J Assist Reprod Genet
DOI 10.1007/s10815-006-9046-0

ANIMAL EXPERIMENTATION

Analysis of the sex ratio in preimplantation embryos from B6.K1 and B6.K2 *Ped* gene congenic mice

Michael J. Byrne · Judith A. Newmark · Carol M. Warner

Received: 12 January 2006 / Accepted: 3 April 2006
© Springer Science+Business Media, Inc. 2006

Abstract *Purpose:* The mouse preimplantation embryo development (*Ped*) gene product, Qa-2, which is the homolog of human HLA-G, influences the rate of preimplantation embryonic development and overall reproductive success. The sex ratio in preimplantation embryos from *Ped* gene congenic mice was examined in order to determine whether embryo sex is a confounding factor in the control of the rate of preimplantation development.

Methods: B6.K1 (*Ped* slow) and B6.K2 (*Ped* fast) congenic mice differ only in the absence (B6.K1) or presence (B6.K2) of the genes encoding Qa-2 protein. We analyzed the sex of B6.K1 ($n = 221$) and B6.K2 ($n = 260$) preimplantation embryos by using Real-Time PCR with primers specific for the X and Y chromosomes.

Results: We found that there was no statistically significant difference in the ratio of male to female preimplantation embryos in either strain.

Conclusions: We conclude that the sex of the embryos is not a confounding factor that affects the *Ped* gene control of the rate of preimplantation development. Therefore, the *Ped* gene is entirely responsible for mediating the faster development of B6.K2 embryos compared to B6.K1 embryos.

Keywords B6.K1 · B6.K2 · HLA-G · IVF · *Ped* gene · Preimplantation embryo · Qa-2 · Sex ratio

There is no confounding effect of the sex of preimplantation embryos on the rate of preimplantation development controlled by the *Ped* gene.

M. J. Byrne · J. A. Newmark · C. M. Warner (✉)
Department of Biology, Northeastern University,
134 Mugar Hall, 360 Huntington Avenue, Boston, Massachusetts, 02115
e-mail: c.warner@neu.edu

Introduction

Survival of mammalian embryos during the preimplantation period is dependent on both environmental and genetic factors. *In vitro* fertilization (IVF) clinics rely upon the analysis of these factors and their effects on embryo development to identify healthy preimplantation embryos. In particular, a fast rate of preimplantation embryo development has been correlated with a higher chance of pregnancy success after IVF (reviewed in [1, 2]). Using the mouse as a model system our laboratory has identified a gene, the preimplantation embryo development (*Ped*) gene that influences the rate of preimplantation embryo development and subsequent embryo survival. The properties of the mouse *Ped* gene, and its human homolog HLA-G, have recently been reviewed [3, 4].

Qa-2 protein is encoded by four almost identical genes, *Q6, Q7, Q8,* and *Q9*, located in the Q region of the mouse major histocompatibility complex (MHC). The presence or absence of these genes and therefore the presence or absence of Qa-2 protein, confers a phenotype of fast or slow preimplantation development, respectively. As in human IVF embryos, mouse embryos with a fast rate of preimplantation development are more likely to give rise to live offspring than embryos with a slow rate of development [5–7]. The *Ped* gene phenotype is intrinsic to the embryos themselves, independent of the uterine environment, as demonstrated by the maintenance of differential cleavage rates when the embryos are cultured *in vitro* in a chemically defined medium [8].

The analysis of the properties of the *Ped* gene has been facilitated by the use of two congenic strains of mice, B6.K1 and B6.K2, which differ only in the presence or absence of the genes encoding Qa-2 protein (Table 1). Embryos from B6.K1 mice have a deletion of the *Q6-Q9* genes, consequently do not express Qa-2 protein, and develop at

Table 1 MHC encoded Q region genes and *Ped* gene phenotype of the B6.K1 and B6.K2 congenic mouse strains

Strain	Q Region genes										*Ped* gene phenotype
B6.K1	*Q1*	*Q2*	*Q3*	*Q4*	*Q5*					*Q10*	slow
B6.K2	*Q1*	*Q2*	*Q3*	*Q4*	*Q5*	*Q6*	*Q7*	*Q8*	*Q9*[a]	*Q10*	fast

[a]Only the *Q9* gene is expressed in B6.K2 preimplantation embryos and is therefore the sole gene that produces Qa-2 protein, the *Ped* gene product, in this strain (9)

a slow rate during the preimplantation period. Embryos from B6.K2 mice have all four Qa-2 encoding genes, but only express the *Q9* gene in preimplantation embryos [9]. Therefore the *Ped* gene is synonymous with the *Q9* gene in the B6.K2 mice. The presence of Qa-2 protein in the B6.K2 embryos results in a fast rate of preimplantation development.

Although the B6.K1 and B6.K2 congenic strains differ only in the expression of the *Ped* gene (*Q9*) product, Qa-2 protein, there is another genetic difference in the embryos from these strains, namely the sex of the embryos. We set up a study to determine whether or not the sex of preimplantation embryos from the B6.K1 and B6.K2 mice could be a confounding factor in influencing the rate of preimplantation development. Nested Real-Time PCR, using primers specific for the X and Y chromosomes, was used to determine the sex of the preimplantation embryos.

Materials and methods

Mice

The congenic B6.K1 and B6.K2 mouse strains were originally obtained from L. Flaherty (Wadsworth Center, Albany, NY) and subsequently bred in our laboratory. The mice were housed according to the NIH guidelines in an AAALAC approved facility with a 14 h day/10 h night cycled room (lights on 0400-1800 EST) with controlled temperature and food and water *ad libitum*. All experiments followed the NIH guidelines. The genetic properties of the B6.K1 and B6.K2 mice are shown in Table 1. Aside from the genes in the Q region of the MHC, all other genes in these congenic strains are identical.

Mouse embryos

Female mice were superovulated with 5 IU eCG (Sigma Chemical Co., St. Louis, MO) at the 9th hr of the light cycle, followed 48 h later by 10 IU of hCG (Sigma). Mice were mated, checked for vaginal plugs, and the plug-positive females were sacrificed by cervical dislocation. Embryos were collected at 89 h and 100 h post-hCG in KSOM^{AA} (potassium simplex optimized medium + amino acids) (Specialty Medium, Phillipsburg, NJ) under 5% CO_2, 5% O_2, 90% N_2 and then washed in KSOM^{AA}. These time points were chosen for the collection of the preimplantation embryos because all morphologically normal embryos were at least at the morula stage of development, thereby having enough cells for the sex determination analysis as described below, and because implantation in mice starts about 104–106 h post-hCG [10]. Individual embryos were transferred in the smallest possible volume to a microfuge tube containing 4 μl lysis buffer [20 mM Tris (pH 8.0), 0.9% Tween 20, 0.9% Nonidet P-40, 0.4 mg/mL of proteinase K] and centrifuged immediately for 60 sec at 14,000 $\times$ *g*. Samples were stored at -20°C until sexing by PCR analysis was performed. Prior to *Ped* gene (*Q9*) PCR analysis, blastocyst stage embryos were heated to 56°C for 45 min followed by 96°C for 10 min to inactivate proteinase K. This step was not necessary for embryo sex determination by PCR.

Isolation of spleen genomic DNA

B6.K1 and B6.K2 control DNA was extracted from male and female spleens. Extraction was performed following the protocol provided with the Qiagen DNeasy Tissue Extraction Kit (Qiagen Inc., Valencia, CA). The purity of the DNA was assessed by determining the 260/280 ratio using a Genosys 8 spectrophotometer (Thermo Spectronic, Madison, WI). The concentration of DNA was determined using a TBS-380 Mini-Fluorometer (Turner Biosystems Inc., Sunnyvale, CA). The DNA concentration of all samples was adjusted to give a total of 20 ng for each Real-Time PCR assay.

Ped gene (*Q9*) detection in splenocytes and blastocysts by Real-Time PCR

Primers designed to amplify a 200 bp fragment of exon 4 of the *Ped* gene (*Q9*) [11] were used at 900 nM each and were synthesized by Sigma-Genosys (Woodlands, TX) (Table 2). Twenty nanograms of splenocyte DNA was used for each splenocyte PCR sample and 5 blastocysts (about 800 pg of genomic DNA) were used for each blastocyst PCR sample for each strain. Briefly, as amplification of a PCR product occurs in the Real-Time PCR instrument, a fluorescent dye in the SYBR Green Master Mix (Applied Biosystems Inc.) binds to the double stranded DNA produced during each cycle. At the end of the PCR protocol the temperature is

Table 2 Primer sequences, melting temperatures and molecular weights of *Ped* gene (*Q9*), *Sry*, and DXNds3 PCR products

Primer	Sequence 5′-3′	Product Melting Temperature (°C)	Molecular Weight
Ped gene (Q9)			
Forward	CAGGTCTTATGGTGCTGTCA	85	200 bp
Reverse	GCATGTGTAATTCTGCTCCTTC		
Sry gene			
Outer		N/A	404 bp
Sry2	TCTTAAACTCTGAAGAAGAGAC		
Sry4	GTCTTGCCTGTATGTGATGG		
Inner		82	147 bp
Sry1	GTGAGAGGCACAAGTTGGC		
Sry3	CTCTGTGTAGGATCTTCAATC		
DXNds3 microsatellite			
Outer		N/A	244 bp
DXNds3	GAGTGCCTCATCTATACTTACAG		
DXNds4	TCTAGTTCATTGTTGATTAGTTGC		
Inner		83	111 bp
DXNds1	ATGCTTGGCCAGTGTACATAG		
DXNds2	TCCGGAAAGCAGCCATTGGAGA		

raised and the dissociation curve of melting temperature versus fluorescence derivative of the product is shown as a peak on the output screen. The thermal cycling conditions consisted of a 10 min hold at 95°C to activate the AmpliTaq Gold DNA Polymerase of the master mix followed by 55 cycles of 95°C for 15 sec to denature the sample, 55°C for 1 min for the annealing phase and 72°C for 30 sec to extend the product with a final step of 4°C for 2 min. The identity of the PCR products was confirmed by first determining the melting temperatures of the products from analysis of dissociation curves generated by the ABI Prism 7000 Real-Time PCR instrument (Applied Biosystems Inc., Foster City, CA), and then by confirming the molecular weights of the products by agarose gel electrophoresis.

Sex determination in splenocytes and embryos by Real-Time PCR

Primers amplifying the polymorphic X chromosome microsatellite locus DXNds3 and the Y chromosome *Sry* gene were used for the sexing nested PCR assay [12–16]. Table 2 presents the sequence, expected size, and melting temperature of the products. Nested PCR performed on male embryos is expected to produce a *Sry* product and a DXNds3 product. The same nested PCR performed on female embryos is expected to produce only the DXNds3 product. Inner and outer primer pairs were synthesized by Sigma-Genosys (Woodlands, TX).

Each embryo lysate was subjected to two separate nested PCR reactions using the Real-Time PCR instrument along with splenocyte genomic DNA from mice whose sex was known serving as controls. The final 50 μl reaction volume consisted of 25 μl SYBR Green Master Mix (containing AmpliTaq Gold DNA Polymerase), 2 μl of the frozen, lysed embryo or 2 μl [100 pg and 50 pg in duplicate] of splenocyte genomic DNA from mice of known sex, and 300 nM of each primer for each outer primer set. The thermal cycling conditions consisted of a 10 min hold at 95°C to activate the AmpliTaq Gold DNA Polymerase of the master mix followed by 50 cycles of 95°C for 15 sec to denature the sample, 60°C for 1 min for the annealing phase and 72°C for 30 sec to extend the product. In the second round of PCR, 5 μl of the first round product were used as template DNA and 300 nM and 300 nM, and 600 nM and 900 nM of the inner primers were used for DXNds3 and *Sry* amplification, respectively. The secondary PCR conditions were the same as the primary PCR conditions with two exceptions: the annealing temperature was raised to 64°C and the cycle number was increased to 55. All samples analyzed showed the presence of DXNds3. The sex of each embryo was determined by the presence (males) or absence (females) of *Sry*.

Statistical analysis

As part of our pre-investigation procedure, we used a statistical power analysis to determine the sample size required for accurate results [17]. We determined that the minimum sample size needed was 50. We used a chi-square analysis to test the hypothesis that the number of male and female embryos should be equal.

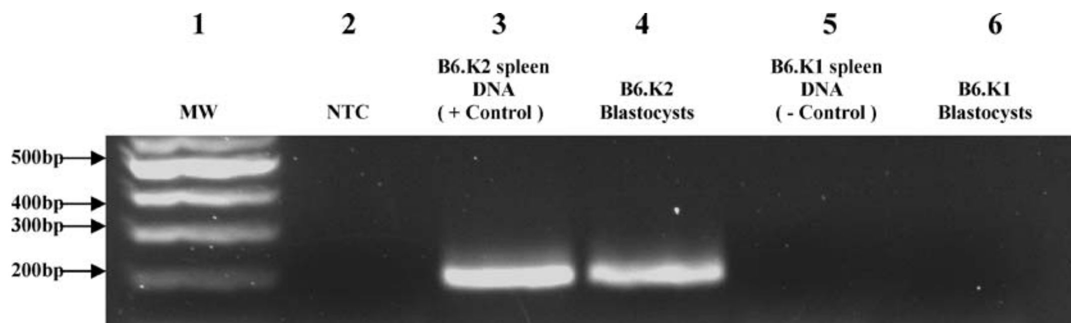

Fig. 1 Agarose gel electrophoresis of the product from Real-Time PCR amplification of the *Ped* gene (*Q9*) in B6.K1 and B6.K2 splenocytes and blastocyst stage embryos. Lane 1, 100 bp DNA ladder (MW); Lane 2, no template control (NTC); Lanes 3 and 4, B6.K2 splenocyte and blastocyst samples, respectively; Lanes 5 and 6, B6.K1 splenocyte and blastocyst samples, respectively

Results

Prior to sexing the embryos, we performed Real-Time PCR on splenocytes and blastocysts from the B6.K1 and B6.K2 strains of mice to confirm that the B6.K1 strain does not possess the *Ped* gene (Q9) while the B6.K2 strain does possess the *Ped* gene (Q9). Figure 1 shows that the *Ped* gene (*Q9*) is present in B6.K2 splenocytes (lane 3) and B6.K2 blastocysts (lane 4) but absent in B6.K1 splenocytes (lane 5) and B6.K1 blastocysts (lane 6).

The sensitivity of the embryo sexing nested Real-Time PCR assay was tested by performing PCR on diluted control samples of both male and female B6.K1 and B6.K2 spleen genomic DNA. These experiments revealed that as little as 15 pg of genomic male and female DNA could be accurately distinguished using the nested Real-Time PCR protocol (data not shown). Since a 16 cell embryo (morula) contains about 80 pg of genomic DNA, the sensitivity of this assay was acceptable for sex determination of the individual embryos used in this study because all embryos tested were at least at the morula stage of development.

We determined the sex of 221 embryos from B6.K1 mice and 260 embryos from B6.K2 mice by using Real-Time PCR. Figure 2 presents Real-Time PCR data from the nested (second) round of PCR. Figures 2a and b show control amplification of B6.K2 spleen genomic DNA for the *Sry* gene (Y chromosome) and the DXNds3 microsatellite (X chromosome), respectively. As expected, amplification of *Sry* occurred only in the male DNA samples, while amplification of DXNds3 occurred in both the male and female DNA samples. Figures 2c and d show the same control samples but include examples of both male ($n = 3$) and female ($n = 3$) embryos. Male embryos showed both *Sry* and DXNds3 amplification while female embryos showed only DXNds3 amplification. Figure 3 is a representative gel showing control PCR products as well as examples of male and female embryo PCR products. Only the male control (lane 2) and the male embryos (lanes 4–6) had a *Sry* product of the expected size (Table 2) while all samples had the DXNds3 product of the expected size. Female embryo samples (lanes 7–9) have a minor band (<50 bp) when subjected to amplification of the *Sry* gene. This is most likely a dimerization between the primers due to a lack of template to bind to in these samples, which also accounts for the small nonspecific hump seen in the dissociation curve of these samples (Fig. 2c).

We compared the number of male embryos to the number of female embryos in each strain by using chi-square analysis. The null hypothesis was that 50% of the total embryos sexed were expected to be male and 50% were expected to be female. The observed values were the actual number of male and female embryos as determined by Real-Time PCR. As shown in Table 3, there was no significant difference in the number of male or female embryos at either time point post-hCG in either strain or when total embryos were compared from the combination of both time points.

Discussion

The B6.K1/B6.K2 model system is ideal for studying the effects of the *Ped* gene on development and reproduction because these mice are genetically identical except for the absence (B6.K1) or presence (B6.K2) of the *Ped* gene. Previous work using the congenic B6.K1 and B6.K2 mice has shown that the presence of Qa-2 protein in the B6.K2 mice confers a faster rate of preimplantation development, larger litter size, larger birth weight, and larger weaning weight to the pups compared to the B6.K1 mice [5, 6]. In addition, embryos that express Qa-2 protein have a higher chance of surviving to birth than those that do not [7]. In this paper we tested the hypothesis that embryo sex might be a confounding factor in mediating the rate of preimplantation cleavage. We found that there was no skewing of the sex ratio in preimplantation embryos from either strain, showing that only the *Ped* gene is involved in the genetic control of the rate of preimplantation development in B6.K1 and B6.K2 mice.

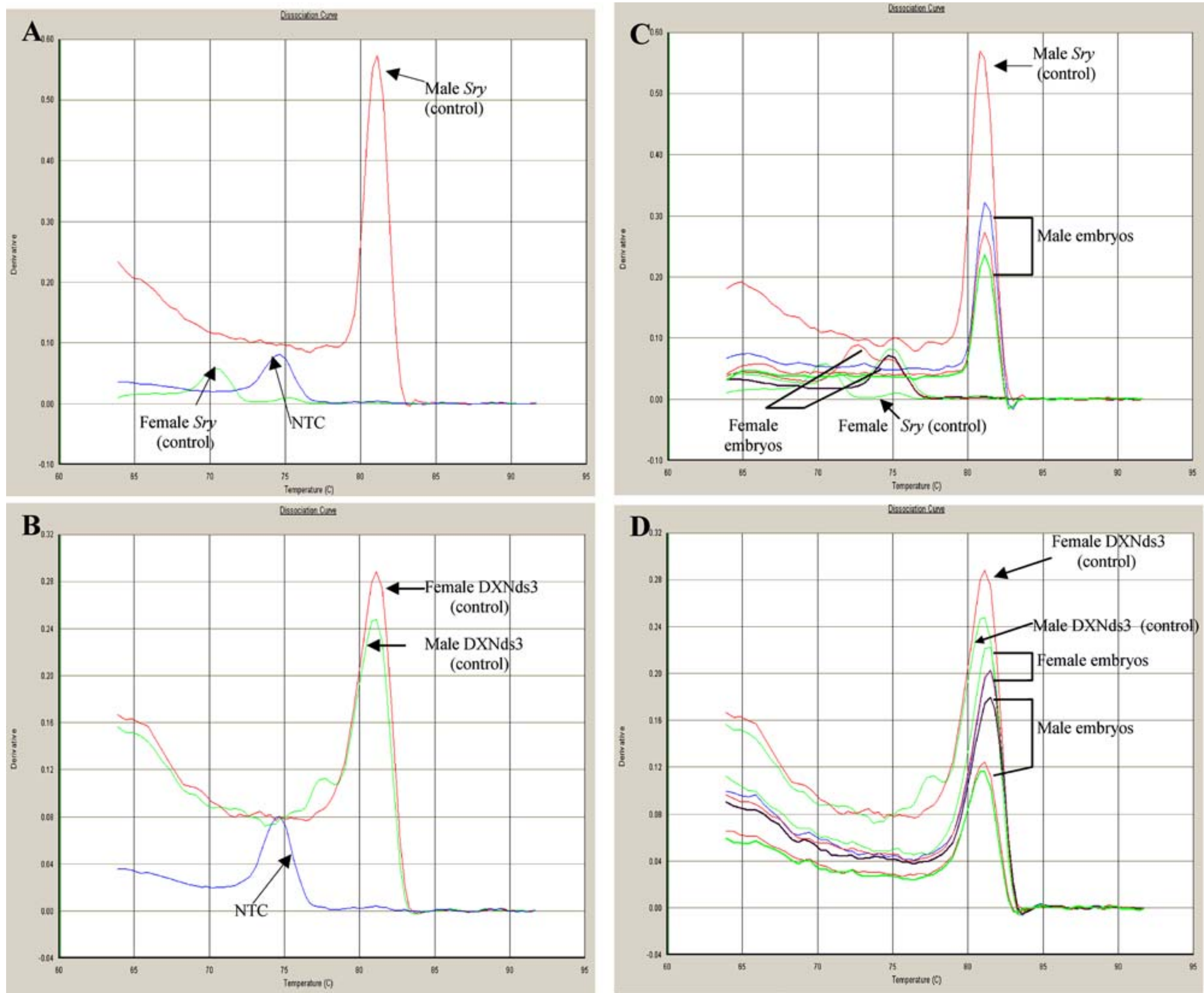

Fig. 2 Dissociation curves of nested Real-Time PCR of *Sry* and DXNds3 amplification. A) B6.K2 control genomic DNA amplification of the *Sry* gene product. B) B6.K2 control genomic DNA amplification of the DXNds3 product. C) B6.K2 control genomic DNA amplification of the *Sry* gene product plus representative male ($n = 3$) and female ($n = 3$) embryo amplification results. D) B6.K2 control genomic DNA amplification of the DXNds3 product plus representative male ($n = 3$) and female ($n = 3$) embryo amplification results. NTC = no template control

Embryo sex has been the subject of a number of studies both on embryos cultured *in vitro* and on embryos allowed to develop *in vivo*. Previous work on *in vitro* cultured preimplantation embryos from the mouse and other species generally supports the idea that male embryos often develop faster than female embryos during the culture period. This phenomenon has been observed in mouse [18–20], cow [21, 22], sheep [23], and human embryos [24–26]. In contrast to these *in vitro* studies, two studies on the sex ratio of preimplantation mouse embryos analyzed after *in vivo* development, show conflicting results. The first study found that female mouse embryos developed faster than male embryos [20], while the second study found that male mouse embryos developed faster than female embryos [27]. In the present study, we also analyzed preimplantation embryos after development *in vivo*, but we have used a rigorously defined genetic system unlike the previous two studies, which used several different mouse strains. Interestingly, our results on the preimplantation sex ratio of the preimplantation embryos from the B6.K1 and B6.K2 mice are consistent with the 50:50 sex ratios that we found at weaning for both of these strains [5].

The human homolog of Qa-2 is HLA-G [28–30]. The presence of soluble isoforms of HLA-G in the culture medium of embryos created after IVF or ICSI has been reported to be correlated with an enhanced chance of pregnancy success [31–35], although this finding has been called into question by others [36–40]. However, regardless of whether or not soluble HLA-G ultimately turns out to be a clinically useful predictor of pregnancy outcome, expression of

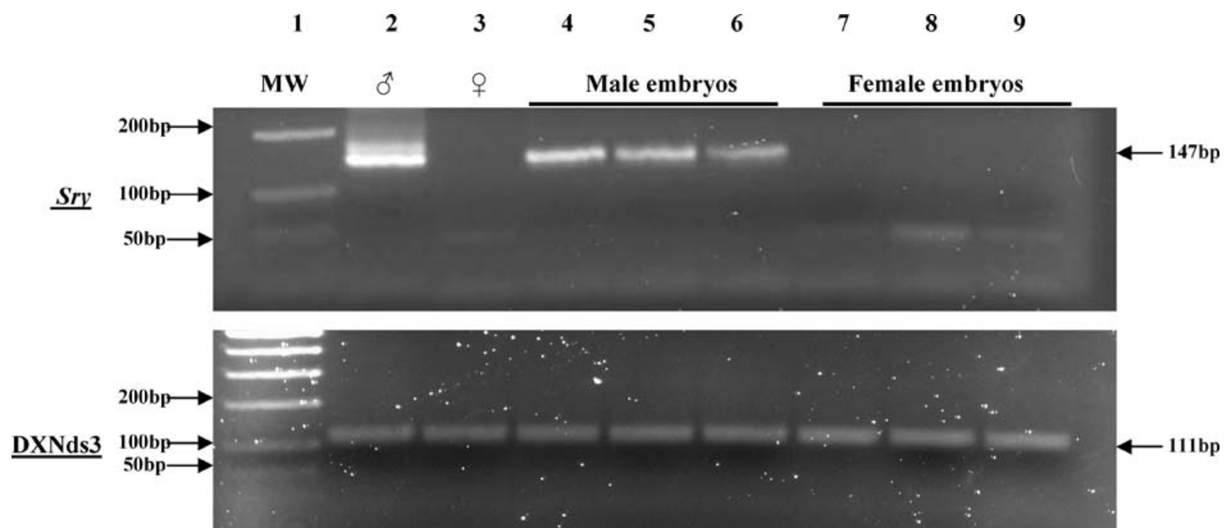

Fig. 3 Representative agarose gel confirming results presented in Fig. 2. Lane 1, 100 bp DNA ladder (MW); Lanes 2 and 3, male and female B6.K2 genomic DNA control samples, respectively; Lanes 4–6, male embryo samples; Lanes 7–9, female embryo samples

membrane-bound HLA-G, as well as membrane-bound Qa-2, is associated with an enhanced rate of preimplantation development and overall reproductive success (reviewed in [41]).

Studies on the rate of development of preimplantation embryos are particularly relevant to the assisted reproduction technology (ART) clinic. It has long been recognized that embryos that develop at a fast rate have a higher chance of leading to pregnancy success (reviewed in [1, 2]). For this reason the *Ped* gene has been of great interest to clinicians working in ART. The B6.K1/B6.K2 mouse system described in this paper should provide an excellent model for future research on how the *Ped* gene product, Qa-2 protein, mediates a fast rate of development independent of the sex of the embryos.

Table 3 Sexing of preimplantation mouse embryos

Mouse Strain	Experiment	Number of Embryos			P value[a]
		Total	Male	Female	
Total embryos sexed					
B6.K1	Total (4 expts)	221	110	111	$P = 0.95$
B6.K2	Total (6 expts)	260	137	123	$P = 0.39$
Embryos sexed 89 h post-hCG					
B6.K1	1	34	14	20	
	2	56	30	26	
	Total	90	44	46	$P = 0.83$
B6.K2	1	9	3	6	
	2	16	8	8	
	3	79	44	35	
	Total	104	55	49	$P = 0.56$
Embryos sexed 100 h post-hCG					
B6.K1	1	67	39	28	
	2	64	27	37	
	Total	131	66	65	$P = 0.93$
B6.K2	1	39	15	24	
	2	29	19	10	
	3	88	48	40	
	Total	156	82	74	$P = 0.52$

[a] P value of males compared to females by chi-square analysis

Conclusions

In conclusion, the present study has shown that in the B6.K1 and B6.K2 congenic strains of mice, which differ only in the presence of the *Ped* gene, there is no statistically significant difference in the sex ratio of their preimplantation embryos. Therefore, genetic control of the rate of preimplantation development in these strains is solely mediated by the *Ped* gene product, Qa-2 protein. The B6.K1 and B6.K2 congenic strains of mice provide a unique opportunity to further elucidate the mechanisms by which the *Ped* gene, and only the *Ped* gene, influences the rate of preimplantation embryo development and pregnancy success.

Acknowledgements We thank Marie Amato for helping with the literature search and we thank Michele Mammolenti for managing the mouse husbandry. This study was supported by NIH grant HD39215.

References

1. Boiso I, Veiga A, Edwards RG. Fundamental of human embryonic growth *in vitro* and the selection of high-quality embryos for transfer. Reprod BioMed Online 2002;5:328–50
2. Wharf E, Dimirakopoulos A, Khalaf Y, Pickering S. Early embryo development is an indicator of implantation potential. Reprod BioMed Online 2004;8:212–18
3. Warner CM, Brenner CA. Genetic regulation of preimplantation embryo survival. Curr Top Dev Biol 2001;52:151–92
4. Warner CM, Newmark JA, Comiskey M, De Fazio SR, O'Malley DM, Rajadhyaksha M et al. Genetics and imaging to assess oocyte and preimplantation embryo health. Reprod Fertil Develop 2004;16:729–41
5. Warner CM, Brownell MS, Rothschild MF. Analysis of litter size and weight in mice differing in *Ped* gene phenotype and the Q region of the H-2 complex. J Reprod Immunol 1991;19:303–13
6. Warner CM, Panda P, Almquist CD, Xu Y. Preferential survival of mice expressing the Qa-2 antigen. J Reprod Fertil 1993;99:145–7
7. Exley GE, Warner CM. Selection in favor of the *Ped fast* haplotype occurs between mid-gestation and birth. Immunogenetics 1999;49:653–9
8. Brownell MS, Warner CM. *Ped* gene expression by embryos cultured in vitro. Biol Reprod 1988;39:806–11
9. Wu L, Exley GE, Warner CM. Differential expression of *Ped* gene candidates in preimplantation mouse embryos. Biol Reprod 1998;59:941–52
10. Dey SK, Lim H, Das SK, Reese J, Paria BC, Diakoku T et al. Molecular cues to implantation. Endocr Rev 2004;25(3):341–73
11. Newmark JA, Sacher F, Jones GS, Warner CM. *Ped* gene deletion polymorphism frequency in wild mice. J Exptl Zool 2002;293:179–85
12. Mardon G, Page DC. The sex-determining region of the mouse Y chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. Cell 1989;56:765–70
13. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 1990;346:245–50
14. Love JM, Knight AM, McAleer MA, Todd JA. Towards construction of a high resolution map of the mouse genome using PCR-analyzed microsatellites. Nucleic Acids Res 1990;8:4123–30
15. Kunieda T, Xian M, Kobayashi E, Imamichi T, Moriwaki K, Toyoda Y. Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. Biol Reprod 1992;46:692–7
16. Greenlee AR, Krisher RL, Plotka ED. Rapid sexing of murine preimplantation embryos using a nested multiplex polymerase chain reaction (PCR). Mol Reprod Dev 1998;14:261–7
17. Cohen and Cohen. Applied multiple regression/Correlation analysis for the behavioral science. 2nd ed. New Jersey: Lawrence Erlbaum Associates, Inc.; 1983, p. 59–61, 529
18. Tsunoda Y, Tokunaga T, Sugie T. Altered sex ratio of live young after transfer of fast- and slow-developing mouse embryos. Gamete Res 1985;12:301–4
19. Valdivia RPA, Kunieda T, Azuma S, Toyoda T. PCR sexing and developmental rate differences in preimplantation mouse embryos fertilized and cultured in vitro. Mol Reprod Dev 1993;35:121–6
20. Peippo P, Bredbacka P. Sex-related growth rate differences in mouse preimplantation embryos in vivo and in vitro. Mol Reprod Dev 1995;40:56–61
21. Avery B, Jorgensen CB, Madison V, Greve T. Morphological development and sex of bovine in vitro-fertilized embryos. Mol Reprod Devel 1992;32:265–70
22. Xu KP, Yadav BR, King WA, Betteridge KJ. Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 1992;31:240–52
23. Bernardi ML, Delouis C. Sex-related differences in the developmental rate of in vitro matured/in vitro fertilized ovine embryos. Hum Reprod 1996;11(3):621–6
24. Pergament E, Fiddler M, Cho N, Johnson D, Holmgren WJ. Sexual differentiation and preimplantation cell growth. Hum Reprod 1994;9:1730–2
25. Menezo YJ, Chouteau J, Torello J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. Fertil Steril 1999;72(2):221–4
26. Tarin JJ, Bernabeu R, Baviera A, Bonada M, Cano A. Sex selection may be inadvertently performed in in-vitro fertilization-embryo transfer programmes. Hum Reprod 1995;10:2992–8
27. Burgoyne P. A Y-chromosomal effect on blastocyst cell number in mice. Development 1993;117:341–5
28. Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL. HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci USA 1996;A93:161–5
29. Comiskey M, Goldstein CY, De Fazio SR, Mammolenti M, Newmark JA, Warner CM. Evidence that HLA-G is the functional homolog of Qa-2, the *Ped* gene product. Hum Immunol 2003;64:999–1004
30. Clements CS, Kjer-Nielsen L, Kostenko L, Hoare HL, Dunstone MA, Moses E et al. Crystal structure of HLA-G: a nonclassical MHC class I molecule expressed at the fetal-maternal interface. Proc Natl Acad Sci USA 2005;102(9):3360–5
31. Fuzzi B, Rizzo R, Criscuoli L, Noci I, Melchiorri L, Scarselli B et al. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. Eur J Immunol 2002;32:311–5
32. Noci I, Fuzzi B, Rizzo R, Melchiorri L, Criscuoli L, Dabizzi S et al. Embryonic soluble HLA-G as a marker of developmental potential in embryos. Hum Reprod 2004;20(1):138–46
33. Sher G, Keskintepe L, Nouriani M, Roussev R, Batzofin J. Expression of sHLA-G in supernatants of individually cultured 46-h embryos: a potentially valuable indicator of 'embryo competency' and IVF outcome. Reprod Biomed Online 2004;9(1):74–8

34. Yie SM, Balakier H, Motamedi G, Librach CL. Secretion of human leukocyte antigen-G by human embryos is associated with a higher in vitro fertilization pregnancy rate. Fertil Steril 2005;83(1): 30–6
35. Sher G, Keskintepe L, Fisch JD, Acacio BA, Ahlering P, Batzofin J et al. Soluble human leukocyte antigen G expression in phase I culture media at 46 hours after fertilization predicts pregnancy and implantation from day 3 embryo transfer. Fertil Steril 2005;83(5):1410–3
36. Warner CM, Comiskey M, Clisham PR, Brenner CA. Soluble HLA-G (sHLA-G)-A predictor of IVF outcome? J Asst Reprod Gen 2004;21:315–6
37. Van Lierop MJ, Wijnands F, Loke YW, Emmer PM, Lukassen HG, Braat DD et al. Detection of HLA-G by a specific sandwich ELISA using monoclonal antibodies G233 and 56B. Mol Hum Reprod 2002;8(8):776–84
38. Noriko S, Horotsugu H, Masanori Y, Takanori S, Motoko O, Katsuhiko H et al. Are in virto fertilized eggs able to secrete soluble HLA-G? Am J Reprod Immunol 2004;52(Suppl 1):8
39. Blaschitz A, Juch H, Volz A, Hutter H, Daxboeck C, Desoye G et al. The soluble pool of HLA-G produced by human trophoblasts does not include detectable levels of the intron 4-containing HLA-G5 and HLA-G6 isoforms. Mol Hum Reprod 2005;11(10):699–710
40. Sargent I. Does ‘soluble’ HLA-G really exist? Another twist to the tail. Mol Hum Reprod 2005;11(10):695–8
41. Hviid TVF. HLA-G in human reproduction: aspects of genetics, function and pregnancy complications. Hum Reprod Update 2005;Nov 9: [Epub ahead of print]