts from this experiment. Both day ($P < 0.01$) and size ($P < 0.05$) effects were detected with no interaction, rendering use of these data to correct hyaluronoglucosaminidase mRNA expression questionable. Results of two other housekeeping mRNA (Ribosomal protein large P2 and (-actin) exhibited a similar decreasing pattern with day of gestation and similar effects of fetal size (not shown).

3.6. Hyaluronoglucosaminidase zymography

Preliminary analysis of fetal placental tissue homogenates using gel zymography indicated that visible clearing of hyaluronan impregnated gels occurred only at pH 3.5, no activity was visible at pH 5.0 or greater (results not shown). Two bands were visible, a minor band at 70 kd molecular weight and a major band at 55 kd molecular weight (not shown). Thus, analyses were performed by incubation of gels at pH 3.5. For both molecular weight forms, there were effects of both day of gestation and size of fetuses ($P < 0.05$), and no interaction was detected (Fig. 8). Both forms increased as gestation advanced and was greater in fetal placentae of the smallest fetuses compared to the largest fetuses.

4. Discussion

This is the first report localizing hyaluronan within the pig placenta and characterizing hyaluronoglucosaminidase gene expression and enzyme activity in the pig fetal placenta during gestation. Results indicate that fetal placental hyaluronan increases dramatically between day 25 and 45 of gestation, which is temporally associated with the major period of fetal placental structural development and with major fetal losses associated with limitations in uterine capacity (Freking et al., 2007). Furthermore, we have demonstrated that hyaluronan localizes to the fetal placental stroma surrounding the trophoblast-endometrial epithelial bilayer on the fetal side of the porcine placenta from day 45 to 105 of gestation. Three cDNAs corresponding to hyaluronoglucosaminidases 1 and 2 were cloned from fetal placental tissue, and gene expression analysis indicated that expression of these forms changed with advancing gestation. Amount of each hyaluronoglucosaminidase mRNA was greater in fetal placentae of the smallest fetuses compared to the largest fetuses, consistent with the proposed role of hyaluronoglucosaminidases in the degradation of hyaluronan necessary for enhanced microscopic fold development in fetal placenta of the smallest fetus compared to the largest fetus during late gestation. Fetal placental hyaluronoglucosaminidase activity followed a similar pattern, and two molecular weight forms were present. These results are consistent with a role for hyaluronan and hyaluronoglucosaminidases in the development of the pig placenta during pregnancy.

The dramatic increase in fetal placental hyaluronan content from day 25 to day 45 of gestation coincided with the development and increasing integrity of fetal placental tissue, again supporting the concept that hyaluronan is a major component. Results from the present study are similar to those of Steele and Froseth (1980) who measured hyaluronan in pig placental tissue from day 50 to 110 of gestation and reported a general increase in fetal placental hyaluronan content during this period. Results from the present study further indicate that a greater increase in fetal placental hyaluronan content occurs in fetal placentae of the smallest fetuses compared to the largest fetuses during very late gestation. Superficially, the increase in hyaluronan content in fetal placentae of the smallest fetuses appeared to contradict our earlier observation that the microscopic folds can penetrate the stroma in fetal placentae of the smallest fetuses. However, our hyaluronan localization results indicate that hyaluronan is located in the stromal tissue between the microscopic folds, and thus fetal placental hyaluronan content is not a measure of the remaining stroma available for placental development. In fact, hyaluronan measurements in the present study suggest that hyaluronan synthesis is increased in the fetal placentae of the smallest fetuses and one could hypothesize that this is a further compensatory mechanism. If this finding is confirmed, it suggests that microscopic fold development is a competition between increased development of the fetal placental stroma and increased penetration of the stroma by the epithelial bilayer. If stromal development is insufficient, this would limit increased

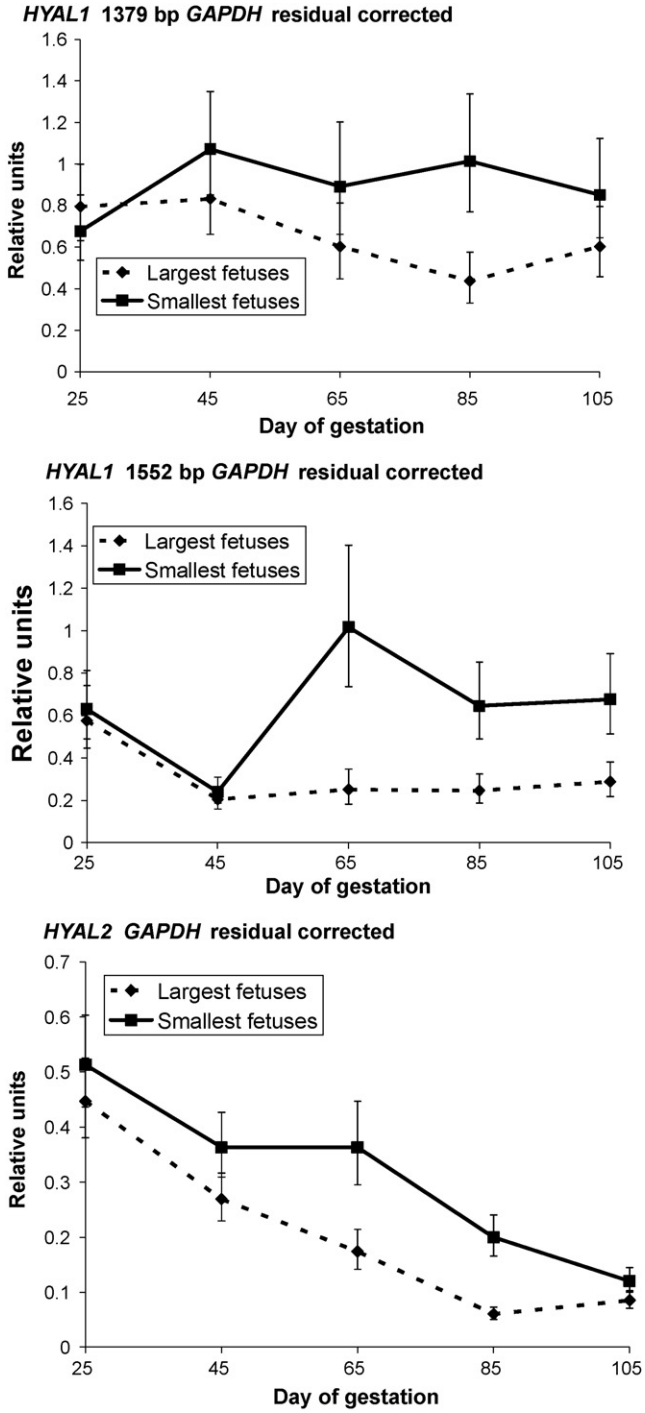

Fig. 7. Least squares means after correction with GAPDH residuals (residual variation remaining in GAPDH data after removing effects of day, size of fetus, and their interaction) for hyaluronoglucosaminidase (HYAL) 1, 1379 bp (top) and 1552 bp (center) forms and hyaluronoglucosaminidase (HYAL) 2 (bottom) are illustrated for fetal placental tissue collected throughout gestation from control line gilts. Means for the 1379 bp form of HYAL1 did not differ between day, but did differ between size of fetuses ($P = 0.01$). Means for the 1552 bp form of HYAL 1 differed between days ($P < 0.05$) and size ($P < 0.01$) of the fetuses, with no interaction. Means for HYAL 2 differed between days ($P < 0.01$) and size of fetuses ($P < 0.01$). Numbers of observations for each graph were 5 (day 25), 5 (day 45), 3 (day 65), 4 (day 85), and 4 (day 105).

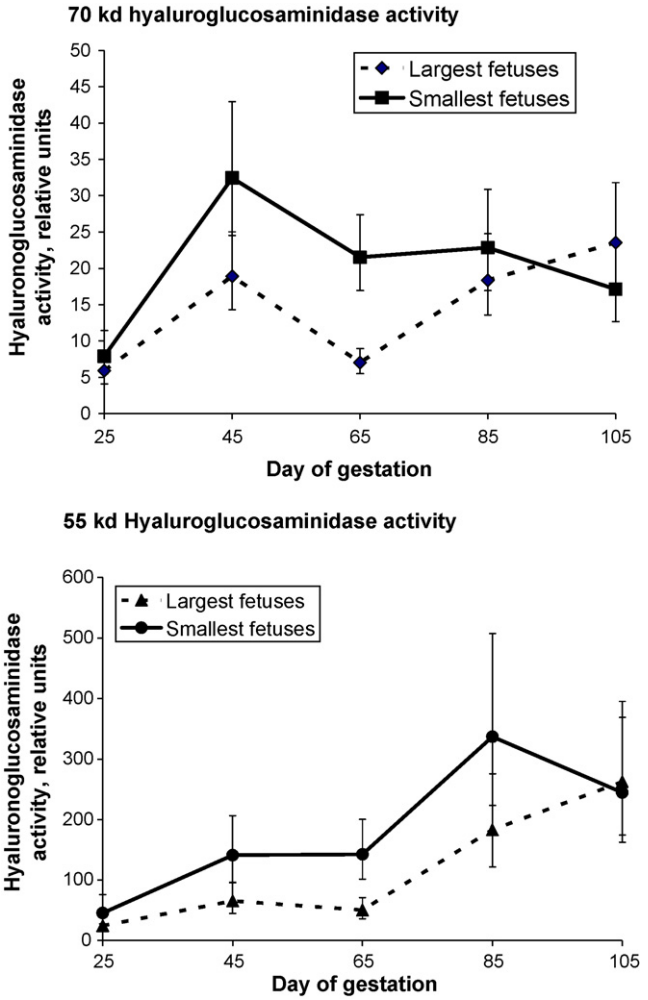

Fig. 8. Least squares means for hyaluronoglucosaminidase activity relative densitometry units for fetal placental tissue samples collected throughout pregnancy from the smallest and largest fetuses in litters of control line gilts from this experiment are illustrated. Day ($P < 0.05$) and size of fetus ($P < 0.05$) effects were detected for both the 70 kd and 55 kd forms. The 55 kd variant was the dominant form throughout gestation. Number of observations were 4 (day 25), 7 (day 45), 9 (day 65), 6 (day 85), and 6 (day 105).

fold development. This hypothesis suggests a role for stromal hyaluronan synthesis, as well as degradation. A cDNA for human hyaluronan synthase 1 and cDNAs for porcine hyaluronan synthase 2 and 3 are available in Genbank, but no cDNAs related to these sequences have been isolated from pig placental EST libraries currently available at the Dana Farber Cancer Institute Porcine Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig). However, recent microarray analysis of placental mRNA indicates that hyaluronan synthase 2 is present in fetal placenta from day 25 to 40 of gestation (BA Freking, personal communication).

Aside from being a component of fetal placental stromal tissue, hyaluronan has other biochemical characteristics that make it of interest to placental development. Hyaluronan plays roles in cancer metastasis, wound repair, tissue morphogenesis, and embryonic development (Heldin, 2003; Laurent and Fraser, 1992; Noble, 2002; Singley and Solursh, 1981; Spicer and Tien, 2004; Toole, 2000). Hyaluronan is composed of varying numbers of repeating β-linked units of glucuronic acid and N-acetyl-glucosamine (Laurent and Fraser, 1992; Toole, 2000). Normally, hyaluronan exists as large polymers in stable (i.e., nongrowing) tissues (Laurent and Fraser, 1992; Toole, 2000). However, through the actions of hyaluronoglucosaminidases, hyaluronan is depolymerized resulting in lower molecular weight forms. Different hyaluronoglucosaminidase enzymes appear to result in different molecular weight forms of hyaluronan (Chao et al., 2007; Lepperdinger et al., 1998, 2001; Stern, 2003; Strobl et al., 1998). Low molecular weight forms of hyaluronan have angiogenic properties (Liekens et al., 2001; West et al., 1985) and a variety of other activities that likely mediate the role of hyaluronan in tissue morphogenesis (Fieber et al., 2004; Rooney et al., 1993; Slevin et al., 2002; Taylor et al., 2004). Thus, degradation products resulting from hyaluronoglucosaminidase activity could contribute to capillary development. Several reports have indicated that vascular development of the pig fetal placenta contributes to the efficiency of nutrient transport (Biensen et al., 1998, 1999; Vonnahme et al., 2001; Wilson

et al., 1998). The combined morphogenic and angiogenic activity of degradation products of hyaluronan suggest the interesting possibility of coordinated epithelial bilayer fold development and capillary development adjacent to the bilayer, both mediated by hyaluronoglucosaminidase activity.

Both hyaluronoglucosaminidase 1 and 2 hydrolyze high molecular weight hyaluronan (Frost et al., 1997; Lepperdinger et al., 1998, 2001; Stern, 2003), are active at low pH, and *in vivo* activity requires CD44, but *in vitro* activity of hyaluronoglucosaminidase 1 does not (Harada and Takahashi, 2007). Thus, the two molecular weight forms of enzyme activity observed using zymography in the current experiment are most likely forms of hyaluronoglucosaminidase 1, due to activity after gel electrophoresis (which would disrupt CD44 interactions). Along with CD44, several other hyaluronan binding proteins have been described and are necessary for the various activities attributed to hyaluronan in biological processes (Knudson and Knudson, 1993; Turley et al., 2002). Receptor for HA-mediated motility (RHAMM) is another protein that binds hyaluronan and mediates cell movement during morphogenesis (Wang et al., 1996). There appears to be some overlap of function between these two binding proteins (Nedvetzki et al., 2004), however other functions controlled by these two proteins are distinct (Savani et al., 2001). Thus, a full understanding of the role of hyaluronan metabolism in placental development will require definition of the roles of hyaluronan binding proteins in the process.

The 1379 bp form of hyaluronoglucosaminidase 1 and the 1421 bp hyalurono-glucosaminidase 2 cDNAs obtained were nearly identical to those previously reported. The finding of similar nucleotide changes in hyaluronoglucosaminidase 1 sequences from other sources suggests that the minor differences in hyaluronoglucosaminidase 1 sequences are likely to be allelic changes. Likewise, the large sequence change in the C terminal end of hyaluronoglucosaminidase 2 is unlikely to be PCR artifact and may also correspond to an allelic difference. Hyaluronoglucosaminidase 2 is a phosphatidylinositol-linked protein (Rai et al., 2001), and the nucleotide changes resulting in amino acid differences in the C-terminal end of this protein may affect phosphatidylinositol linkage. Phosphatidylinositol linked proteins contain a hydrophobic signal sequence in the C-terminal region (Ikezawa, 2002; Sharom and Lehto, 2002). The leucine, threonine, tryptophan substitution of tyrosine, proline, glycine represents a relatively conservative change, however, it may influence the efficiency with which hyaluronoglucosaminidase 2 is attached to phosphatidylinositol in plasma membranes.

To our knowledge, the 1552 bp form of hyaluronoglucosaminidase 1 cDNA obtained from placental tissue differs from other cDNA isolated from pigs. The retained intronic region is similar to a region retained in a previously reported porcine liver hyaluronoglucosaminidase 1 mRNA available in GenBank (AK232421), although the porcine liver hyaluronoglucosaminidase 1 mRNA contains a further exon not included in the 1552 bp hyaluronoglucosaminidase 1 cDNA. A human hyaluronoglucosaminidase 1 cDNA with a similar structure has been isolated from pooled spleen/pancreas tissues (GenBank accession # BC025774.1). These differences are the result of differential splicing of the hyaluronoglucosaminidase 1 gene, which has been described by others (Lokeshwar et al., 2002). Inclusion of putative intron 5 in the 1552 bp form introduced a premature stop codon, resulting in a putative truncated hyaluronoglucosaminidase 1 protein. According to Chao et al. (2007), the deleted region in the putative amino acid sequence coded by the 1552 bp form corresponds to an EGF-like domain of the hyaluronoglucosaminidase 1 protein and does not involve the active site. Chao et al. (2007) suggested that this region controls binding to cofactors and may regulate activity of the enzyme, including binding to CD44. The consequences of deleting this region on the control of hyaluronoglucosaminidase activity remain to be determined.

The real time rtPCR results of expression analysis of all three mRNAs for hyaluronoglucosaminidase indicated differences in gene expression associated with fetal size. In addition, expression of hyaluronoglucosaminidase 2 and the 1552 bp form of hyaluronoglucosaminidase 1 indicated day of gestation effects. These results are in agreement with hyaluronoglucosaminidase activity results generated using zymography. This is consistent with a role for hyaluronoglucosaminidase in the fold development during gestation and in the greater development of the folds that occurs in placentae of small fetuses reported (Vallet and Freking, 2007).

In summary, results from the present study indicate that hyaluronan is a component of the stromal tissue of the developing pig fetal placenta, and changes in hyaluronoglucosaminidase gene expression and protein are consistent with a role for hyaluronoglucosaminidases in placental structural development and angiogenesis. It is possible that polymorphisms in either the hyaluronoglucosaminidase 1 or 2 genes, by virtue of changes in fetal placental hyaluronoglucosaminidase activity during pregnancy, may influence placental development, placental efficiency, litter size, and preweaning mortality in pigs.

Acknowledgements

Research supported by USDA-ARS CRIS Project No. 5438-31000-084-00D. The authors wish to acknowledge the technical help of A. Paus, S. Hassler, and T. Gramke in the performance of these experiments and L. Parnell for help in preparation of the manuscript.

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