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Preimplantation genetic haplotyping: 127 diagnostic cycles demonstrating a robust, efficient alternative to direct mutation testing on single cells


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Pamela Renwick is a consultant clinical scientist and is head of the molecular genetic group within the Centre for Preimplantation Genetic Diagnosis at Guy's and St. Thomas' Hospital; this service provides preimplantation genetic diagnosis (PGD) for couples at risk of inherited single-gene defects. She introduced the approach of preimplantation genetic haplotyping in 2006 which won the centre a national Hospital Doctor award for innovation. She has recently joined the steering committee of the ESHRE PGD consortium.

Abstract Preimplantation genetic diagnosis using whole genome amplification and a haplotyping approach (PGH) was first described in 2006 and suggested as an efficient alternative to single-cell PCR for monogenic disorders. DNA from single cells was amplified using multiple displacement amplification; the resulting products were then tested using disease-specific PCR multiplexes applied under standard laboratory conditions to determine the haplotypes in the embryo. This study reports on a total of 127 completed biopsy cycles for 101 couples at risk of: autosomal recessive disease (71 cycles, 53 couples including one germ-line mosaic carrier), autosomal dominant disease (31 cycles, 26 couples including one germ-line mosaic carrier), X-linked recessive disease (18 cycles, 16 couples including one germ-line mosaic carrier), X-linked dominant disease (six cycles, five couples) and a double inheritance of both autosomal and X-linked recessive diseases (one cycle, one couple). Of these, 107 cycles reached embryo transfer. Overall success rates were: fetal heart beat-positive pregnancies (FHB+)/biopsy cycle = 28%; FHB+/embryo transfer = 34%; FHB+/couple = 36%; 26 babies born, 13 ongoing pregnancies. These data demonstrate that PGH provides a robust, efficient and successful alternative to single-cell PCR for monogenic diseases. 

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KEYWORDS: haplotype analysis, monogenic disease, PGD, PGH, whole genome amplification

Introduction

PGD for single-gene defects has been carried out worldwide since 1990; early attempts involved identification of male embryos for couples with sex-linked disease (Handyside et al., 1990). Since then, specific mutation tests have been developed for an ever-widening range of diseases (Goossens et al., 2008). The development and application of these specific tests requires design and optimisation of protocols that are robust at the single-cell level. These protocols include such techniques as restriction site assays and minisequencing (Burlet et al., 2005; Fiorentino et al., 2003; Renwick and Ogilvie, 2007) for direct mutation analysis. Most PGD centres now also routinely include analysis of up to three linked markers, requiring family-specific test design (Sermon and De Rycke, 2007). Optimisation includes testing a series of single cells in order to estimate amplification efficiency and allele drop-out (ADO). For application in a clinical diagnostic setting it is recommended to achieve >90% amplification and <10% ADO (Thornhill et al., 2005).

An alternative approach, preimplantation genetic haplotyping (PGH) has been proposed (Renwick et al., 2006). PGH relies on amplification of the whole genome from a single cell to give microgram quantities of DNA, which allows the testing of multiple loci using standard DNA-based PCR protocols. Multiplex microsatellite amplification was used in the published cases to haplotype and diagnose embryos for one couple with cystic fibrosis and one with Duchenne muscular dystrophy. Other groups have now introduced whole genome amplification and indirect haplotyping into their PGD programmes (Qubbaj et al., 2008; Ren et al., 2007).

Since 2006, PGH has been applied in over 100 biopsy cycles at the study centre. An overview of these cycles is reported here, and the advantages and disadvantages of PGH are discussed.

Materials and methods

Microsatellite markers close to the disease gene (preferably within 3 centimorgans of each end of the gene) were chosen and tested for informativity. In the absence of sufficient published microsatellite markers, novel ones were identified using the UCSC genome browser (<http://genome.ucsc.edu/>), see Supplementary Table. For each disease, a generic multiplex PCR of appropriate markers was designed using up to four fluorescent primer tags. Each work-up involved linkage analysis carried out by genotyping both partners and other family members of known carrier/affected status, as appropriate. The same generic PCR multiplex was used for every family with the same disease. Given available DNA, each couple took approximately 2 days to work up. Haplotypes were constructed, ensuring a minimum of two informative markers on each side of the gene (or mutation, if known). Using these criteria, 95% of couples requesting PGD were able to proceed. Figure 1 shows a haplotype pedigree of parental alleles and resulting embryos' genotypes for a typical autosomal recessive PGH cycle.

All PGH cycles were carried out under licence from the Human Fertilisation and Embryology Authority. Following ovarian down-regulation, stimulation and oocyte retrieval (Grace et al., 2006), single blastomeres were biopsied from

day-3 embryos. Whole genome amplification was carried out according to previously published protocols (Renwick et al., 2006). The amplified DNA was subjected to PCR using the multiplex PCR developed for the specific familial single-gene defect. PCR products were separated using an ABI 3730 Genetic Analyzer (Applied Biosystems, USA), and the products for each embryo were scored for the presence of the low-risk haplotypes. Following genetic diagnosis, one or two suitable embryos were selected on morphological criteria, and transferred on either day 4 or day 5 with cryopreservation of any remaining suitable embryos.

Table 1 shows the composition of the PCR multiplexes for each of the diseases for which PGH was offered. For dystrophin, which shows a high recombination frequency across the gene, all of the markers are intragenic. Table 2 shows the number of different mutations for each disease in the couples tested in this cohort.

Results

Table 3 summarizes the PGH cycles carried out at the study centre over a 2-year period. A total of 127 biopsy cycles were carried out for 101 couples, where the average age of the female partner was 34.5 years (range 23–41 years). Eleven different conditions were tested. One couple was at risk from two different conditions (haemophilia A and cystic fibrosis).

Of these 127 cycles, 42% were for autosomal recessive disease (25% risk of an affected pregnancy); 39% were for autosomal dominant disease, or autosomal recessive with one partner affected (50% risk). 14% were for X-linked recessive disease (25% risk); 5% were for X-linked dominant disease, (50% risk). 107 biopsy cycles reached embryo transfer. Overall success rates were: fetal heart beat-positive pregnancies (FHB+)/biopsy cycle = 28%; FHB+/embryo transfer = 34%; FHB+/couple = 36%; 26 babies, 13 ongoing pregnancies. Where prenatal diagnosis and/or testing at birth have been carried out, all results have been concordant with the PGH results.

Overall, for all inheritance modes, 91% (612/672) of embryos were diagnosed, of which 53% (324/612) were transferable. For couples with 75% risk of embryos being unaffected, 338/374 (90%) were diagnosed, of which 66% (223/338) were found to be transferable. For couples with 50% risk of embryos being unaffected, 267/291 (92%) were diagnosed, of which 36% (97/267) were found to be transferable. These figures may be lower than expected because of the presence of abnormal results (query aneuploid) accounting for 11% of the total number of embryos diagnosed. An adjustment can be made excluding embryos diagnosed as abnormal (query aneuploid): for couples with 75% risk of embryos being unaffected, 72% (223/309) were suitable for transfer; for couples with 50% risk of embryos being unaffected, 42% (97/230) were suitable for transfer. Following this adjustment, there is no significant difference between the observed and expected number of embryos suitable for transfer in either group. Embryos not suitable for transfer either had high-risk haplotypes, an abnormal diagnosis or had poor morphology. In addition, five out of 612 embryos (0.82%) gave results that could not be considered for transfer, due either to high ADO (resulting in insufficient informative markers) or to crossing-over between

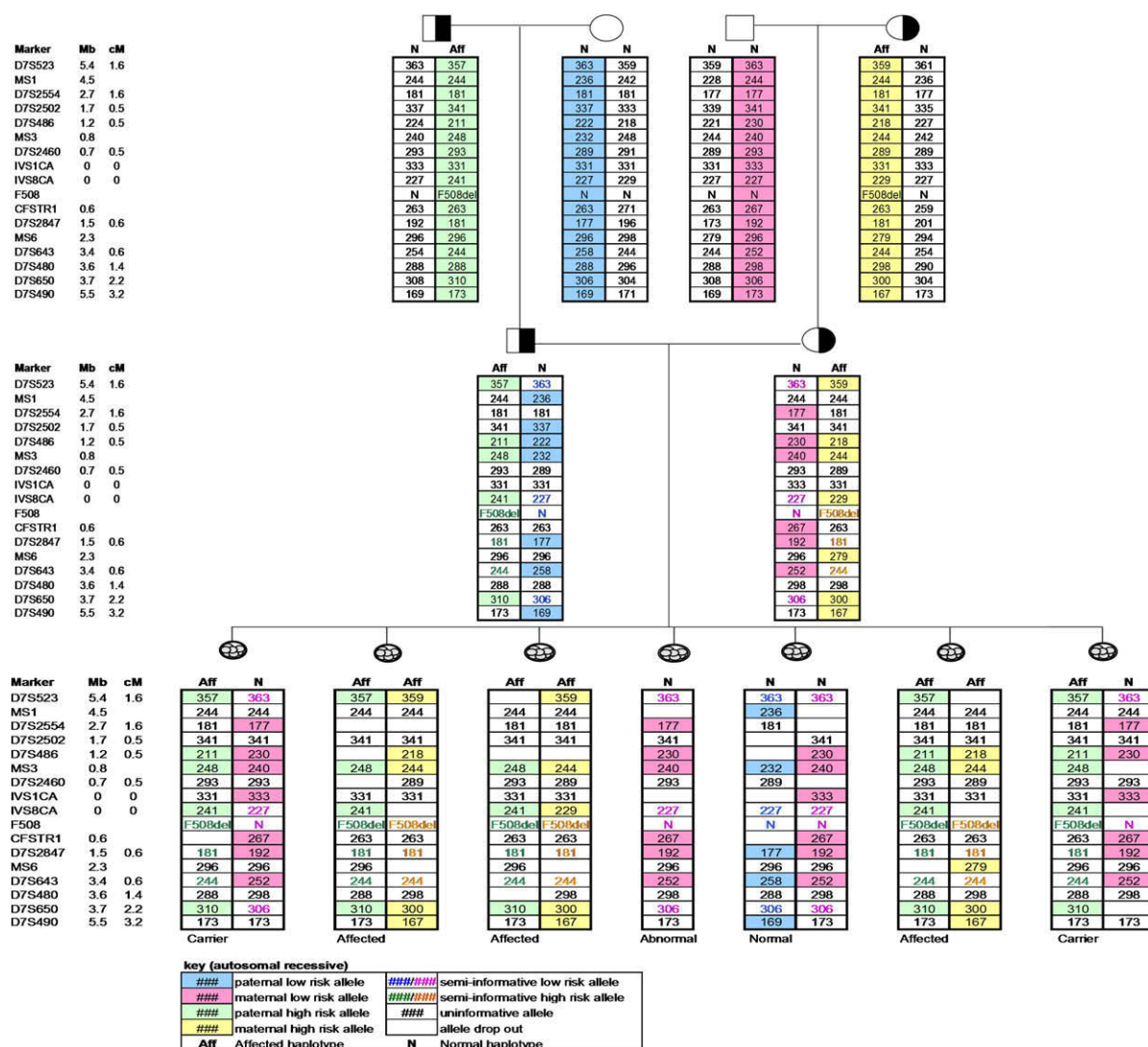


Figure 1 Preimplantation genetic haplotyping (PGH) case for cystic fibrosis in a couple known to be carriers of the p.Phe508del mutation who do not have a previous affected child; parental genotypes were therefore used to deduce the high- and low-risk haplotypes. Fully informative alleles are shown in coloured boxes; partially informative alleles (with known risk, but of unknown parental origin) are shown as coloured numbers. Alleles that are shared between high- and low-risk haplotypes are uninformative for the construction of haplotypes and shown in white boxes. Empty boxes show allele drop-out. Seven embryos (bottom row) were tested in the PGH case and identified two carriers, one non-carrier, three affected embryos and one abnormal (probably monosomy 7) embryo.

the flanking markers closest to the gene so risk could not be assigned to the recombinant haplotype.

Multiple displacement amplification (MDA) failed to produce amplified product for PCR in 9% of embryos. The average ADO rate following MDA was 27% (range 0–78%). Both MDA failure and ADO were probably dependent on the quality of the embryos tested.

Novel alleles are expected with microsatellite markers and can result from meiotic or somatic mutation. Novel alleles were observed in six out of 612 embryos (0.98%); so were a rare event. A novel allele would be seen for one allele at one marker so sufficient information would be available from surrounding markers to identify the haplotype.

Of the 164 embryos transferred, 98.8% had informative markers on both sides of the gene to determine the low-risk haplotype (misdiagnosis risk <1%).

Discussion

The development of PGD tests for single-gene disorders is challenging and can be time-consuming. By amplifying single-cell DNA using whole genome amplification, the PGH approach incorporates information from multiple polymorphic markers and thus identifies high-risk haplotypes, creating a generic test for any single-gene disorder. Whilst multiple markers can be tested at the single-cell level without prior genome amplification (Rechitsky et al., 2004; Spits et al.,

Table 1 Composition of the linkage multiplex PCR for each of the diseases for which PGH was offered.

Disease	Gene	Chromosome	Markers		
			Centromeric	Intragenic	Telomeric
Alport syndrome (X-linked)	<i>COL4A5</i>	Xq22.3	6 (2.8; 1)	2	7 (3.9; 3.3)
Cystic fibrosis	<i>CFTR</i>	7q31.2	7 (5.4; 1.6)	2	7 (5.5; 3.2)
Duchenne/Becker muscular dystrophy	<i>DMD</i>	Xp21.1	0	16 (11)	0
Familial partial lipodystrophy	<i>LMNA</i>	1q22	3 (2.9; 2.3)	1	4 (3.6; 4.6)
Fragile X syndrome	<i>FMR1</i>	Xq27.3	7 (3; 6.1)	0	8 (3; 7)
Haemophilia A	<i>F8</i>	Xq28	8 (3.9; 3.3)	3	7 (0.8; 0)
Epidermolysis bullosa (Herlitz junctional)	<i>LAMA3</i>	18q11.1	4 (6.9; 7)	2	4 (6.2; 6.7)
Epidermolysis bullosa (Herlitz junctional)	<i>LAMB3</i>	1q32.2	8 (2.7; 4)	2	5 (1.5; 4.8)
Huntington disease	<i>HTT</i>	4p16.3	8 (3.8; 11.3)	1	6 (2.4; 3.3)
Prader–Willi syndrome	<i>SNRPN</i>	15q11.2	3 (2.5; 2.5)	1	3 (1.5; 2.5)
Sickle-cell disease	<i>HBB</i>	11p15.4	8 (2.5; 3.6)	0	11 (3.9; 3.9)
Spinal muscular atrophy	<i>SMN1</i>	5q13	6 (3; 2)	1	7 (3; 3)

Values are number (max. Mb; centimorgan from gene) or number (centimorgan from gene).

Table 2 The number of different mutations in the genes for each disease in the couples tested in this cohort.

Disease	Gene	Number	Mutations	Couples
Alport syndrome (X-linked)	<i>COL4A5</i>	2	c.2880–10T>G, p.Gly3924Thr	2
Becker muscular dystrophy	<i>DMD</i>	2	p.Lys3312Glu, 45_48del	2
Cystic fibrosis	<i>CFTR</i>	13+	p.Phe508del, c.1138_39insG, p.Gly542X, p.Gly551Asp, p.Asp1152His, c.1717–1G>A, p.Trp1282X, c.3659delC, p.Arg345His, c.2657+2_2657+3insA, c.2052delA, p.Arg117His, p.Ser1235Arg + 3 unidentified	38
Duchenne muscular dystrophy	<i>DMD</i>	11	51_53del, 12_16del, p.Glu692 fs, 8_41del, 45del, p.Arg768X, 48_50del, 19dup, 3_7del, 2_5dup, p.Ile749 fs	13
Epidermolysis bullosa (Herlitz junctional)	<i>LAMA3</i>	1	c.7657delTA	1
Epidermolysis bullosa (Herlitz junctional)	<i>LAMB3</i>	2	p.Arg42X, p.Arg635X	3
Familial partial lipodystrophy	<i>LMNA</i>	1	p.Arg482Trp	1
Fragile X syndrome	<i>FMR1</i>	1	Expansion	3
Haemophilia A	<i>F8</i>	2	c.4339_4340insG, c.2696delG	2
Huntington disease	<i>HTT</i>	1	Expansion	24
Prader–Willi syndrome	<i>SNRPN</i>	1	IC deletion	1
Sickle-cell disease	<i>HBB</i>	1	HbS	6
Spinal muscular atrophy	<i>SMN1</i>	1	SMN1 deletion	6

2006), this approach involves labour-intensive PCR protocols, and is usually carried out alongside direct mutation testing, requiring extensive family-specific work-up.

PGH for the cases reported here used whole genome amplification by MDA; this technique is known to be associated with a high ADO rate (Burlet et al., 2006; Handyside et al., 2004). Whilst high ADO is unacceptable in testing for specific mutations, due to the possibility of

misdiagnosis or non-diagnosis, the strength of PGH lies in the number of markers used in each test (around 15); where there is amplification of at least one informative marker on each side of the gene, the risk of misdiagnosis is equal to the probability of a double recombination event between these informative markers (<1.0%). Drop-out of some markers in these cases is therefore extremely unlikely to result in misdiagnosis, due to redundancy of informative markers.

Table 3 Summary of the PGH cycles carried out at the study centre over a 2-year period.

<i>Disease according to prior risk of an affected child</i>	<i>Biopsy cycles</i>	<i>Cycles to ET</i>	<i>Couples</i>	<i>Embryos biopsied</i>	<i>Results obtained</i>	<i>Suitable for transfer</i>	<i>HCG+</i>	<i>FHB+</i>	<i>FHB+/biopsy cycle</i>	<i>FHB+/ET</i>	<i>FHB+/couple</i>	<i>FHB</i>	<i>Deliveries</i>	<i>Babies born</i>	<i>Ongoing pregnancies</i>
<i>Autosomal (25%)</i>															
Cystic fibrosis	34	29	26	193	175	115	14	13	38	45	50	15	8	10	4
Epidermolysis bullosa (Herlitz junctional)	7	6	4	27	26	18	1	1	14	17	25	1	0	0	1
Sickle-cell disease	4	3	4	16	16	11	2	1	25	33	25	1	0	0	1
Spinal muscular atrophy	8	8	6	33	29	18	2	2	25	25	33	2	2	2	0
Total	53	46	40	269	246	162	19	17	32	37	43	19	10	12	6
<i>X-linked (25%)</i>															
Duchenne/Becker muscular dystrophy	17	17	15	101	88	58	12	8	47	47	53	10	6	7	2
Haemophilia A	1	1	1	4	4	3	1	0	0	0	0	0	0	0	0
Total	18	18	16	105	92	61	13	8	44	44	50	10	6	7	2
<i>Autosomal (50%)</i>															
Sickle-cell disease	2	1	2	11	10	3	0	0	0	0	0	0	0	0	0
Cystic fibrosis	16	12	11	87	80	27	6	3	19	25	27	4	2	3	1
Huntington disease	29	22	24	150	139	51	11	7	24	32	29	8	3	3	4
Prader–Willi syndrome	1	1	1	5	5	2	1	1	100	100	100	1	1	1	0
Familial lipodystrophy	1	1	1	2	2	2	0	0	0	0	0	0	0	0	0
Total	49	37	39	255	236	85	18	11	22	30	28	13	6	7	5
<i>X-linked (50%)</i>															
Alport syndrome	3	2	2	26	22	7	0	0	0	0	0	0	0	0	0
Fragile X syndrome	3	3	3	10	9	5	0	0	0	0	0	0	0	0	0
Total	6	5	5	36	31	12	0	0	0	0	0	0	0	0	0
<i>Double disease (56%)</i>															
Haemophilia A + cystic fibrosis	1	1	1	7	7	4	0	0	0	0	0	0	0	0	0
Grand total	127	107	101	672	612	324	50	36	28	34	36	43	22	26	13

Values are number or percentage. ET, embryo transfer; FHB+, fetal heart beat-positive pregnancy; HCG+, positive human chorionic gonadotrophin test.

Where there are informative markers on one side of the gene only, the risk of misdiagnosis rises to the rate of recombination between the gene and the nearest informative marker. In one case, after discussion and consent from the couple, one embryo with a 4% misdiagnosis rate was transferred (being the only embryo suitable for transfer); however, the protocol has since been revised such that only embryos with less than 2% misdiagnosis risk are considered for transfer.

The average ADO in this data set was 27% (range 0–78%); despite this, the percentage of embryos for which no diagnosis could be made due to ADO or recombination was 0.82%. The total figure for embryos with transferable results was 53%. This compares favourably with data from single-cell PCR cycles for single-gene disorders (Goossens et al., 2008), showing that the high ADO following MDA is not a contraindication for the use of this technology.

The implementation of PGH has meant that once a disease-specific multiplex PCR has been developed, family-specific test design for other couples at risk from the same disease is no longer necessary. Table 2 shows the mutations present in the couples who underwent PGH. These couples carried a total of 38 different mutations, for which at least 38 individual family-specific tests would have been required for PGD using direct mutation testing. The table shows that these couples were treated using only 12 different test designs, demonstrating the inherent efficiency of PGH as a diagnostic tool. The disparity between the number of test designs required using the two different approaches is expected to increase as more cycles are carried out.

Couples with X-linked recessive diseases, which had been previously tested by female embryo selection using fluorescence in-situ hybridization, and X-linked dominant diseases can now be offered PGH, allowing the transfer of normal males; this is ethically more acceptable than discarding all male embryos (50% of which are likely to be normal). In addition, it increases the number of transferable embryos, and therefore the chances of establishing successful pregnancies. Further ongoing development of the PGH service is currently targeting sex-linked conditions which are still generally tested by fluorescence in-situ hybridization (Goossens et al., 2008).

One disadvantage of PGH is the need for material from informative family members in order to construct haplotypes for embryo diagnosis. This has been problematic for some of the families, in particular those with sickle-cell disease; this is because, for many of these, relevant family members are not available. In some cases, it was possible to use sperm haplotyping, but this is a labour-intensive procedure which does not always produce informative results. Thus there may be cases where direct mutation testing on DNA from a single cell is the only option. It has been suggested that incorrect assignation of disease-carrying haplotype may occur if there are no affected children available for testing and recombination may occur in an intervening generation. However, even when phase is determined using a previously affected child, a crossover event may have occurred, meaning that the child has a recombinant haplotype. This will become apparent during diagnosis of the case and the parental phase can be corrected. The frequency of crossing-over is dependent on the genetic distance of the markers from the gene, so is more commonly seen at more distal markers and does not affect the diagno-

sis of the embryos. For all genes tested, there are markers within 1 centimorgan of the gene, so the likelihood of a crossover having occurred between the marker and the gene is less than 1%. In addition, if further family members are available they can be tested in a pre-case work-up to confirm the haplotypes constructed for the couple.

Pregnancy rates for the PGH couples (Table 3) in this study are similar to those for couples treated at the same centre for chromosome rearrangements by FISH, and higher than the average rates for single-gene defects reported to the European Society for Human Reproduction and Embryology PGD consortium (Goossens et al., 2008). Couples referred for Duchenne/Becker muscular dystrophy exclusion were particularly successful, with a fetal heart beat-positive pregnancy rate per couple of 53%. These pregnancies occurred within an ongoing strategy to reduce multiple pregnancies and, out of 107 cycles that reached embryo transfer, 64 (60%) had only one embryo transferred, most often electively.

It is concluded that PGH is a robust and successful technique for the exclusion of single-gene defects in embryos from at-risk couples. It has significant advantages over other commonly-used technologies; test development for new diseases is fast and economical and does not require the use of specialized single-cell facilities, as the MDA products can be handled under standard laboratory conditions. In addition to the diseases for which data is published here, PCR multiplexes have been developed for myotonic dystrophy and von Hippel Lindau's disease and a single test panel has been developed for X-linked hydrocephalus, Rett's syndrome, adrenoleukodystrophy and incontinentia pigmenti which lie within a gene-rich region on Xq27–28. These PCR multiplexes are now available for PGH for at-risk couples. Further expansion of this repertoire of diseases is in progress.

Acknowledgements

The authors thank their fellow members of the Guy's and St. Thomas' Assisted Conception Unit and Centre for Preimplantation Genetic Diagnosis and their colleagues in the molecular and cytogenetics laboratories. Funding was provided by Guy's and St. Thomas' Charity and Evelina Children's Hospital. The authors also acknowledge Prof John McGrath and Dr Hiva Fassihi for their involvement with the Herlitz junctional epidermolysis bullosa work which was kindly funded by the Dystrophic Epidermolysis Bullosa Research Association (DebRA UK).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.rbmo.2010.01.006](https://doi.org/10.1016/j.rbmo.2010.01.006).

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Declaration of interest: The authors report no financial or commercial conflicts of interest.

Received 27 February 2009; refereed 27 March 2009; accepted 16 December 2009.