Comparative Organization and Gene Expression Profiles of the Porcine Pseudoautosomal Region

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Abstract

The pseudoautosomal region (PAR) has important biological functions in spermatogenesis, male fertility and early development. Even though pig (*Sus scrofa*, SSC) is an agriculturally and biomedically important species, and its genome is sequenced, current knowledge about the porcine PAR is sparse. Here we defined the PAR in SSCXp/Yp by demarcating the sequence of the pseudoautosomal boundary at X:6,743,567 bp in intron 3–4 of *SHROOM2* and showed that *SHROOM2* is truncated in SSCY. Cytogenetic mapping of 20 BAC clones containing 15 PAR and X-specific genes revealed that the pig PAR is largely collinear with other mammalian PARs or Xp terminal regions. The results improved the current SSCX sequence assembly and facilitated distinction between the PAR and X-specific genes to study their expression in adult and embryonic tissues. A pilot analysis showed that the PAR genes are expressed at higher levels than X-specific genes during early development, whereas the expression of PAR genes was higher at day 60 compared to day 26, and

higher in embryonic tissues compared to placenta. The findings advance the knowledge about the comparative organization of the PAR in mammals and suggest that the region might have important functions in early development in pigs.

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The mammalian pseudoautosomal region (PAR) is a region of true sequence homology between the X and the Y chromosome [Galtier, 2004; Blaschke and Rappold, 2006; Raudsepp et al., 2012]. Sequences in the PAR are diploid, undergo recombination in males and females, and are not subject to dosage compensation by X inactivation in females [Ellis and Goodfellow, 1989; Brown and Greally, 2003]. The physical domain of the PAR lies between the terminal ends of the sex chromosomes and the pseudoautosomal boundary (PAB) – a border across which sequence homology between the sex chromosomes reduces and recombination stops [Blaschke and Rappold, 2006; Flaquer et al., 2008].

The PAR is necessary for sex chromosome pairing, recombination and proper segregation in male meiosis [Ellis and Goodfellow, 1989; Blaschke and Rappold, 2006; Mangs and Morris, 2007; Flaquer et al., 2008] and is

therefore critical for spermatogenesis, male fertility and the formation of a normal zygote. Additionally, the gene content of the PAR might also have an important role during development as evidenced by modeling abnormal early development using aneuploid embryonic [Urbach and Benvenisty, 2009] and induced pluripotent stem cells [Li et al., 2012] and by comparing the incidence of viable XO individuals across species with different PARs [Raudsepp et al., 2012]. Furthermore, because the PAR is a recombination hotspot [Filatov, 2004; Flaquer et al., 2008, 2009] and an elevated recombination rate has been associated with DNA methylation, it is possible that some PAR genes might be subject to genomic imprinting [Luedi et al., 2007; Sigurdsson et al., 2009].

Despite the important biological properties of the PAR and the availability of draft sequence assembly for the X chromosome in most domestic and many wild mammals (Ensembl, <http://www.ensembl.org/index.html>; UCSC, <http://genome.ucsc.edu/>), the current knowledge about the organization of the eutherian PAR is limited. The region is sequenced or relatively well-studied only in humans/primates [Skaletsky et al., 2003; Ross et al., 2005; Hughes et al., 2010, 2012] and mice [Ellison et al., 1996; Perry et al., 2001; Alföldi, 2008]. In other species, a variable degree of information for the PAR is available [reviewed by Raudsepp et al., 2012]: the approximate physical domain and gene content of the region is known for most domestic species and predicted for the porpoise [Van Laere et al., 2008] and armadillos [Sciurano et al., 2012]. However, the exact sequence of the PAB has so far been determined only in cattle/bovids [Van Laere et al., 2008]. Collectively, the data indicate that the PAR varies in size and gene content between species, suggesting that orthologous X-linked genes might be pseudoautosomal in one, and non-pseudoautosomal in another species.

Even though pig (*Sus scrofa*, SSC) is considered one of the most important agricultural species, as well as an important biomedical model for humans [Kuzmuk and Schook, 2011; Swine Genome Sequencing Consortium, 2012], current knowledge of the porcine PAR lags behind those of other domestic animals. While the recently published porcine genome sequence assembly [Swine Genome Sequencing Consortium, 2012] includes sequence information for 144 Mb of the pig X chromosome, the PAB is not defined, thus precluding distinction between the PAR and X-specific sequences. So far, the confirmed PAR loci in pigs include cytogenetically mapped genes *KALI*, *PRKX* and *STS* [Quilter et al., 2002], a marker pCMS14 [Ellegren et al., 1994] and a paternally imprinted QTL for loin weight [Duthie et al., 2009].

In this study we generate an ordered physical map of the porcine PAR and precisely demarcate the region by delineating the PAB sequence. Additionally, we carry out a pilot study comparing the expression of selected PAR and X-specific genes in adult and embryonic porcine tissues.

Materials and Methods

Selection of Genes to Demarcate the Pig PAR and PAB

Putative pig PAR genes were selected using the following sources: (i) the sequence assembly of the pig X chromosome (Sscrofa10.2; Ensembl, <http://www.ensembl.org/index.html>), (ii) tentative location of the PAB in pigs [Raudsepp et al., 2012], and (iii) the gene content of human [Ross et al., 2005], horse [Raudsepp and Chowdhary, 2008b], cattle [Das et al., 2009] and dog [Young et al., 2008] PARs for comparative information. As a result, 13 pig genes were selected to tag the terminal, middle and distal regions of the putative porcine PAR, as well as the adjacent X-specific region (table 1).

Primer Design and Optimization

Overgo and PCR primers for the putative PAR genes (online suppl. table 1; for all online supplementary material, see www.karger.com/doi/10.1159/000351310) were designed from the sequences of the pig genome assembly Sscrofa10.2 and from Roche 454 sequences of a flow-sorted SSCY (our unpublished data; flow-sorted Y was kindly provided by M.A. Ferguson-Smith). PCR primers were also designed for 20 BAC end sequences (BES) (online suppl. table 2; see below). Overgo primers were designed manually using NCBI guidelines (<http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechOvergo.shtml>), and Primer3 software [Rozen and Skaletsky, 2000] was used for constructing the PCR primers. The latter were optimized on male and female pig genomic DNA.

Pig CHORI-247 BAC Library Screening and BAC DNA Isolation

Overgo primers were radioactively labeled and hybridized to male pig (ID#Duroc 243-2) genomic BAC library CHORI-247 (<http://bacpac.chori.org/library.php?id=473>) high-density filters as described elsewhere [Gustafson et al., 2003; Avila et al., 2012]. Positive BAC clones were identified and picked from the library. The BAC clones corresponding to individual genes were identified by PCR using gene-specific primers (online suppl. table 1) and BAC cell lysates as a template. Isolation of DNA from individual BACs was carried out with the Plasmid Midi Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of BAC DNA were evaluated by gel electrophoresis and Nanodrop spectrophotometry.

Chromosome Preparations and Fluorescence in situ Hybridization

Fluorescence in situ hybridization (FISH) was used to determine whether the genes belonged to the PAR, i.e. mapped to both the X and Y chromosomes, or were sex chromosome-specific. Metaphase and interphase chromosome spreads were prepared

Table 1. Cytogenetically mapped PAR and X-specific genes and markers (in map order)

Gene/marker symbol	Type of sequence	Map location		Sequence in SSCX (Sscrofa10.2)	Sequence in SSCY; GenBank accession No.	Corresponding CHORI-247 BACs
		FISH	Sscrofa10.2			
<i>CRLF2</i>	gene	<i>Xp/Yp</i>	<i>Un</i>	GL893451.1: 12,561–27,485	n/a	579F8, 640H13
<i>CSF2RA</i>	gene	<i>Xp/Yp</i>	<i>Un</i>	GL896316.1: 21–12,375	contig 3607; SAMN01815296	579F8, 640H13
<i>SLC25A6</i>	gene	<i>Xp/Yp</i>	<i>Xqter</i>	X: 113,743,301–113,746,206	n/a	395M18, 635O11
<i>ARSF</i>	gene	<i>Xp/Yp</i>	<i>Un</i>	GL894117.1: 2,759–20,486	contig 5800; SAMN01815296	339A1, 640O13
<i>MXRA5</i>	gene	<i>Xp/Yp</i>	<i>Xqter</i>	X: 143,912,804–143,940,427	contig 3441; SAMN01815296	48N19, 515M22
<i>PRKXY</i>	gene	<i>Xp/Yp</i>	<i>Xqter</i>	X: 144,130,681–144,164,818	contig 8474; SAMN01815296	26B13, 108N16
<i>SW949^a</i>	microsatellite	<i>Xp/Yp</i>	<i>Xqter</i>	X: 144,124,593–144,124,775	n/a	26B13, 108N16
<i>HDHD1</i>	gene	Xp/Yp	Xpter	X: 4,016,053–4,053,447	contig 7092; SAMN01815296	436O11
<i>PNPLA4</i>	gene	Xp/Yp	Xpter	X: 4,788,455–4,823,753	contig 1004; SAMN01815296	435B16
<i>KAL1</i>	gene	Xp/Yp	Xpter	X: 5,366,015–5,562,572	contig 4917; SAMN01815296	152P13
<i>TBL1X</i>	gene	Xp/Yp	Xpter	X: 6,423,780–6,538,073	n/a	6D24, 14P3, 703A8, 204D23
<i>GPR143</i>	gene	Xp/Yp	Xpter	X: 6,541,513–6,575,981	n/a	6D24, 14P3, 703A8, 204D23
<i>SHROOM2</i>	gene	Xp/Yp	Xpter	X: 6,595,889–6,801,781	contig 00202; SAMN01815296	82K13
<i>CH242-24K2.1</i>	lncRNA gene	Xp	Xpter	X: 6,959,367–6,960,018	n/a	94N3, 326C23
<i>CH242-24K2.2^a</i>	lncRNA gene	Xp	Xpter	X: 7,015,573–7,018,840	n/a	94N3, 326C23

Discrepancies between FISH and sequence maps are in bold italic. Un = unassigned; lncRNA = long non-coding RNA.

^a Markers found by BES analysis.

from short-term peripheral blood lymphocyte cultures of a male pig according to standard protocols [Raudsepp and Chowdhary, 2008a]. The BACs containing putative PAR genes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP using Biotin- or DIG-Nick Translation Mix (Roche) and hybridized in combinations of 2 or 3 to metaphase and interphase chromosomes as previously described [Raudsepp and Chowdhary, 2008a]. Relative order of closely located PAR genes was determined by interphase FISH using multiple 3-probe combinations and dual color experiments. Images for a minimum of 10 metaphase spreads and 30 interphase cells were captured for each experiment, and analyzed with a Zeiss Axioplan2 fluorescence microscope equipped with Isis V5.2 (MetaSystems GmbH) software.

BAC End Sequencing, Chromosome Walking and Sequence Tagged Site Content Mapping

End sequencing of the BAC clones was carried out using the standard T7 (5'-CGCTAATACGACTCACTAT AGGGAGA-3') and SP6 (5'-CCGTCGACATTTAGGTGACACTATAG-3') primers and BigDye terminator chemistry (Applied Biosystems). The BES were quality-trimmed, aligned for possible overlaps using Sequencher V 4.8 software (Gene Codes Corporation), evaluated for the presence of repeats (RepeatMasker; <http://www.repeatmasker.org/>), and BLAST (Ensembl) analyzed to identify corresponding sequences in the pig genome assembly Sscrofa10.2 (table 2). Non-repetitive BES were used to design primers (see above) for additional BAC screening and for sequence tagged site (STS) content mapping. Chromosome walking was carried out around the putative PAB using BES primers of clone 82K13 which contains the *SHROOM2* gene. New overlapping clones were isolated from the library and arranged into a contig (fig. 1) by STS content mapping (reciprocal PCRs with BES primers). STS content mapping was also used to detect possible overlaps between all other BAC clones.

Sequence Analysis of the PAB

Demarcation of the PAB sequence was carried out by aligning SSCX sequences from 1 to 7.5 Mb (Sscrofa10.2), sequence contigs of the flow-sorted Y chromosome (our unpublished data) and all BES developed in this study. Alignments and sequence analyses were done with Sequencher 4.8 software, BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and RepeatMasker.

Tissues Samples, DNA and RNA Extraction

Genomic DNA samples from adult male and female pigs and porcine embryos were obtained from peripheral blood or embryonic tissues (see below) using standard protocols [Birren et al., 1997]. For gene expression analysis, 8 adult tissues (brain, heart, kidney, lung, liver, spleen, skeletal muscle, and testis) were collected from necropsies. Embryonic tissues were procured via blunt dissection of uterine horns after separation of the fetus and the placenta. Placenta and whole embryos were collected from three 26-day pregnancies (males), while placenta and a panel of 10 tissues (brain, heart, gonads, kidney, liver, lung, skeletal muscle, spleen, small intestine, and stomach, representing ectoderm, mesoderm, and endoderm) were obtained from three 60-day-old embryos (2 males and 1 female). The 26-day and 60-day time points were selected because pig embryos complete organ differentiation after 75 days [McPherson et al., 2004]. The embryos were sexed by PCR (data not shown) using porcine *SRY* primers (5'-3' F: GTGAAGACAACCTTTC-CAAACG, R: GCCACTCTCTCTACCACTTCC; product size 230 bp). The tissues were stored in RNAlater (Ambion) at -80°C until use. Total RNA from tissues was isolated with RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The samples were treated with DNaseI (Ambion), quality checked with BioAnalyzer 2100 (Agilent) and quantified with a Nanodrop spectrophotometer.

Ethics Statement

Procurement of porcine tissues was in accordance to the United States Government Principles for the Utilization and Care of

Table 2. Summary information for the CHORI-247 BACs that were used for mapping the PAR and PAB

BAC ID	Gene content	FISH	BES-SP6: repeats and BLAST	SP6 GenBank accession	BES-T7: repeats and BLAST	T7 GenBank accession
6D24	<i>GPR143, TBL1X</i>	Xp/Yp PAR	pig CH242-144C16 on chromosome X (100%)	JY172533	pig CH242-236H7 on chromosome X (100%)	JY172534
14P3	<i>GPR143, TBL1X</i>	Xp/Yp PAR	pig CH242-144C16 on chromosome X (100%)	JY172535	LINE/L1; WTSI_1061-62J14 on chromosome Y (966, 100%); pig CH242-236H7 on chromosome X (100%)	JY172536
26B13	<i>PRKXY</i> , SW949	Xp/Yp PAR	pig CH242-62E3 on chromosome X (100%); pig CH242-266M18 on chromosome X (94%)	JY172537	pig CH242-62E3 on chromosome X (100%)	JY172538
48N19	<i>MXRA5</i>	Xp/Yp PAR	no significant similarity	JY172539	pig CH242-228K3 on chromosome X (100%)	JY172540
82K13	<i>SHROOM2</i>	Xp/Yp PAR	SINE/tRNA-Glu; pig CH242-236H7 on chromosome X (100%); cattle <i>SHROOM2</i> , 2e-16	JY172541	LINE/L1; pig CH242-156O11 on chromosome X (100%)	JY172542
94N3	<i>PIG CH242-24K2.1, PIG CH242-24K2.2</i>	Xp	LINE/L1; pig CH242-24K2 on chromosome X (97%)	JY172543	SINE/tRNA-Glu; pig CH242-156O11 on chromosome X (99%)	JY172544
108N16	<i>PRKXY</i>	Xp/Yp PAR	SINE/tRNA-Glu; pig CH242-266M18 on chromosome X (100%); pig CH242-231E5 on chromosome X (100%)	JY172545	SINE/tRNA-Glu; no significant similarity	JY172546
152P13	<i>KAL1</i>	PAR	pig CH242-507C8 on chromosome X (99%), pig <i>KAL1</i> , 6e-55	JY172547	LTR/ERV1; pig CH242-507C8 on chromosome X (99%)	JY172548
204D23	<i>GPR143, TBL1X</i>	Xp/Yp PAR	LINE/L1; pig WTSI_1061-5C19 on chromosome Y (100%); pig CH242-144C16 on chromosome X (100%)	JY172549	low complexity, SINE/tRNA-Glu; pig CH242-236H7 and pig CH242-156O11 on chromosome X (100%), cattle <i>SHROOM2</i> , 8e-09	JY172550
326C23	<i>CH242-24K2.1, CH242-24K2.2</i>	Xp	pig CH242-24K2 on chromosome X (96%)	JY172551	SINE/tRNA-Glu, LINE/L1; pig CH242-156O11 on chromosome X (96%)	JY172552
339A1	<i>ARSF</i>	Xp/Yp PAR	SINE/MIR; <i>Homo sapiens</i> 959-kb contig between <i>AML1</i> and <i>CBRI</i> on chromosome 21q22, 4e-38	JY172553	<i>Sus scrofa</i> clone KVL9136 microsatellite sequence, 2e-54	JY172554
395M18	<i>SLC25A6</i>	Xp/Yp PAR	SINE/tRNA-Glu, LINE/L1, (CAAA) ₈ ; pig CH242-266M18 on chromosome X (81%)	JY172555	no significant similarity	JY172556
435B16	<i>PNPLA4</i>	Xp/Yp PAR	pig CH242-236E18 on chromosome X (100%)	JY172557	pig CH242-207C4 on chromosome X (100%)	JY172558
436O11	<i>HDHD1A</i>	Xp/Yp PAR	LTR, SINE/tRNA-Glu; pig E-95J7 on chromosome X (100%)	JY172559	LTR/ERV1-MaLR, SINE/tRNA-Glu; pig CH242-88J14 on chromosome X (97%)	JY172560
515M22	<i>MXRA5</i>	Xp/Yp PAR	pig CH242-266M18 on chromosome X (100%)	JY172561	LINE/L1; no significant similarity	JY172562
579F8	<i>CRLF2, CSF2RA</i>	Xp/Yp PAR	<i>Sus scrofa</i> mRNA, clone: ITT010044E04, expressed in intestine, 6e-31	JY172563	SINE/tRNA-Glu, (CA) ₁₈ ; no significant similarity	JY172564
635O11	<i>SLC25A6</i>	Xp/Yp PAR	LINE/L1; no significant similarity	JY172565	LTR/ERV1-MaLR; no significant similarity	JY172566
640H13	<i>CRLF2, CSF2RA</i>	Xp/Yp PAR	LTR/ERV1; no significant similarity	JY172567	SINE/tRNA-Glu, LTR/ERV1; <i>Sus scrofa</i> mRNA, clone: ITT010044E04, expressed in intestine, 4e-12	JY172568
640O13	<i>ARSF</i>	Xp/Yp PAR	pig CH242-215D15 on chromosome 7 (76%)	JY172569	LTR/ERV1; pig CH242-228K3 on chromosome X (100%)	JY172570
703A8	<i>GPR143, TBL1X</i>	Xp/Yp PAR	SINE/tRNA-Glu; pig WTSI_1061-35G12 on chromosome Y (100%); pig CH242-144C16 on chromosome X (100%)	JY172571	CH242-236H7 on chromosome X (98%); <i>Sus scrofa</i> mRNA, clone: ADR010054D04, expressed in adrenal gland, 0.0	JY172572

BES = BAC end sequence.

Vertebrate Animals Used in Testing, Research and Training and was approved by Animal Use Protocol AUP No. 2008-103 at Texas A&M University.

Gene Expression Analysis

Primers for reverse transcriptase (RT) and quantitative RT-PCR (qRT-PCR) were designed from exonic sequences of 15 genes: 9 from the PAR and 6 from X-specific regions (online suppl. ta-

ble 3). Expression profiles of 8 genes (5 PAR and 3 X-specific) were analyzed by RT-PCR in a panel of adult porcine tissues. RT-PCR reactions were carried out in 15 µl volume using Superscript III One-Step RT-PCR System and Platinum TaqDNA polymerase (Invitrogen) as previously described [Das et al., 2010; Paria et al., 2011]. Genomic controls were analyzed simultaneously with the RNA samples and housekeeping genes *ACTB* and *GAPDH* were used as internal controls. Next, expression levels of all 15 genes

Fig. 1. Gene map of the pig PAR and Xp. **A** Sequence map of SSCXp from Sscrofa10.2 draft assembly (Ensembl); arrows with numbers indicate gaps in the current assembly. **B** FISH map of the pig PAR and adjacent X-specific region. **C** Metaphase and interphase images showing the location or relative order of PAR genes. Note that *SCL25A6* (red) and *MXRA5* (green) at Xp/Yp (far left) are assigned to Xq in Sscrofa10.2.

were compared between the 26-day- and 60-day-old placenta and embryos/embryonic tissues by qRT-PCR. The qRT-PCR assays were carried out with SYBR Green PCR kit (Roche) on a LightCycler® 480 (Roche Diagnostics) according to the manufacturer's instructions and the results were analyzed with LightCycler 480 Software v1.5. The genes were tested in duplicate reactions in triplicate experiments and the expression of the PAR and X-specific genes was analyzed by absolute quantification method as described elsewhere [Bustin, 2000; Nolan et al., 2006] (Illumina Technical Note: http://www.illumina.com/Documents/products/technote/tech-note_eco_absolutequant_sybr_green.pdf). Standard curves for each gene were prepared from cDNA of known template concentrations, such as 200, 100 and 50 ng. Gene expression (copies/μl) was calculated based on the cDNA concentration (ng/μl), the lengths of the DNA fragments (bp; e.g. *AFF2*: 199 bp, *KAL1*: 194 bp, etc., see online suppl. table 3), the number of targets per DNA fragment (copies), the Avogadro constant ($NA = 6.0221415 \times 10^{23} \text{ mol}^{-1}$), and the average weight of a double-stranded base pair (650 kDa). The expression of any unknown sample was then determined by interpolation of the PCR signal into this standard curve and presented as the number of transcripts (copies/μl) in test samples.

Results

A Cytogenetic Map of the Pig PAR

Twenty BAC clones corresponding to 13 putative pig PAR genes (tables 1, 2) were isolated from the CHORI-247 BAC library. BES analysis identified 2 additional markers: a microsatellite SW949 close to *PRKXY* in clone 26B13 and 2 long non-coding (lnc) RNA genes *CH242-24K2.1/2.2* in clones 94N3 and 326C23 (tables 1, 2). Mapping of the 20 BAC clones to male pig metaphase spreads by FISH

assigned 18 clones and 13 genes/markers to the PAR at Xpter/Ypter, and 2 clones with the lncRNA genes to Xpter only, as outlined in figure 1B. Relative order of closely located BACs was determined by interphase FISH (fig. 1C). A linearly ordered cytogenetic map with 15 genes/markers was thus constructed for the porcine PAR and the adjacent X-specific region (fig. 1B, C). Comparison of the FISH map with the pig genome sequence assembly (fig. 1A) showed that the 6 most distal PAR genes, i.e. *CRLF2*, *CSF2RA*, *SLC25A6*, *ARSF*, *MXRA5*, *PRKXY*, and marker SW949, were assigned to contigs Un or to Xqter in Sscrofa10.2 (table 1; fig. 1). However, these genes were unambiguously mapped to Xpter/Ypter by FISH (fig. 1C). Further, alignment of PAR gene sequences, PAR BES, Sscrofa10.2 and sequence contigs of a flow-sorted pig Y chromosome (our unpublished data) showed that 8 PAR genes were present in both the X and the Y chromosome (table 1), thus confirming the FISH results. The results also indicated that the PAB lies within or immediately next to BAC 82K13, which contains *SHROOM2* and is the most proximal clone that FISH mapped to both sex chromosomes (figs. 1, 2).

We infer that the pig has a single PAR which is located terminal in the short arms of the sex chromosomes, has a gene content and order very similar to other mammalian PARs or Xp terminal regions [Raudsepp et al., 2012] and extends approximately up to *SHROOM2*.

Demarcation of the PAB Sequence

In order to precisely define the PAB, we constructed a contig of overlapping BAC clones across the region

Fig. 2. Demarcation of the porcine PAB. Red vertical arrow from **A** to **C** shows the increasing resolution of the PAB. **A** BAC contig map spanning the putative PAB in SSCX. Green and orange rectangles denote STS markers that were used to align the BAC clones. The location of known genes is denoted by green circles. The PAB is narrowed down to a 128,154-bp region between markers 204D23T7 and 94N3SP6. **B** Location of the PAB (black circle) in *SHROOM2* intron 3–4. **C** Sequence alignment of PAB-X and PAB-Y in *SHROOM2* intron 3–4: 100% alignment in the PAR (left, green shade) and reduced sequence similarity in sex chromosome-specific regions (right; red and blue shades).

around *SHROOM2* (fig. 2A). The contig comprised 7 clones, 4 of which (14P3, 6D24, 703A8 and 204D23) were located in the PAR and 2 (94N3 and 326C23) in the X-specific region. BAC clone 82K13 containing the *SHROOM2* gene bridged the 2 regions. STS content analysis and BES (table 2) alignments with SSCX sequence assembly (Sscrofa 10.2) narrowed the putative PAB-X down to a 128,154-bp region between BES 204D23-T7 and 94N3-SP6 (fig. 2A; online suppl. fig. 1). In Sscrofa10.2 this sequence corresponded to X:6,692,098–6,820,252 bp and contained the proximal part of *SHROOM2*, from exon 2 onwards (fig. 2B). Because chromosome walking did not identify any Y chromosome BACs, the sequence of the putative PAB-X was aligned with the sequences of a flow-sorted Y chromosome (table 1; our unpublished data). We identified Y contig 00202 (828 bp) which showed 97–100% similarity to a part of *SHROOM2* intron 3–4, whereas sequence similarity abruptly dropped to less than 50% from position X:6,743,567 bp onwards, thus demarcating the porcine PAB (fig. 2C; online suppl. fig. 1). Notably, SINE/tRNA^{Glu} elements spanned the PAB in both X and Y chromosomes as revealed by RepeatMasker analysis of the PAB sequences. Alignment of the genomic sequence of *SHROOM2* (Ensembl) with the assembled sequences of flow-sorted SSCY (our unpublished data) and with the sequences of SSCY fosmid library WTSI_1061 (NCBI, <http://www.ncbi.nlm.nih.gov/nuccore?term=Pig%20DNA%20sequence%20from%20clone%20WTSI>) showed no evidence of the presence of *SHROOM2* exons 4–10 in SSCY.

We conclude that the porcine PAB is located in intron 3–4 of *SHROOM2*, so that the first 3 exons of the gene are located in the PAR, the remaining exons (4–10) are X-specific, and the gene is truncated in the Y chromosome. Based on the sequence position of *SHROOM2* in the current X chromosome sequence assembly, we propose that the porcine PAR is at least 6.7 Mb in size.

Expression Analysis of the Pig PAR and X-Specific Genes

Demarcation of the PAB not only determined the size and the gene content of the PAR, but also indicated which genes in pigs are functionally diploid on both sex chromosomes, and which undergo X inactivation in females [Brown and Greally, 2003]. Such discrimination might be important because recent studies in human embryonic and induced pluripotent stem cells show that functional diploidy for the PAR genes might be critical for normal embryonic development [Urbach and Benvenisty, 2009; Li et al., 2012]. That is why we carried out a pilot study profiling the expression of the PAR and X-specific genes in pigs.

As a first step, expression of 5 randomly selected PAR genes and 3 X-specific genes was studied by RT-PCR in a panel of 8 adult porcine tissues, i.e. brain, heart, kidney, lung, liver, spleen, skeletal muscle, and testis (online suppl. fig. 2). All genes, regardless of their location, showed nearly ubiquitous expression. Of these, the X-specific *NOX1* had an overall low expression level compared to other genes, and the transcription of PAR genes

Fig. 3. Expression profiles of selected PAR and X-specific genes in pig embryo and placenta at days 26 and 60 of development. y-axis = expression level; x-axis = linear order of the studied genes in the pig X chromosome and the tissues studied per each gene.

Table 3. Expression of pig PAR (bold italic) and X-specific (italic) genes in early development

Gene symbol	26 days		60 days										
	placenta	embryo	placenta	brain	heart	gonads	kidney	spleen	skeletal muscle	lung	liver	small intestine	stomach
<i>SHOX</i>	5.09	64.67	1.02	54.00	21.46	15.54	78.34	3.91	94.35	55.97	8.81	21.11	0.90
<i>SLC25A6</i>	0.26	1.05	2.04	1.15	0.89	0.61	0.12	0.60	3.67	2.52	0.04	0.18	0.88
<i>MXRA5</i>	0.84	0.01	0.02	14.39	0.12	0.54	0.39	0.16	0.19	0.88	0.28	0.20	1.20
<i>PRKXY</i>	0.28	0.42	0.05	0.50	0.19	0.86	0.41	0.25	0.15	0.22	0.86	0.29	0.56
<i>HDHD1</i>	19.23	32.81	51.89	4.86	69.43	15.32	83.00	12.66	6.34	6.57	57.98	112.60	3.44
<i>PNPLA4</i>	28.21	2.37	1.28	119.29	1.19	3.01	16.06	0.17	1.71	2.61	1.61	2.02	0.20
<i>KAL1</i>	3.85	56.55	21.13	1.07	6.39	1.54	9.61	31.12	5.26	0.78	26.74	14.28	1.27
<i>GPR143</i>	5.87	29.34	43.81	0.47	12.41	3.50	3.89	8.90	120.40	17.47	1.75	10.34	2.01
<i>SHROOM2</i>	4.82	21.43	30.38	1.92	47.61	5.85	1.80	12.76	7.41	7.25	0.74	4.91	25.05
<i>WWC3</i>	0.04	0.02	0.02	0.03	0.01	0.06	0.04	0.01	0.05	0.03	0.48	0.06	4.39
<i>MID1</i>	0.01	0.02	0.49	0.01	0.05	0.13	0.04	1.77	0.01	0.04	0.14	0.10	0.57
<i>ARI</i>	0.01	0.25	0.94	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.10
<i>NOX1</i>	0.29	0.55	0.05	0.24	0.34	0.85	3.32	0.13	6.73	3.43	0.16	0.74	8.10
<i>AFF2</i>	22.58	11.36	16.75	28.80	14.09	39.12	30.51	24.00	22.86	32.03	30.51	45.35	0.82
<i>IDS</i>	0.49	0.42	0.42	0.18	0.21	0.22	0.37	0.82	0.25	0.30	0.14	0.18	0.27

Absolute expression (copies/μl) of each gene across the tissue panel is categorized as: very high >100 (red); high >50–100 (yellow); medium >5–50 (green); low 1–5 (blue), and very low or absent <1 (blank).

KAL1 and *MXRA5* was prominent in the brain. Because the expression of PAR and non-PAR genes was similar, no qRT-PCR experiments were conducted in adult tissues.

Next, the expression of 15 genes (9 from the PAR and 6 from the X-specific region; table 3; fig. 3) was quantified by qRT-PCR in embryonic tissues and the placenta. Absolute

expression was calculated for the placenta and whole embryo from day 26, and for the placenta and embryonic heart, liver, lung, skeletal muscle, brain, spleen, kidney, gonads, small intestine, and stomach from day 60 (table 3). While the selected PAR genes were confined to the terminal end of SSCXp, the 6 X-specific genes were distributed all over the X chromosome – from next to the PAB (*WWC3*)

up to the distal end of the long arm (*AFF2* and *IDS*), as indicated by their approximate Mb position in the sequence map (fig. 3, online suppl. table 3). Despite this, there was a clear difference in the expression of the 2 groups of genes: 6 out of 9 PAR genes (70%) showed high- to medium-level expression both in the embryo and placenta, while the expression of non-PAR genes in most embryonic tissues and placenta was very low or absent (fig. 3; table 3). Among the X-specific genes only *AFF2* (alias *FMR2*) showed consistent medium-level expression, while transcripts of 3 PAR genes, viz., *SLC25A6*, *MXRA5* and *PRKXY*, were absent in almost all tissues (table 3; fig. 3).

Overall, the absolute expression of PAR genes was higher in 60-day-old embryonic tissues and placenta compared to embryos and placenta at 26 days of development. The only exceptions were *SHOX* and *KAL1* that were highly expressed (>50–100 copies/μl) also in the 26-day-old embryos. While the expression of *KAL1* reduced to medium or low at day 60, *SHOX* retained high expression levels in 60-day-old brain, kidney, skeletal muscle and lung (table 3; fig. 3). At both time points, the expression of PAR genes was higher in embryos/embryonic tissues compared to placenta. We did not observe gender differences in the expression of the PAR or X-specific genes, which was expected because, by definition, PAR genes are functionally diploid in both sexes and the X-inactivation mechanism should equal the expression of X-specific genes between mammalian males and females [Brown and Greally, 2003; Blaschke and Rappold, 2006].

In summary, we observed higher expression of most of the PAR genes compared to the X-specific genes at early stages of embryonic development in pigs, whereas the expression of PAR genes was higher at day 60 compared to day 26, and higher in embryonic tissues compared to placenta.

Discussion

The Pig PAR and the Current Pig Genome Sequence Assembly

The study represents an important addition and improvement to the current sequence assembly of the porcine X chromosome. By constructing a medium-density cytogenetic map for the pig PAR, we unambiguously showed that the sequences of *CRLF2*, *CSF2RA*, *SLC25A6*, *ARSF*, *MXRA5*, *PRKX*, and *SW949* truly belong to Xp and not to Xq or ChrUn as they are in Sscrofa10.2 (fig. 1). Cytogenetic mapping of these and

other PAR genes to the terminal ends of Xp and Yp (fig. 1) also confirmed and expanded earlier map data [Quilter et al., 2002]. Most importantly, the discovery of the PAB sequence in *SHROOM2* intron 3–4 clearly defined the physical boundaries of the PAR in both sex chromosomes (fig. 2).

The proposed 6.7-Mb size of the porcine PAR as defined in this study, however, is a rough estimate and may increase with the completion of the sequence assembly. For example, the sequence map of the terminal 7 Mb of SSCXp contains 17 gaps (fig. 1), some of which are likely due to the incorrect assignment of PAR sequences to Xq. FISH mapping of genes from *CRLF2* to *PRKXY* and building a BAC contig over the PAB facilitate filling gaps 3–9 and 14–17, respectively (fig. 1). Because of the gaps in the SSCX sequence assembly, the actual size of the porcine PAR might be about 9 Mb which is in accordance with the genomic location of *SHROOM2* at 9.7 Mb in the human X chromosome [Ross et al., 2005].

Difficulties to assemble the PAR have also been observed in other species, including those with finished sequence assemblies like humans, where only 80% of PAR1 sequence is available to date [Ross et al., 2005] or mice, where the small 700-kb PAR is still not completely sequenced [Alföldi, 2008]. This is attributed to the specific properties of the PAR sequences, such as increased number of segmental duplications and elevated rate of inter-specific sequence polymorphism [Meroni et al., 1996; Ross et al., 2005]. The latter might have affected PAR sequence assembly in pigs more than in other species because the pig genome has been sequenced by a hybrid approach of whole genome shotgun and BAC sequencing, whereas BACs originated from more than one library and individual [Archibald et al., 2010; Swine Genome Sequencing Consortium, 2012].

Comparison of the Pig and Other Eutherian PARs

Demarcation of the porcine PAB in *SHROOM2* at X:6,743,567 bp includes pig among the mammalian species where the sequence of the PAB has been determined and the PAR is precisely delineated. These include human/primates [Skaletsky et al., 2003; Ross et al., 2005; Hughes et al., 2010, 2012], mouse [Palmer et al., 1997; Perry et al., 2001] and cattle/ruminants [Van Laere et al., 2008]. Though the genomic locations of these PABs are different, they share some striking similarities: all contain SINE elements (tRNAGlu in pigs; Alu in human/chimp, and Bov-tA1/A2 in cattle) and truncate a gene on the Y chromosome (*SHROOM2* in pigs; *GPR143* in cattle/ruminants; *XG* in human/primates, and *Mid1* in mouse)

(fig. 4). These hallmarks of the PAB across evolutionarily diverged species suggest that retrotransposition into a gene is a common and effective evolutionary mechanism to establish a recombination barrier between the sex chromosomes.

In addition to the species with sequenced PAB, approximate location of the boundary and the size of the PAR are known for 6 more mammals, i.e. horse [Raudsepp and Chowdhary, 2008b], porpoise [Van Laere et al., 2008], cat [Murphy et al., 2007], dog [Young et al., 2008], alpaca [Raudsepp et al., 2012], and armadillos [Sciurano et al., 2012]. It appears that eutherian PARs are more alike than previously thought [Das et al., 2009], and the size of the pig PAR is similar, yet not identical, to the PARs of other cetartiodactyls, as well as carnivores (fig. 4). Recent immunocytogenetic studies of meiosis in armadillos indicate that species of Xenartha, one of the basal eutherian orders [Meredith et al., 2011], might have the largest known PAR (8.4–13.6 Mb; fig. 4) among mammals, providing the first ‘live’ evidence to the theory that the ancestral eutherian PAR extended proximally up to *AMELX* or further on the X chromosome [Iwase et al., 2003; Park et al., 2005]. Thus, the first and the best studied PARs of human [Ross et al., 2005] and mouse [Ellison et al., 1996], and the PAR of the horse [Raudsepp and Chowdhary, 2008b] are different from other species (fig. 4). Despite this, the map information for the PAR and the adjacent X-specific region in all species studied so far, except mouse, provide compelling evidence about the overall evolutionary conservation and collinearity of this region in eutherian mammals [reviewed by Raudsepp et al., 2012]. Though minor differences exist, like the presence of the odorant binding protein (*OBP*) gene in the PAR of pigs (fig. 1), cattle and dogs (NCBI; <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>), but not in other species.

Functional Profiles of Pig PAR Genes in Adult and Embryonic Tissues

Even though eutherian X chromosomes are largely collinear, different location of the PAB implies that the gene content of eutherian PARs varies (fig. 4), so that a different set of genes undergo or escape X inactivation and are functionally haploid or diploid, respectively, in different species. Current information about the spatio-temporal expression, molecular functions and genotype-phenotype correlations of PAR genes is limited mainly to *SHOX* and a few other PAR1 loci in humans [Mangs and Morris, 2007; Flaquer et al., 2008]. Because the PAR and PAB in other eutherians have been demarcated only re-

Fig. 4. Comparative status of the gene content and size of the pig and other eutherian PARs. The human Xp sequence map at the left includes only the genes from Sscrofa10.2 that were mapped in this study. The genes at known eutherian PABs are shown in bold.

cently, knowledge about the functional profiles of PAR genes in these species is sparse. The preliminary expression profiles of the PAR and X-specific genes in porcine adult and embryonic tissues, as presented in this study, are thus among the first attempts to improve this knowledge in domestic animals.

Our pilot findings indicate elevated expression of the PAR genes compared to X-specific genes in porcine embryos (fig. 3; table 3), suggesting that the PAR genes might be important in early development. Initial evidence for this was provided by the studies of human XX and XO embryonic stem cells showing that XO cells which are haploinsufficient for the PAR and other genes that escape X inactivation, are differentially expressed in placenta [Urbach and Benvenisty, 2009]. Very recently, another human study used induced pluripotent stem cells (iPSCs) from XO individuals to model abnormal early development [Li et al., 2012] and showed that cell lines derived from XO iPSCs display lower expression level of PAR genes than normal controls. Indirectly,

these findings are supported by the observations that viable XO individuals can be found in species with small PARs (human, horse, mouse) but are rare or absent in species with large PARs (pig, cattle, dog, cat) [Raudsepp et al., 2012], as if haploinsufficiency for too many PAR genes causes embryonic lethality. However, in contrast to the notion of Urbach and Benvenisty [2009] that PAR genes might be necessary for placenta differentiation, the results of this study did not show higher expression of PAR genes in placenta compared to embryos or embryonic tissues (fig. 3; table 3). It is possible that there are differences in developmental gene regulation between species, such as human and pig. Within the limited scope of our study involving 9 PAR and 6 X-specific genes, it is also likely that the full spectrum of PAR gene expression was not documented.

Finally, it must be noted that the selection of genes for expression analysis was solely based on their PAR or non-PAR status, and not on their functions. This is probably why very low expression was detected for the X-specific NADPH oxidase 1 (*NOX1*) in adult tissues (online suppl. fig. 2): the gene is known to be expressed mainly in adult colon [Szanto et al., 2005] and this tissue was not included in the panel. More intriguing was the almost complete absence of the expression of the porcine PAR-linked protein kinase (*PRKXY*) in embryonic but not in adult tissues (table 3, online suppl. fig. 2). The X-specific ortholog of the gene is known to be expressed in human and murine embryonic and adult tissues, and the gene is considered important for morphogenesis and cellular differentiation

[Li et al., 2005; Li et al., 2009]. Whether the low expression of *PRKXY* in pig development is related to its PAR status or other factors needs future studies.

Summary

This is the first detailed structural and comparative analysis of the porcine PAR and the first pilot study of the expression of PAR genes in domestic species. While the findings improve our overall knowledge about the organization of this region across mammals, and give a hint about the possible role of PAR genes in development, many aspects of the mammalian PAR remain enigmatic. For example, how does the PAR or non-PAR status affect the function of orthologous genes in different species? Complete sequencing and comprehensive functional analysis of the region in species with very large and very small PARs is needed to further unveil the roles of the PAR in mammalian biology.

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