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# Psg22-null mouse embryos develop normally under normoxic and hypoxic conditions of pregnancy

**John M Williams, Tania Bezak, Mrinmoy Das, Zhenfei Ning, Eric F Lucking, Vincent P Kelly, Patrick Harrison, Paul Young, Mary J O'Connell, Peter Dockery, Ken D O'Halloran, Tom Moore**

School of Biochemistry and Cell Biology, University College Cork; School of Biochemistry and Immunology, Trinity College Dublin; School of Microbiology, University College Cork; Department of Physiology, University College Cork; School of Biology, University of Leeds; Department of Anatomy, National University of Ireland Galway

**Correspondence**
t.moore@ucc.ie

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## Abstract

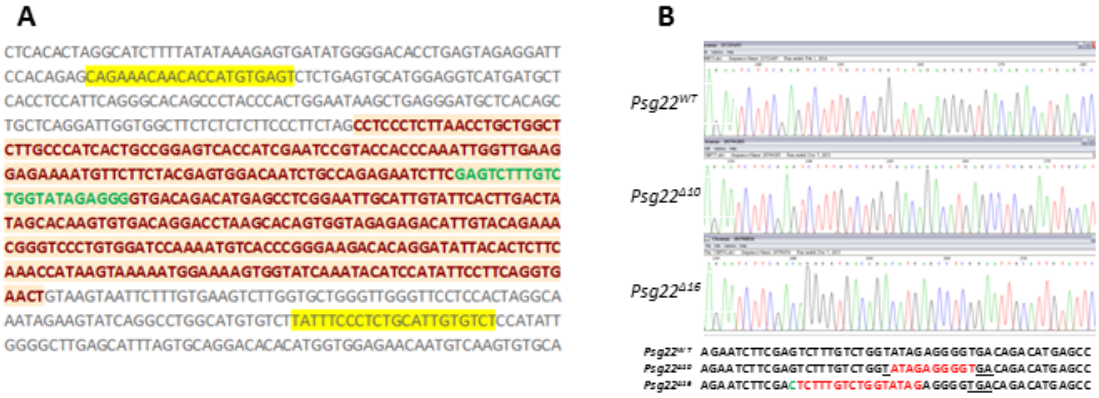
Pregnancy-specific glycoproteins are secreted by immunoglobulin superfamily members encoded by multigene families in eutherian mammals with haemochorial placentation. They are expressed predominantly in placental trophoblast and exhibit immunomodulatory, anti-platelet, and pro-angiogenic functions. An inversion of *Psg22* in the mouse locus is associated with relatively high *Psg22* expression in the first half of the pregnancy. Bioinformatic analyses of 17 mouse strains indicated that *Psg22* inversion arose at least 1.7 MYA. We used CRISPR-Cas9 mutagenesis to generate *Psg22*-null mutants, two of which were analysed in detail ($Psg22^{\Delta 10}$and $Psg22^{\Delta 16}$). Both mutants contain frame-shifting deletions in exon 2, resulting in premature stop codons, and Psg22 mRNA was virtually undetectable. Both mutants are fertile and there was no distortion of Mendelian ratios in heterozygous crosses. Housing of pregnant females in a hypoxic (11% $O_2$) environment for 5 (E5–E10) or 10 (E5–E15) days did not induce differential growth or survival of *Psg22* wildtype and null mutant genotypes. Our results indicate that *Psg22* is dispensable for embryonic development and reproduction under laboratory conditions. As PSGs are secreted into maternal blood, future work will focus on whether *Psg22* deficiency alters maternal physiology.

## Introduction

Pregnancy-specific glycoproteins (PSG) are abundantly secreted by placental trophoblast into the maternal bloodstream during human pregnancy, and their deregulation is implicated in gestational disease [1] [2]. PSGs aremembers of the immunoglobulin superfamily and are closely related to the predominantly membrane-anchored carcinoembryonic antigen-related cell adhesion molecules (CEACAM) [3] [4]. PSGs are encoded by 17 genes in the mouse and 11 in the human [5] [6]. *PSG* gene families are rapidly evolving and PSG protein domain organisation differs between the mouse and human, with no discernible orthologous relationships. However, PSG functions appear to be conserved with evidence of roles in immune regulation, TGFβ1 activation, platelet regulation, and angiogenesis [7] [8] [9] [10] [11] [6]. Therefore, mouse *Psg* gene mutants may provide models of human gestational disease. PSG mRNA in the first half of mouse pregnancy is almost exclusively derived from *Psg22* in trophoblast giant cells (TGC), which may be due toinversion of the *Psg22* gene and duplication of a long noncoding RNA (lncRNA) within the *Psg* locus [12] [13]. This expression pattern facilitates ablation of mouse *Psg* expression at this developmental stage without having to delete multiple *Psg* genes.

## Objective

We used CRISPR-Cas9 genome editing to produce and characterise mouse *Psg22*-null mutants. We examined offspring genotype ratios at birth in normal pregnancy and in pregnancy with hypoxic stress, and we analysed placental weights and structure at E17 of gestation.

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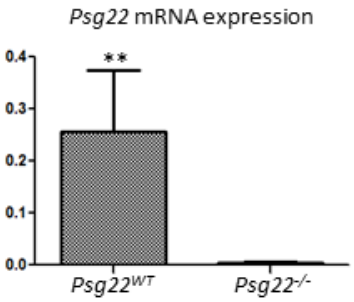


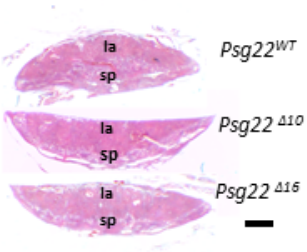

D

| | Offspring genotype | Psg22Δ10 | Psg22Δ16 |
|---|---|---|---|
| Normoxia | +/+ | 26.09% (12) | 23.19% (16) |
| | +/- | 47.82% (22) | 50.72% (35) |
| | -/- | 26.09% (12) | 26.09% (18) |
| Hypoxia | +/+ | 33.33% (10) | 27.91% (12) |
| | +/- | 50.00% (15) | 48.84% (21) |
| | -/- | 16.67% (5) | 23.25% (10) |

E

| Psg22 genotype | Psg22WT | Psg22Δ10 | Psg22Δ16 |
|---|---|---|---|
| Vv Spong:total | 36.79 ± 0.92 | 35.30 ± 2.12 | 36.81 ± 0.17 |
| Vv Labyr:total | 63.21 ± 0.92 | 64.69 ± 2.12 | 63.18 ± 0.17 |
| Spong/Labyr ratio | 1.726 ± 0.072 | 1.846 ± 0.17 | 1.716 ± 0.012 |
| Placental weight (g) | 0.0978 ± 0.003 | 0.0916 ± 0.001 | 0.102 ± 0.008 |

F




a

## Figure Legend

**Figure 1. Psg22-null mutants are viable and fertile.**

**(A)** *Psg22* exon 2 (red text) with flanking intronic sequences (plain text). CRISPR-Cas9 target sequence (green text) and primers used to amplify targeted region (yellow highlight).

**(B)** Wildtype ($Psg22^{WT}$) and mutant ($Psg22^{\Delta 10}$, $Psg22^{\Delta 16}$) DNA sequences at exon 2 CRISPR-Cas9 target site. Deleted regions are in red. G⟺C substitution in green. Sequences comprising premature stop codons (TGA) are underlined.

**(C)** Psg22 mRNA expression in wildtype and mutant E17 placentas. Combinedrepresentative data from one sample from each mutant ($n$=2); wildtype ($n$=2). Data are mean ± S.E.M; ** $p$<0.01, paired t-test ($t$(DF): $t$(5)=-5.318).

**(D)** Progeny genotype ratios from crosses of heterozygous parents under normoxic and hypoxic conditions did not depart from Mendelian expectations; Chi-square test, $p$>0.05 for all comparisons. Number of progeny in parenthesis.

**(E)** Wildtype ($n$=3) and mutant ($n$=3 for each mutant) E17 placenta weights, and ratios of spongiotrophoblast to labyrinthine trophoblast. There was no difference between genotypes for any of the parameters measured, One-way ANOVA, $p$>0.05 (weight: $F$(2,9)=0.780; spong-total:$F$(2,9)=0.524; lab-total: $F$(2,9)=0.524; ratio: $F$(2,9)=0.569.

**(F)** Representative examples of wildtype and mutant E17 placentas. la, labyrinthine trophoblast; sp, spongiotrophoblast. Scale bar = 1 mm.

**Bioinformatics**

A dataset of 17 mouse strains [14] [15] was assembled from the Sanger Institute website (ftp://ftp-mouse.sanger.ac.uk/REL-1504-Assembly), as follows: 129SI_SvlmJ, AJ, AKR_J, Balb_cJ, C3H_HeJ, C57BL_6NJ, Cast_EiJ, Caroli_EiJ, CBA_J, DBA_2J, FVB_NJ, LP_J, NOD_ShiLtJ, NZO_HlLtJ, PWK_PhJ, Spretus_EiJ, WSB_EiJ. The region known to contain the *Psg* cluster, i.e. region 15,000,000–22,000,000 on chromosome 7, was extracted from all mouse strains from the whole-genome file using a custom Bash script. *Psg* gene sequences were obtained from Ensembl [23] for the mouse reference strain C57BL/6J, and these were used as the query sequences in the subsequent sequence similarity searches. Nucleotide BLAST [24] was used to obtain *Psg* gene sequences from all mouse strains using the annotated *Psg* sequences from C57BL/6J. Coordinates from the BLAST search allowed localisation of the queried *Psg* genes on either leading or lagging strand in the different strains.

**CRIPSR-Cas9 vector cloning and validation**

*Psg22* targeting oligonucleotides were selected using CHOPCHOP (https://chopchop.rc.fas.harvard.edu) and were chosen from exon 2 of the *Psg22* to disrupt the ORF. Oligonucleotides (Eurofins MWG Operon, Eberberg, Germany) were cloned into px458 CRISPR vectors (www.addgene.org) using Zhang laboratory protocol (www.genome-engineering.org/crispr/) [25]. The px458-Psg22 vector was tested in the NIH-3T3 cell line using transient transfection with 3 µL Turbofect transfection reagent. 72 h post-transfection, cells were harvested and genomic DNA was extracted using QIAGEN DNeasy Blood and Tissue kit. 10 ng DNA was used as template for PCR reaction using *Psg22* specific primers spanning the CRISPR target site; the resulting PCR products were analysed for successful genome editing using Surveyor nuclease *Cel* I assay kit (Integrated DNA Technologies, www.idtdna.com).

***Psg22* -mutant mouse production**

Male parental B6D2F1 mice and female B6D2F1 zygote donor mice were obtained from DBA/2 and C57BL/6J breeding pairs. B6D2F1 females were superovulated at 6–10 weeks of age by intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG) 48 h apart. Directly after hCG injection, the superovulated females were mated to B6D2F1 males. CD1 pseudopregnant females were set up in the afternoon of day 1 by mating with vasectomised CD1 males. The following morning (day 0.5 postcoitum; embryonic day 1, E1), B6D2F1 zygotes were harvested and placed in M16 drops in a humidified 5% $CO_2$ incubator at 37℃. The vector px458-Psg22 was diluted to 5 ng/µL in microinjection buffer (8 mM Tris-HCl, 0.15 mM EDTA). Pronuclear microinjection was carried out in a drop of M2 medium under mineral oil. The same day, 72 microinjected zygotes were surgically transferred into the oviducts of 3 CD1 pseudopregnant females. Each CD1 female was bilaterally transferred with 24 microinjected zygotes. 25 weaned offsprings were ear-clipped and genotyped. Genotyping primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify a 606-bp *Psg22*- specific product spanning the CRISPR-Cas9 target site. Genotyping primers were Psg22-2F 5′-CAGAAACAACACCATGTGAGT-3′ and Psg22-2R 5′-AGACACAATGCAGAGGGAAATA-3′. PCR products were purified (QIAGEN PCR purification kit) and directly sequenced (GATC, Koln, Germany).

**Hypoxia treatment of pregnant mice**

Commencing at E5 of pregnancy, female mice were housed in standard cages placed in commercial environmental chambers with precise control of ambient oxygen concentration (Oxycycler, Biospherix, NY) [19] [22] which was maintained at 11% balance nitrogen for 5 or 10 consecutive days (until E10 or E15, respectively). On removal from hypoxia, mice were housed under normal husbandry conditions in room air until E17 or E18.

**Quantitative reverse transcription polymerase chain reaction**

First-strand cDNA was synthesised using 1 µg total RNA in a 20 µL reaction using random hexamer priming and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative RT-PCR (qRT-

PCR) primers were designed to give unbiased amplification of Psg22 transcripts: Psg22-QRT-F 5′-CGCATGGCCAGTTGGCCATT-3′ and Psg22-QRT-R 5′-AAAGCGGGGGAAATAGTTGTAGTA-3′. Hypoxanthine guanine phosphoribosyltransferase (*Hprt*) expression was used to normalise mRNA input and cDNA synthesis efficiency using primers: Hprt-F: 5'-CTATAAGTTCTTTGCTGACCTGCT-3'; Hprt-R: 5'-ATCATCTCCACCAATAACTTTTATGT-3'. qRT-PCR was carried out in triplicated 20 µL reactions using SYBR Green PCR Master Mix (Applied Biosystems, UK), 1 µL cDNA, and primers at 600 mM using the ABI PRISM 7900HT instrument. PCR cycle was initial denaturation (95℃ for 10 min), amplification and quantification repeated for 40 cycles (95℃ for 45 s, 60℃ for 30 s, and 72℃ for 60 s with a single continuous fluorescence measurement), followed by a melting curve program (60–95℃, with a heating rate of 1℃ per 30 s and a continuous fluorescence measurement). Mouse E15 placental cDNA was used to produce the standard curve. PCR products were identified by generating a melt curve, and results were expressed as mean *Psg22* expression relative to mean *Hprt* expression.

**Histology**

Placental weights were measured after fixation in 4% paraformaldehyde (in phosphate buffered saline at pH 7.4). Samples were then dehydrated and embedded in paraffin wax. 5 mm thick sections were cut on a Leica RM2125RT microtome and stained with hematoxylin/eosin and viewed using an adapted Zeiss Jena Microfilm Reader fitted with a 17.5x projector lens. Simple point counting methods were used to estimate the volume fraction of spongiotrophoblast and the labyrinthine trophoblast compartments [26] [27]. Statistical analyses were implemented in Microsoft Excel.

## Results & Discussion

To determine whether inversion of *Psg22* relative to flanking *Psg* genes is a recent event confined to a limited number of laboratory strains, or an older variant that may predate divergence of major mouse strains and indeed species, we surveyed the Mouse Genomes Project [14] [15]. *Psg* locus genomic sequences of 17 mouse strains were analysed by sequence similarity searches, genomic location, and alignment. We found *Psg22* inversion in 16 of 17 mouse strains analysed (129SI_SvlmJ, AJ, AKR_J, Balb_cJ, C3H_HeJ, C57BL_6NJ, Cast_EiJ, CBA_J, DBA_2J, FVB_NJ, LP_J, NOD_ShiLtJ, NZO_HlLtJ, PWK_PhJ, Spretus_EiJ, WSB_EiJ), including the most early diverging *M. spretus*; the *Psg22* transcript could not be clearly identified in the *M. caroli*/EiJ strain due to poor sequence quality. This phylogenetic distribution most likely indicates an inversion of *Psg22* at least 1.7 million years ago that has been maintained in the mouse clade. Therefore, this inversion may underpin a placenta phenotype or influence fetal growth or survival.

We have produced *Psg22*-null mutant mouse strains by pronuclear microinjection of fertilised oocytes with a CRISPR-Cas9 pX458 vector targeting a site in the *Psg22* open reading frame (ORF) in the exon 2 (Fig. 1A), which was previously tested for activity in the NIH-3T3 embryonic fibroblast cell line (data not shown). 72 microinjected B6D2F2 zygotes were transferred to oviducts of 3 CD1 pseudopregnant female recipients, and 25 offsprings were ear-clipped for identification and DNA extraction. PCR genotyping was performed using *Psg22* gene-specific primers flanking the CRISPR target site, followed by Cel I assay and direct sequencing of Cel I assay-positive PCR products. 8 progeny had mutations, and BLAST alignment indicated that 5 were genetic mosaics, 2 had deletions of 10 and 16 bp, respectively, and 1 had a 16 bp deletion just 3′ of a 1 bp substitution.

We selected 2 mutant founders for breeding: one with a 10 bp deletion ($Psg22^{\Delta 10}$) and one exhibiting a 16 bp deletion 3' of a 1 bp substitution ($Psg22^{\Delta 16}$) (Fig. 1B). Both mutations are predicted to cause a frameshift resulting in a premature stop codon and were associated with greatly reduced mRNA levels (Fig. 1C), possibly due to nonsense-mediated decay.

Both founders were backcrossed to the C57BL/6 strain for two generations (to give an average of 87.5% C57BL/6 background) before intercrossing heterozygous male and female littermates of the $Psg22^{\Delta 10}$ and $Psg22^{\Delta 16}$ strains to determine genotype transmission ratios. Combining the data from the two *Psg22*-mutant strains, there were 115 offsprings: 28 homozygous wildtype, 30 homozygous null, and 57 heterozygotes, consistent with Mendelian expectations, and suggesting that *Psg22* is not essential for the development (individual strain data and the statistics are given in figure 1D). Subsequent

breeding of the *Psg22*$^{\Delta 10/\Delta 10}$ and *Psg22*$^{\Delta 16/\Delta 16}$ homozygotes confirmed that these genotypes are fertile (data not shown). This suggests that *Psg22* is dispensable for fertility and reproduction under laboratory conditions.

Previous reports indicate that reproductive phenotypes may be elicited by applying stressors such as hypoxia to pregnancy in mouse strains with the mutations of placenta-expressed genes [16] [17] [18]. We intercrossed heterozygous male and female littermates of the *Psg22*$^{\Delta 10}$ and *Psg22*$^{\Delta 16}$ strains as described above and, using environmental chambers, applied hypoxic stress (11% $O_2$) to pregnant females for either 5 days (E5–E10) or 10 days (E5–E15). At E17 or E18, pregnant [females were sacrificed and embryos were genotyped and corresponding placentas were fixed, weighed, and analysed using stereological techniques. Similar to unstressed pregnancies, there was no deviation from expected Mendelian ratios (Fig. 1D), and there was no difference in placental weight and anatomy among the genotypes (Fig. 1E and F), indicating that *Psg22* expression is dispensable for successful pregnancy under hypoxic conditions.

## Conclusions

We show that the inverted orientation of the *Psg22* gene relative to flanking *Psg* genes is conserved in multiple mouse strains, suggesting that it originated at least 1.7 million years ago. Unlike the Prolactin family gene *PLP-A* [17], *Psg22*-null mutants are viable under both normoxic and hypoxic conditions of pregnancy, with no distortion of Mendelian ratios in heterozygous crosses and no detectable placental phenotype.

## Limitations

We did not analyse Psg22 protein expression in our *Psg22* mutants due to lack of a validated anti-Psg22-specific antibody. However, this deficit is offset by our finding of very low levels of Psg22 mRNA in the mutants. We did not analyse maternal gestational physiology in *Psg22* mutant strains. However, unlike human PSGs that are present at high levels in maternal blood, mouse PSGs appear to be rapidly degraded in the maternal bloodstream and do not exhibit significant steady-state levels [13] [18] [19], suggesting that a significant effect on maternal physiology may be unlikely.

*PSG* gene families may have evolved due to maternal-fetal conflict, and a prediction of this theory is that high expression levels of conflictor genes may result from mutually antagonistic interactions, but with a small net effect on phenotype [20] [21]. A corollary is that, notwithstanding high expression, deletion of the entire *Psg* locus may not result in a significant mutant phenotype. This argument is supported by the observation of high *Psg* mRNA expression in the placental trophoblast with rapid turnover of Psg protein in the maternal circulation [12] [19] [22]. Therefore, a more informative experiment may be to overexpress the Psg proteins in transgenic mouse models under the hypothesis that excess Psg protein may swamp maternal mechanisms for removing Psg from the circulation.

## Additional Information

### Methods

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## Supplementary Material

Please see https://sciencematters.io/articles/201611000023.


## Funding Statement

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## Ethics Statement

Mouse procedures were conducted at UCC and TCD following institutional ethical review and under HPRA authorisations B100/3801 and B100/4429 (https://www.hpra.ie/homepage/veterinary/scientific-animal-protection).

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