TIMELINE

Prenatal and pre-implantation genetic diagnosis

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Abstract | The past decade has seen the development of technologies that have revolutionized prenatal genetic testing; that is, genetic testing from conception until birth. Genome-wide single-cell arrays and high-throughput sequencing analyses are dramatically increasing our ability to detect embryonic and fetal genetic lesions, and have substantially improved embryo selection for *in vitro* fertilization (IVF). Moreover, both invasive and non-invasive mutation scanning of the genome are helping to identify the genetic causes of prenatal developmental disorders. These advances are changing clinical practice and pose novel challenges for genetic counselling and prenatal care.

Worldwide, millions of individuals are affected by dominant or recessive genetic mutations that cause highly penetrant, early-onset severe phenotypes, or late-onset life-threatening phenotypes. Overall, the Online Mendelian Inheritance in Man (OMIM) database currently reports more than 4,600 phenotypes with a genetic cause. Indeed, every individual carries alleles that in a homozygous state could cause recessive disorders[1]. In addition, 0.17% of the human population are carriers of an apparently balanced translocation that often causes infertility or recurrent miscarriages (owing to embryonically lethal segmental aneuploidies in the conceptuses), or severe birth defects in offspring[2]. Furthermore, the incidence of aneuploidies in conceptuses increases with maternal age[3], and in the past four decades the age of women giving birth for the first time has continuously risen in Western societies[4,5].

To avoid the transmission of pathogenic genetic variants and to enable early detection of genetic disorders, prenatal genetic testing is offered. In the case of a fetus with developmental anomalies detected by ultrasonography, genetic testing can potentially provide an accurate diagnosis and enable parents to make an informed decision about the pregnancy. For couples who are known carriers of mutant alleles, pre-implantation genetic diagnosis (PGD) enables the detection of genetic disorders in embryos that have been fertilized *in vitro*, thereby avoiding the transmission of these disorders to offspring. Moreover, a preliminary meta-analysis of several small randomized clinical trials suggested that pre-implantation genetic screening (PGS) for aneuploidies improves pregnancy outcome following *in vitro* fertilization (IVF)[6].

In this Timeline article, we provide an overview of the evolution of both prenatal and pre-implantation genetic testing (FIG. 1). We then expand on the current and near-future development and implementation of tools for genome-wide single-cell and cell-free fetal DNA (cfDNA) analysis, and discuss both their power and limitations. Finally, we speculate on future developments in prenatal and pre-implantation genetic diagnostics.

Prenatal genetic testing

The first human prenatal genetic test was performed in 1966, when karyotyping of cultured cells obtained from amniotic fluid sampling provided a chromosomal view of the fetus[7]. The following year, the first prenatal diagnosis of a chromosomal abnormality was achieved[8]. The introduction of chromosome-banding techniques in the 1970s led to an increase in resolution and enabled the detection of segmental chromosomal imbalances. Over the next 40 years, karyotyping became the gold standard for prenatal diagnosis for the genome-wide detection of genomic rearrangements, despite its inherent limitations. These limitations include the need for an invasive procedure to obtain a tissue sample, culturing of cells, visual screening for numerical or structural chromosome anomalies (which requires skilled analysts and is time consuming), and limited resolution. Hence, there has been an unceasing quest for improved DNA-based molecular genetics techniques.

Fluorescence *in situ* hybridization (FISH) was the first test that allowed aneuploidy detection in interphase nuclei, which eliminated the need to culture cells and provided results in a mere 2 to 3 days[9]. Other molecular diagnostic tests, such as quantitative fluorescence PCR (QF-PCR)[10] and multiplex ligation-dependent probe amplification (MLPA)[11], subsequently competed with FISH. However, these techniques suffer the disadvantage that they interrogate only specific genomic loci, which need to be selected a priori. Anomalies that are visible on ultrasound images or prior family history can often provide a clinical diagnosis to direct FISH- or PCR-based tests to a specific locus in the genome. However, in cases in which the clinical indication is imprecise, these methods often fail to identify the causal genetic alteration.

The development of array comparative genomic hybridization (aCGH) provided a comprehensive, rapid, genome-wide screening strategy for the detection of DNA copy number imbalances in patients with developmental disorders[12]. This application paved the way for the use of this technology for prenatal testing[13]. In aCGH, fetal DNA and reference DNA are differentially labelled with fluorescent dyes and are co-hybridized to complementary single-stranded DNA molecules on the array. Following competitive hybridization, the observed fluorescence intensities on the probes allow the determination of a relative DNA copy number profile in the fetus, the resolution of which relies on the type and

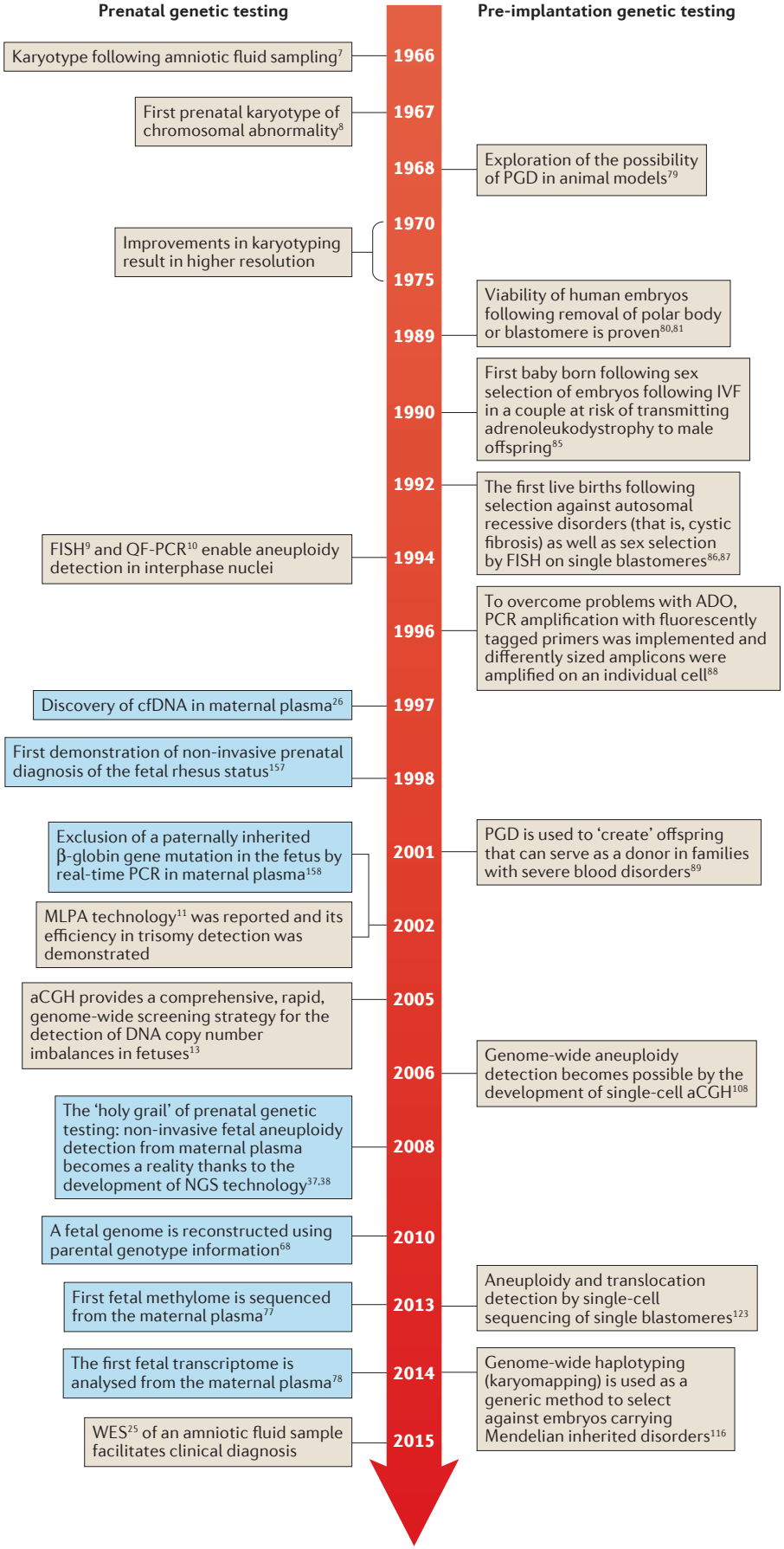


Figure 1 | **Timeline of prenatal and pre-implantation genetic diagnostics.** A selection of key milestones in the implementation of genetic tests for invasive (grey) and non-invasive (blue) prenatal (left side) and pre-implantation (right side) diagnosis. aCHG, array comparative genomic hybridization; ADO, allele drop out; cfDNA, cell-free fetal DNA; FISH, fluorescence *in situ* hybridization; IVF, *in vitro* fertilization; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; PGD, pre-implantation genetic diagnosis; QF-PCR, quantitative fluorescence PCR; WES, whole-exome sequencing.

number of targets on the array[14]. The first clinical prenatal arrays used long (~150 kb) sequences of human genomic DNA — cloned into bacterial artificial chromosomes (BACs) — as targets and interrogated the human genome every 1 Mb (REF. 13). Subsequently, these arrays have been largely replaced with higher-resolution array platforms containing artificially synthesized oligonucleotides as targets. Modern array platforms enable thorough interrogation of specific loci or the entire human genome, and even allele-specific characterization of single-nucleotide polymorphisms (SNPs); they are commonly referred to as oligonucleotide arrays and SNP arrays, respectively, or as chromosomal microarrays in general (TABLE 1).

Currently, invasive prenatal testing is indicated for all fetuses with structural malformations detected by ultrasonography. There is a general consensus that chromosomal microarrays should be the first-tier cytogenetic test for prenatal diagnosis[15–17], as they provide a higher diagnostic yield, detecting both larger and submicroscopic copy number variants (CNVs) that are undetectable by conventional karyotyping. Recent prospective studies report that chromosomal microarrays increase the detection rates of pathogenic CNVs by 6–8% compared with karyotyping in the presence of ultrasound anomalies[15,17,18]. Furthermore, arrays provide the ability to detect a number of microdeletion and microduplication syndromes, some of which may cause severe childhood developmental disorders but may not show any fetal abnormalities using classic ultrasound techniques[15]. In the absence of ultrasound abnormalities, chromosomal microarrays were reported to increase the detection rate above karyotyping by 1–2%[15,19]. Clinically significant findings unrelated to the initial referral reason are detected in 0.5–3.6% of cases[17].

Most developmental disorders are not caused by CNVs, however, but by single-nucleotide variants (SNVs) or insertions or deletions (indels)[20]. Whole-exome sequencing (WES) studies of children and adults with developmental disorders have shown a diagnostic yield of about 25%[21,22]. Hence, the use of WES in the prenatal setting is being explored. WES of 30 non-aneuploid fetuses and neonates with diverse physical abnormalities that were first identified by prenatal ultrasonography identified 35 *de novo* SNVs, small indels, deletions or duplications, of which three (10% of the cohort) were pathogenic[23]. In five other cases (17%), *de novo* or inherited recessive or X-linked variants in plausible candidate genes were identified. In another study, WES was performed on DNA extracted from chorionic villi or amniocytes in 24 fetuses with unexplained ultrasound findings[24]. In 21% of the cases, WES provided definitive diagnoses.

Clinical implementation of fetal WES during pregnancy still poses several challenges. Studies to date have been retrospective, and analyses have yet to be performed in a timeframe that would enable parents to make informed decisions regarding the pregnancy. Clinical implementation will require the development of large-scale and rapid analytical and interpretation pipelines. In addition, to facilitate the interpretation of genetic variants determined prenatally, it will be necessary to develop a vastly more detailed knowledge base on the genetic causes of prenatal developmental disorders. Nevertheless, feasibility in principle has been demonstrated[25], and large-scale studies have been launched[26].

Non-invasive prenatal testing

Owing to the risk of procedure-induced miscarriage and the technical difficulties associated with invasive testing, non-invasive prenatal testing (NIPT) to determine the genetic constitution of the fetus has long been considered the 'holy grail'. It has been known for some time that fetal cells are present in the maternal circulation, albeit at very low numbers, meaning that their enrichment and subsequent culture has remained challenging[27]. Clearly, single-cell 'omics' techniques show great potential for NIPT if technical challenges of single-cell genome analysis (BOX 1) are overcome and methods for the isolation of fetal cells from maternal blood improve and sufficient yields can be obtained consistently.

Table 1 | **Prenatal diagnostic tests**

	Karyotyping	FISH	Microsequencing	aCGH	SNP array	Targeted NIPT	Genome-wide NIPT
Genetic lesion							
Monogenic disorders	–	–	+	–	–	–	–
Whole-chromosome aneuploidy	+	+/–*	–	+	+	–	+
Segmental chromosomal aneuploidies	+/–‡	+	–	+	+	+/–	+/–
Submicroscopic deletions or duplications	+/–‡	+	–	+	+	–	–
Uniparental disomy	–	–	–	–	+	–	–
Methodology							
Invasive testing required (AF or CVS)	+	+	+	+	+	–	–
cfDNA extraction required	–	–	+/–	–	+/–	+	+

+, possible; –, not possible; +/–, possible but requires specific work-up; aCGH, array comparative genomic hybridization; AF, amniotic fluid; cfDNA, cell-free fetal DNA; CVS, chorionic villus sampling; FISH, fluorescence *in situ* hybridization; NIPT, non-invasive prenatal testing; SNP array, single-nucleotide polymorphism array. *Limited by number of fluorochromes. ‡Limited by size.

Cell-free fetal DNA. With the discovery of circulating cfDNA in maternal plasma in 1997, non-invasive prenatal diagnosis first became feasible[28,29]. cfDNA was shown to be trophoblast-derived[30], fragmented DNA with a size distribution of 140–180 bp (REF. 31) that is present from 3% to as much as 20%[32] against a high maternal cfDNA background. cfDNA analysis has enabled fetal sex assessment[33], fetal rhesus D blood group genotyping[34], the detection of monogenic disorders[35,36] and of aneuploidy, albeit not until the development of next-generation sequencing (NGS).

Non-invasive fetal aneuploidy detection by NGS applies counting statistics to up to tens of millions of both maternal and fetal sequencing reads to identify subtle changes in the amount of reads mapping to particular chromosomes as a result of fetal aneuploidy[37,38] (FIG. 2). In addition to whole-genome sequencing, targeted sequencing approaches allow aneuploidy detection of chromosomes 13, 18, 21, X and Y. With these methods, a set of loci across the chromosomes of interest are selected for NGS. Two approaches are commonly used: targeted counting[39] and SNP-based methods[40,41] (FIG. 2). In the first group, the loci of interest are selected by hybridization of custom-designed ligation oligonucleotides, amplification by multiplex PCR and subsequent sequencing. In the second category, the loci of interest are selected for subsequent enrichment based on their SNP content. Enrichment can be performed either by capture probes or by a highly multiplexed PCR (up to 20,000 amplicons)[42]. Statistical differences in the number of reads or SNP distributions to the chromosome-specific loci are deduced (FIG. 2).

Targeted genome strategies have also been used to analyse the epigenetic differences between fetal and maternal cfDNA nucleic acids[43], or to determine the allelic ratio of the genes on chromosome 21 that are exclusively expressed in the placenta and not in maternal haematopoietic cell lines[44]. Several large-scale clinical validation studies have been performed, each showing high sensitivity and specificity of non-invasive fetal aneuploidy detection by NGS[39,45,46]. These methods also enable the specific detection of sex chromosome aneuploidies. This may be of questionable clinical utility given the lower accuracy compared with trisomy 21 detection[47–49] due to maternal chromosome X copy number mosaicism[50]. Moreover, the variability in phenotype of many disorders of sex development — for example, monosomy X (Turner syndrome), XXY (Klinefelter syndrome), XYY and XXX individuals, some of which are identified only in adulthood owing to fertility problems — render prenatal genetic counselling and subsequent decision making particularly challenging[51].

NIPT was initially aimed at the detection of trisomy 13, 18 and 21, but random sequencing also enables the detection of other fetal aneuploidies, segmental imbalances and even submicroscopic

Box 1 | **Technical challenges of single-cell genome analysis**

Current genome-wide screening platforms require several nanograms of input material, necessitating whole-genome amplification (WGA) of the 7 picograms of DNA of a single cell before analysis. Three main principles for WGA are commonly used: PCR, multiple displacement amplification (MDA) or a combination of MDA with PCR (MDA–PCR). Unfortunately, none of the available WGA methods is unbiased. The span of the genome that is effectively amplified, the amplification bias due to local concentration differences in guanine and cytosine bases (% G+C bias), as well as the prevalence of chimeric DNA molecules, allele drop outs (ADOs), preferential allelic amplifications and nucleotide misincorporations during amplification vary substantially between different WGA approaches. A major challenge in single-cell genome analysis is discriminating such WGA artefacts from genuine genetic variants present in the cell before WGA. Artefacts such as unevenness in amplification and chimeric DNA molecules may be misinterpreted as real DNA copy number and structural variants in the cell, respectively. Similarly, ADO and preferential allelic amplification events across heterozygous single-nucleotide variants (SNVs) in the cell as well as base misincorporations following WGA may lead to erroneous SNV calls in the single cell. As a consequence, a particular WGA method may be preferred over another depending on the desired genetic readout from the cell[122,123,148,149].

MDA is often the preferred method for both detecting and genotyping SNVs[123,148–150]. The resultant WGA products can cover more than 90% of the genome following sequencing[149], ADO rates as low as approximately 14% have been reported[114], and the bacteriophage Φ29 DNA polymerase that is commonly used for MDA has an error rate an order of magnitude lower than most polymerases used in other WGA methods, which often lack proof-reading capacity[149]. However, copy number profiles after MDA can be 'noisy' (REFS 114,122,123) and chimeric DNA-amplification products contort the original genomic architecture of the cell[123,151]. Lowering MDA reaction volumes and/or the amount of DNA amplification by using microfluidics has been shown to further reduce this amplification bias[149,152,153]. PCR- and MDA–PCR-based WGA products are preferable for copy number profiling[114,123,149] as the amplification bias is less strongly influenced by additional cycles of the WGA reaction[149]. Although these methods have also been used for SNP genotyping[122,123] and base mutation detection[122], they generally yield lower genome coverage and less faithful nucleotide copying[123,149].

CNVs[52–57]. The incidence of atypical chromosomal aneuploidies is about 0.3%[52,58–60]. In an important subset of aneuploidies, the chromosomal imbalance may be confined to the placenta and, therefore, may not adversely affect fetal development[61]. However, there is a risk of placental insufficiency and fetal growth restriction owing to the abnormal placental karyotype[61], as well as a risk of mosaic fetal aneuploidy and/or fetal uniparental disomy (UPD) owing to a trisomy rescue.

Given the clinical importance of segmental aneuploidies and microdeletion syndromes (for example, 1p36 deletion (OMIM 607872), cri-du-chat (OMIM 123450), DiGeorge syndrome (OMIM 188400), Wolf–Hirschhorn (OMIM 194190), Prader–Willi (OMIM 176270), Angelman (OMIM 105830) and Miller–Dieker lissencephaly (OMIM 247200)), several groups are exploring analytical methods to increase the resolution achievable by NIPT, for which proof-of-concept has been provided[57,62,63]. However, test sensitivity is a function of fetal fraction, read depth and size of the fetal CNVs; with current sequencing depths, the positive and negative predictive values are too low for a generalized introduction of this test into the clinic[61,62].

Evolution of prenatal genetic testing. With further cost reductions and technological and algorithmic advances, the resolution and scope of non-invasive testing will increase. NIPT is already becoming a screening test and replacing less accurate biochemical tests[64–66]. Increased sequencing depth will allow the accurate detection of genetic disorders, eventually reaching the resolution of current array analyses on invasive prenatal samples. Several groups have demonstrated proof-of-principle that non-invasive cfDNA analysis enables the reconstitution of the total fetal genome sequence[31,67,68], an approach that could eventually lead to non-invasive prenatal whole-genome sequencing and the detection of *de novo* mutations. This will ultimately make invasive sampling obsolete.

Considering that cfDNA is a result of apoptosis- or necrosis-related cell death of maternal cells of diverse tissues[69,70], its constitution is representative of the whole body. As a consequence, cfDNA screening offers the potential to monitor fetal and maternal health beyond mere aneuploidy detection. Already, several reports show that the presence of (presymptomatic) cancers can be detected by NIPT[71–73]. In addition, random genome-wide cfDNA profiling also detects maternal constitutional CNVs[52,74]. These can be relevant for pregnancy management, the fetus or future reproductive choices[75].

Recent studies also show the potential to analyse the plasma beyond genome analyses[76]. In addition to the fetal genome, the fetal methylome[77] and transcriptome[78] have been obtained by deep sequencing. Their analysis will yield valuable information on both fetal and maternal health. Taken together, we anticipate that genomic analyses of maternal plasma will substantially improve overall pregnancy management.

Pre-implantation genetic diagnosis

In parallel to prenatal genetic testing, an alternative form arose for the genetic analysis of IVF-derived embryos. In 1968, Robert Edwards and Richard Gardner reported the successful transfer of sexed pre-implantation rabbit blastocysts, which foreshadowed the advent of human PGD[79]. Subsequently, the groundwork for the clinical application of PGD for various conditions began.

PGD requires the application of assisted reproductive techniques. Following gamete collection from both partners, IVF is performed. In 1989, it was first demonstrated that the polar body or one or more embryonic cells could be biopsied from the cleavage-stage embryo or the trophectoderm without compromising embryonic viability[80,81]. The developing embryo can thus be tested for the inheritance of a risk allele (or alleles) causing a particular disorder based on the genetic analysis of the polar body, a single or a pair of blastomeres, or a small number of trophectoderm cells that were biopsied from a human embryo[82,83] (FIG. 3a). Traditionally, DNA imbalances are detected by FISH, and nucleotide mutations in the single cell are detected by a multiplex PCR diagnostic test followed by microsequencing[82] (FIG. 3b; TABLE 2). Embryos diagnosed as being free of the disease being tested or having a balanced genetic constitution can subsequently be transferred to the woman's uterus on day 4 or 5 after fertilization (FIG. 3c). Approximately 24% of implanted embryos will make it to term[84].

In 1990, the first successful human PGD analyses were performed on polar bodies biopsied from fertilized oocytes[81], which allow screening only for the inheritance of a maternal mutation, and on blastomeres[85]. Using PCR amplification of Y-chromosome-specific sequences, sex selection was

performed on embryos following IVF in a couple at risk of transmitting recessive X-linked mental retardation[85] — in this case, adrenoleukodystrophy — to male offspring. The first live births following selection against autosomal recessive disorders (that is, cystic fibrosis)[86], as well as sex selection by FISH on single blastomeres[87], were reported 2 years later.

Despite initial successes, it soon became clear that in some cases the target sequence failed to amplify. To overcome this problem, PCR amplification using fluorescently tagged primers was implemented for single-cell analysis[88]. By using different fluorescent tags or designing differently sized amplicons, a multiplex PCR can be performed on an individual cell.

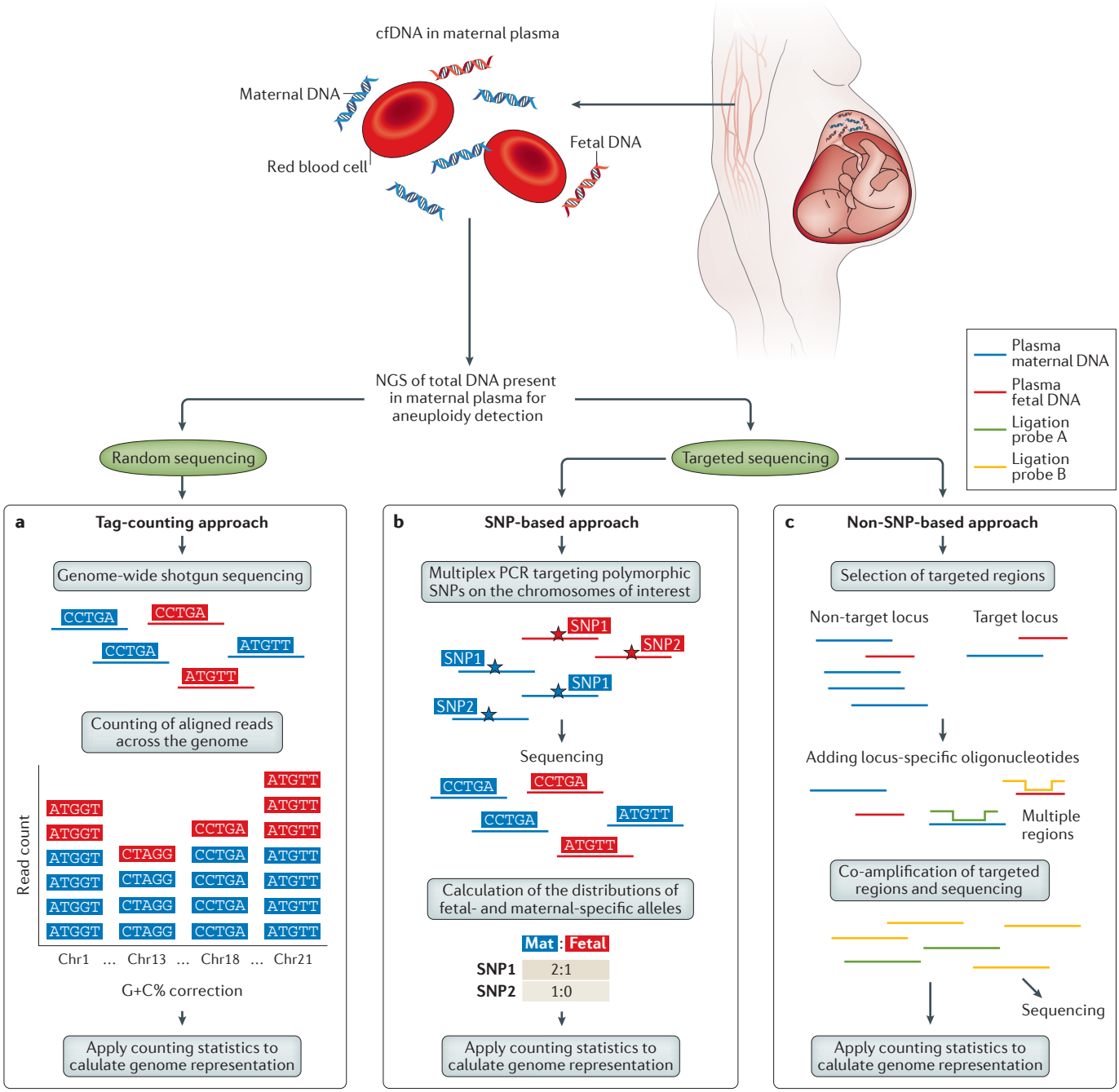


Figure 2 | **Cell-free fetal DNA aneuploidy screening methods.** Two major strategies are widely implemented in routine cell-free fetal DNA (cfDNA) aneuploidy testing; random and targeted sequencing. **a** | Random sequencing applies counting statistics on the reads that map to the human reference genome. **b** | In targeted sequencing, selected loci with single-nucleotide polymorphisms (SNPs; red and blue stars) are analysed. The maternal (mat) genotypes are used to model the allelic distributions for every ploidy scenario and based on the actual SNP distributions the likelihood for each hypothesis is calculated. **c** | In an alternative targeted sequencing approach, selected fragments are enriched, sequenced and mapped to the human genome. A chromosome proportion metric is calculated by applying counting statistics. Chr, chromosome; NGS, next-generation sequencing. Adapted with permission from REF. 159, Elsevier.

Typically, one amplified fragment contains the mutation that is associated with the disorder and one or more fragments contain polymorphic markers that are closely linked to that mutation. Microsequencing of these fragments allows the identification of the inherited parental allele and indicates cases in which allele drop out (ADO) is likely to have taken place, thereby increasing the PGD accuracy by 1,000-fold.

PGD is now offered mainly to couples carrying autosomal dominant, recessive or X-linked Mendelian disorders, chromosomal rearrangements or mitochondrial disorders. In addition, PGD has been used to 'create'

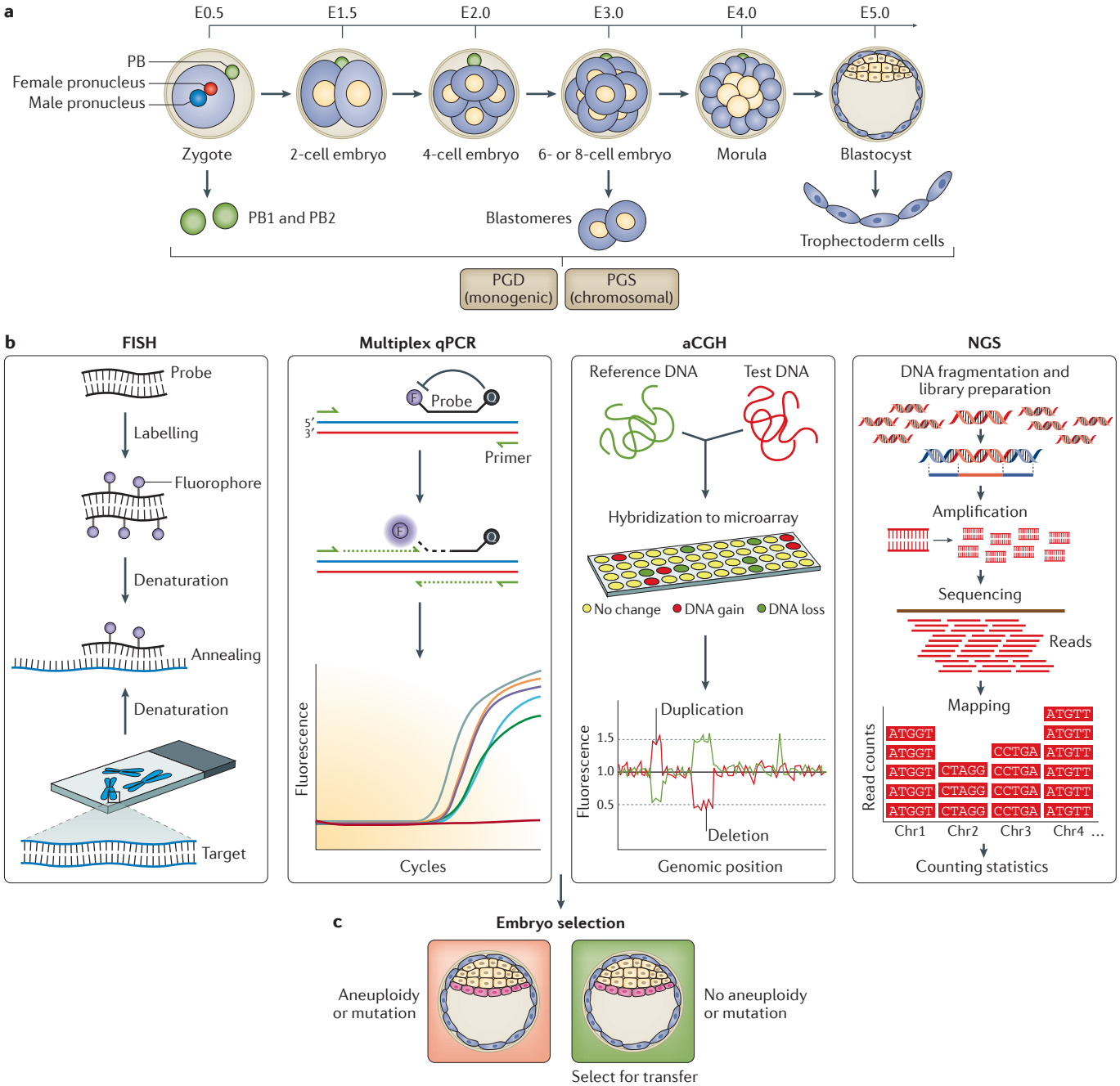


Figure 3 | **Pre-implantation genetic diagnosis and screening. a** | The developmental stages (for example, embryonic day 1.5 (E1.5)) of an *in vitro* fertilization (IVF)-derived embryo and the type of cells used for pre-implantation genetic diagnosis (PGD) and pre-implantation genetic screening (PGS). The samples can be polar bodies (PBs), a single or pair of blastomeres or a small aggregate of cells from the trophectoderm. **b** | The methods routinely applied in diagnostic services are fluorescence *in situ* hybridization (FISH; for aneuploidy screening and/or diagnosis), multiplex quantitative PCR (qPCR; for mutation detection and/or genotyping), array comparative genomic hybridization (aCGH; for aneuploidy screening and/or diagnosis) and low-coverage next-generation sequencing (NGS; for aneuploidy screening). **c** | Embryos are prioritized for transfer to the uterus based on their genotypes (PGD) or their chromosome content (PGS). Chr, chromosome, F, fluorophore; Q, quencher. Part **b**, far-left panel, adapted from REF. 160, Nature Publishing Group. Part **b**, middle-right panel, adapted from REF. 161, Nature Publishing Group.

offspring that can serve as a donor in families with severe blood disorders. In 2001, the first child selected by pre-implantation human leukocyte antigen (HLA) genotyping was born, so that he could become a donor of haematopoietic stem cells for his sister who had Fanconi anaemia[89]. Since the clinical implementation of PGD, the European Society of Human Reproduction (ESHRE) PGD Consortium has recorded the number of PGD cycles performed in Europe and elsewhere over the past 10 years. In 2010, the most recent year for which numbers are available, 2,753 PGD cycles were performed[90], of which 1,071 were for chromosomal abnormalities, 108 were for X-linked disorders and 1,574 were for Mendelian disorders.

Pre-implantation genetic screening

PGS, also known as PGD for aneuploidy screening (PGD-AS), analyses whether a single cell or a small number of cells biopsied from a pre-implantation embryo is euploid before transferring it to the uterus. Under the premise that a major cause for the low success rate of IVF (~30%)[91] was the increased presence of aneuploid embryos[92], especially those resulting from meiotic chromosomal segregation errors, the technique has been mainly offered to couples with advanced maternal age, recurrent implantation failure, recurrent miscarriages or severe male factor infertility[93]. As such, PGS aims to increase the pregnancy rates per embryo transferred and the live birth rates following IVF, and to prevent abnormal pregnancies[94–96]. First-generation PGS was performed by FISH using a limited number of probes targeted to specific chromosomes[97] and has since been replaced by multiplex quantitative PCR (qPCR) targeting all chromosomes[98] or single-cell genome-wide assays applying chromosomal microarrays[99–102] (TABLE 2). Despite its promise, randomized prospective clinical trials failed to show a significant increase in the baby-take-home rate following PGS[96,103,104]. This has been attributed to both technical and biological factors. The limited set of probes used in FISH discloses ploidy information relevant to only a specific, predetermined set of chromosomes, but no information regarding the copy number status of the rest of the genome. Adding to this limitation, the common chromosomal instability (CIN) observed in human cleavage-stage embryos[105] (BOX 2) challenges the biological paradigm upon which PGS is based. As CIN is a post-zygotic event resulting in mosaic embryos, one or two single blastomeres derived from a cleavage-stage embryo may not be proper representatives of the remaining sister cells in the embryo[95]. In addition, mitotic chromosome segregation errors[99,105,106] in human cleavage-stage embryos do not necessarily impair normal embryonic development, and transfer of mosaic embryos comprising aneuploid and diploid cells can result in normal euploid offspring[107].

Current PGS research focuses on genome-wide aneuploidy screening of biopsies from the trophectoderm, which may better represent the ultimate genetic constitution of the embryo. Trophectoderm biopsies have also become the preferred method for obtaining embryonic material; whole-genome amplification (WGA) methods perform better on trophectoderm biopsy samples, as these contain 5–10 cells compared with a single blastomere obtained on day 3 post fertilization, when the pre-compacted embryos contain 6–8 cells. Moreover, biopsies of the trophectoderm are less harmful to the overall developmental potential of the embryo. Owing to recent improvements in cryopreservation, biopsied embryos can be vitrified with little loss of viability, and the traditional time pressure for fresh embryo transfer following PGD or PGS is alleviated. This allows more detailed and time-demanding genome-wide analyses of both blastomere and blastocyst biopsies.

Table 2 | **Pre-implantation genetic diagnosis and screening tests**

	Microsequencing	QF-PCR	FISH	qPCR	aCGH	Genome-wide haplotyping	Low-coverage sequencing
Genetic lesion							
Monogenic disorders	+	+	–	–	–	+	–
Combination of monogenic and chromosomal disorders	–	–	–	–	–	+	–
Whole-chromosome aneuploidy	–	+/–*	+/–‡	+	+	+	+
Balanced chromosomal rearrangements	–	–	–	–	–	+	–
Unbalanced translocations	–	+/–*	+	+/–	+/–§	+	+
Complex rearrangements	–	–	+/–‡	±	+/–§	+	+/–§
Submicroscopic deletions	–	+/–*	+	–	–	+	–
Submicroscopic duplications	–	+/–*	–	–	–	+	–
Uniparental disomy	–	+/–*	–	–	–	+	–
Mechanistic origin of trisomies (mitotic vs meiotic)	–	–	–	–	–	+	–
Familially inherited	+	+	+	+	+	+	+
De novo mutations	+	–	+	+	+	–	+
Methodology							
WGA required	–	–	–	–	+	+	+

+, possible; –, not possible; ±, possible but requires specific work-up; aCGH, array comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; QF-PCR, quantitative fluorescence PCR; qPCR, quantitative PCR; WGA, whole-genome amplification. *Limited by primer set. ‡Limited by number of fluorochromes. §Limited by size.

Genome-wide screening of single cells

The development of single-cell diagnostic tests targeted at a particular mutation in individual families is labour intensive, costly and time consuming, leading to long waiting lists for couples that undergo this procedure. Moreover, some mutations (for example, small deletions, duplications or complex chromosomal rearrangements) are practically impossible to diagnose using current PGD strategies. Novel genome-wide screening approaches, such as microarrays and genome sequencing, have begun to overcome these limitations (TABLE 2).

Single-cell array comparative genomic hybridization. The feasibility of genome-wide aneuploidy detection in single fibroblasts, white blood cells and human

Box 2 | **Novel biological insights provided by single-cell haplotyping**

Single-cell haplotyping enables the concurrent analysis of the nature and origin of segmental aneuploidies and meiotic recombination sites. Analyses of polar bodies, zygotes, blastomeres and trophectoderm have changed the paradigms of chromosomal behaviour during early embryonic development. Here, we summarize the main recent discoveries. First, the embryonic cleavage stage is characterized by chromosomal instability (CIN). Analysis of all the blastomeres of cleavage-stage embryos has revealed that in most of them not only is there mosaicism for whole-chromosome aneuploidies, but also frequent segmental chromosomal aneuploidies[105]. These include deletions, duplications and amplifications that were reciprocal in sister blastomeres, including the occurrence of breakage–fusion–bridge cycles. The high incidence of chromosomal rearrangements indicates that the regular cell cycle control and genome maintenance mechanisms may not be fully operational. Chromosomally abnormal cells are probably selected against during further development into the blastocyst stage, a period during which transcription is activated and large scale apoptosis is observed. The survival of different cell lines probably underlies the confined placental mosaicism and somatic mosaicisms in constitutional disorders[154]. Overall, CIN can explain the low fecundity in humans, and post-zygotic CIN has been identified as a major cause of constitutional chromosomal disorders. Second, analysis of both the polar bodies (PB1 and PB2) and the zygote revealed that, rather than errors in meiosis I and meiosis II, premature sister chromatid segregation is the main cause for meiotic aneuploidy[155]. A new reverse chromosome segregation pattern in which both homologues separate their sister chromatids in PB1 at meiosis I was discovered. The most frequent non-canonical segregation pattern led to the formation of a PB1 that contained two non-sister chromatids. As a consequence, following meiosis II the oocyte and the PB2 each contained a non-sister chromatid. It is inferred that sister chromatids of both homologues separated first in meiosis I, followed by the separation of non-sister chromatids in meiosis II. It was also demonstrated that higher maternal recombination rates protect against trisomies. Moreover, a chromosomal drive against non-recombinant chromatids at meiosis II was discovered. When two sister chromatids segregate at meiosis II, non-recombinant chromatids seem to be preferentially driven into the PB2 and thus eliminated from the human germ line. Hence, meiotic recombination not only affects homologue segregation at meiosis I but also the fate of sister chromatids at meiosis II. The mechanism underlying the latter phenomenon remains to be uncovered. A third novel phenomenon that has been uncovered is the development of chimerism and mixoploidy by non-canonical cell division of the zygote, in which entire parental genomes segregate into different cell lineages during zygotic cleavage[156]. The persistence of those cell lines during development is the probable cause of molar pregnancies, chimerism and mixoploidy in mammals. The mechanistic nature of these novel discovered phenomena remains to be explored.

blastomeres was first demonstrated in 2006 using single-cell aCGH on arrays containing large insert BAC targets[108] and subsequently on oligonucleotide arrays and SNP arrays[105,109]. Owing to biases in WGA (BOX 1), the resolution ranges from 1 Mb to 4 Mb, far lower than when DNA is obtained from a large number of cells[110]. These initial demonstrations that DNA copy number profiling and SNP genotyping of single cells following WGA was possible, triggered the field of single-cell genomics. Since then, these technologies have rapidly become standard practice in PGD laboratories, particularly for aneuploidy detection[111–113].

With aCGH (both BAC and oligonucleotide arrays) only copy number changes can be profiled, whereas SNP arrays were shown to enable both aneuploidy detection and genotyping[114,115]. SNP arrays genotype an individual by interrogating hundreds of thousands and up to millions of SNVs, which are polymorphic in the human population. There are two main genotyping chemistries, which can be classified according to the design of the probes on the array. In the first approach, allele-specific probes encompassing the SNP are deposited on the array, which is hybridized with a labelled human DNA sample. Genome-wide genotyping of SNPs in the sample is possible on the basis of fluorescence intensities observed above background for the specific probes used for each variant allele of the SNP. Alternatively, the probes on the array are designed to hybridize one base upstream of the SNP locus in the DNA sample, which is followed by fluorescent one-base extension to enable SNP genotyping. After imaging, genotyping algorithms that interpret the fluorescent signals of each probe provide the overview of all homozygous and heterozygous SNPs interrogated by the array.

Single-cell haplotyping. The genotype of a diploid DNA sample is determined by two haplotypes; that is, the chain of variants on the maternally and paternally inherited chromosomes. Knowledge of familial genotypes as well as the segregation pattern of the disorder in parents and relatives allows the identification of the parental haplotype that carries the disease-causing mutation (FIG. 4). Subsequently, the inheritance of this risk haplotype and the linked mutation can be inferred within an embryo following IVF by genotyping and haplotyping one or more cells biopsied from the embryo; thus, unaffected embryos can be selected for.

Two genome-wide single-cell haplotyping methods using SNP arrays have been developed and clinically implemented: karyomapping[116] and single-cell haplotyping and imputation of linked disease variants (siCHILD)[117] (FIG. 4). These approaches offer a generic approach for the detection of monogenic disorders and address most of the disadvantages of FISH- or PCR-based PGD tests. First, both tests are generic, which circumvents per family or per locus optimization, as the analysis is performed on a genome-wide level at high resolution. Second, ADO is less of a concern than with direct mutation detection in the embryo biopsy sample. Even if a part of the polymorphic markers does suffer ADO, a significant part of the markers flanking the locus of interest will not undergo ADO. Third, the sites of meiotic homologous recombination — a process that can break down linkage of a pathogenic variant with its nearby SNPs — can be precisely identified. Fourth, the tests enable the distinction of chromosomally normal and abnormal embryos. Fifth, the tests can determine the meiotic or mitotic origin of trisomies based on the recombination pattern on inherited chromosomes and, finally, it is possible to detect UPDs. One shortcoming of these haplotyping approaches is that in addition to parental DNA, DNA from family members is required for phasing of the disease mutation with the flanking SNPs, which is often not available or accessible (FIG. 4). Also, a large fraction of pathogenic variants arise *de novo* in the prospective parent[20], and thus no carrier family members exist.

To overcome these issues, generic strategies for genome-wide haplotyping are currently being pioneered. One approach uses long-fragment read technology[118]. In this approach, genomic DNA is diluted such that long parental DNA fragments are distributed into physically distinct pools. Each pool with only a fraction of the haploid genome is subsequently converted to a barcoded short-read sequencing library. Following pooling and sequencing of the barcoded libraries of the same DNA sample, the genetic variants can be assigned to a single haplotype[118]. This approach was shown to allow for haplotyping of embryo biopsy samples[119]. A second approach physically

separates individual chromosomes through a microfluidic device. The individual chromosomal DNA is subsequently amplified, and the genetic variants can be analysed by SNP arrays or NGS. This direct phasing allows mapping of the genetic variants on individual chromosomes, thus enabling the reconstitution of the personal whole-genome haplotypes[120]. More recently, by sequencing the first and second polar bodies, as well as the oocyte pronuclei from the same female egg donors, the genomes of these donors could be haplotyped[121]. The drawback of this approach is that it allows haplotyping of the maternally inherited genome only.

Single-cell sequencing. The rapid reduction of sequencing costs over the past decade has rendered single-cell sequencing an attractive alternative to chromosomal microarray approaches. Following successful copy number profiling of single cells from 2012 to 2014 (REFS 122–125), PGS and PGD single-cell sequencing rapidly became a reality[126]. Low-coverage sequencing (<0.5× coverage) of single-cell genomes following WGA enables the detection of segmental aneuploidy, with resolutions similar to or even surpassing aCGH depending on sequencing depth per cell and selected genomic bin size for copy number analysis[127]. The reliability of single-cell DNA copy number profiles can be further increased by using additional data sources from the same cell as SNP B allele frequency (BAF) values, which can be determined from the sequence read depth[124] or separate SNP array data[117]. Alternatively, BAF values can be obtained from discordantly mapping read pairs, which can be determined by mapping paired-end sequences of a single-cell WGA product. Indeed, real copy number changes are corroborated by a characteristic skewed SNP–BAF signature, as well as by discordant read pairs, in line with the architecture of the DNA copy number change[123].

Low-coverage sequencing for aneuploidy detection is price competitive with arrays and, as a consequence, is being implemented in diagnostic laboratories, whereas the cost for deep sequencing for structural variation and SNV detection remains prohibitively high. The development of new genome analysis pipelines, or the optimization and adaptation of existing pipelines, that are able to accurately and timely analyse the massive data sets produced from single-cell technologies will be of crucial importance, as will strategies to reduce the number of sequencing reads needed for genome-wide SNP typing.

Evolution of pre-implantation genetic testing. As a consequence of the technological advancements in single-cell DNA amplification and single-cell genome analysis, PGD and PGS methods now enable faster, more accurate analyses and have the potential of increasing IVF success rates. PGS is suggested not only to improve the success rates of embryo survival in patients with advanced maternal age, but also to improve the efficacy of IVF in general[6,128]. Currently, a randomized clinical trial is underway to compare the outcomes of standard IVF treatment with aneuploidy

Glossary

Allele drop out
(ADO). The failure to detect an allele in a sample or the failure to amplify an allele.

Amniocytes
Cells of the fetus that are suspended in the amniotic fluid.

Aneuploidies
The presence of abnormal numbers of chromosomes in a cell. In human cells, this is typically when a cell contains either 45 or 47 chromosomes, instead of the expected 46.

Assisted reproductive techniques
Clinical approaches that are used to help infertile couples achieve a normal pregnancy. These include ovarian stimulation protocols using exogenous hormones, *in vitro* fertilization, intracytoplasmic sperm injection and pre-implantation genetic diagnosis.

B allele frequency
(BAF). A metric that is used to analyse the data derived from single-nucleotide polymorphism genotyping platforms and is defined as the proportion of allele B occurrence compared with the total allele A and allele B occurrences.

Blastocysts
A blastocyst is a specific stage in embryonic development. On day 5 post fertilization the structure comprises a cavity, the blastocoel, with an inner cell mass; that is, the cells that subsequently contribute to the embryo and also extra-embryonic structures surrounded by a layer of trophoblast cells that provide the fetal component of the placenta.

Blastomeres
Cells produced by cleavage of the zygote after fertilization.

Breakage–fusion–bridge cycles
Mechanisms of chromosome instability involving repeated cycles of telomeric breakage and fusion of the sister chromatids. As a consequence, the fused sister chromatids are pulled towards opposite poles during anaphase and are broken apart creating new breakpoints.

Chimeric
A condition in which an organism contains genetically distinct cell lines (that is, different parental genomes).

Chorionic villi
Villi that sprout from the chorion in the placenta to provide maximum contact area with maternal blood, allowing for efficient exchange of gasses and nutrients needed for fetal development.

Chromosomal instability
(CIN). An elevated rate of chromosome missegregation or breakage per cell division leading to aneuploidy or segmental aneuploidy.

Haplotyping
The determination of the set of alleles for consecutive loci that are present on the same chromosome.

Mixoploidy
A condition in which an organism contains cell lines with different ploidy levels (for example, diploid and triploid).

Molar pregnancies
Pregnancies in which the trophoblast proliferates like a non-cancerous tumour and grows into a swollen chorionic villi mass in the uterus known as a hydatidiform mole.

Penetrance
The conditional probability of a phenotype (specifically, the probability of being affected with disease) given an underlying genotype.

Polar body
During oogenesis the primary and secondary oocyte divide asymmetrically; that is, most of the cytoplasm is segregated into one daughter cell (which becomes the egg or ovum) and the remaining cytoplasm goes to the smaller polar bodies. In humans, the first polar body is formed following the first meiotic division of the primary oocyte (which occurs near ovulation), and a second polar body is formed following the second meiotic division of the secondary oocyte (which occurs with fertilization).

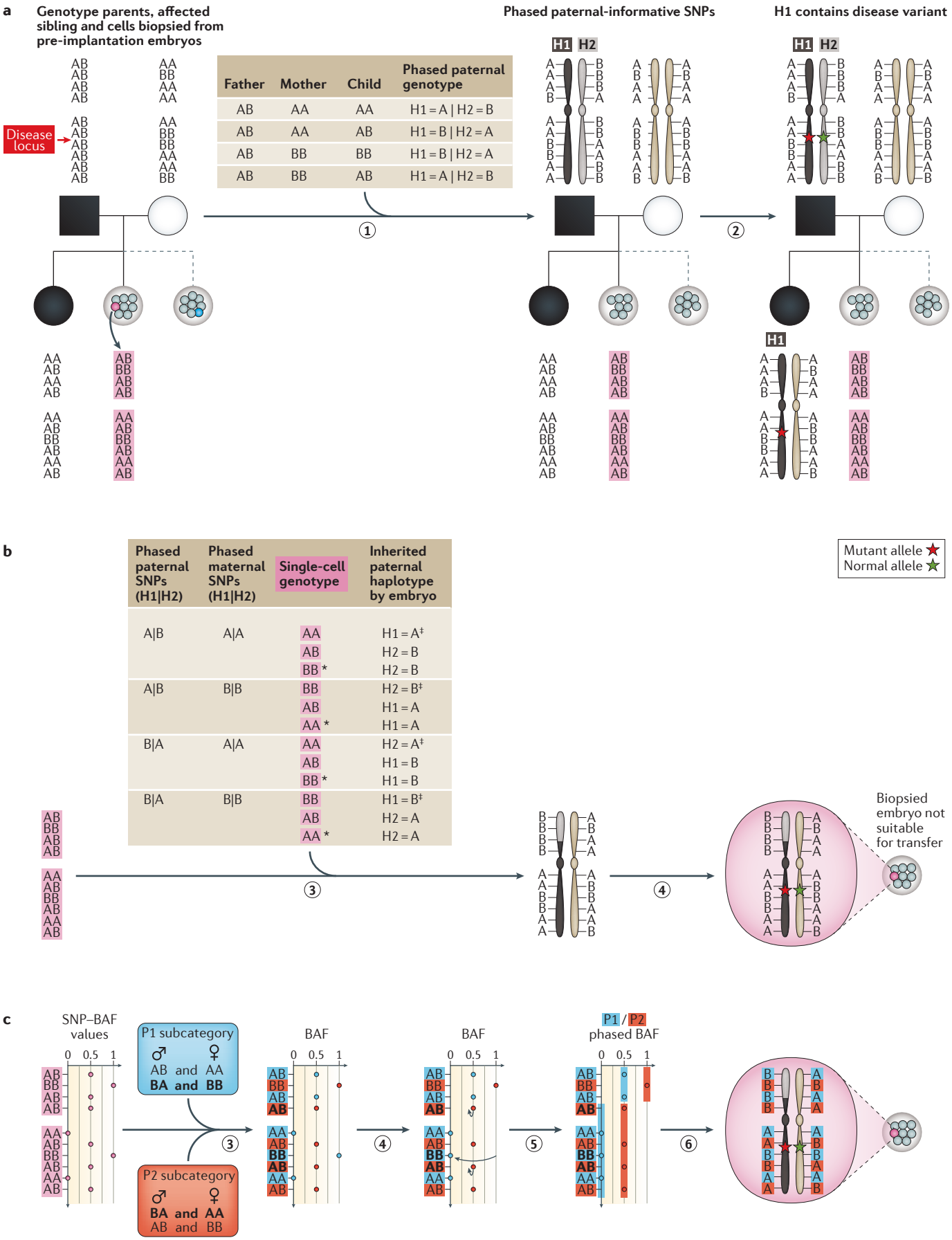
Read pairs
In paired-end sequencing, a technology in which both ends of a short linear DNA molecule are sequenced, read pairs are mapped to a reference genome with a discordant orientation or distance between them, which can pinpoint structural variants.

Trisomy rescue
A phenomenon in a trisomic zygote (which contains three copies of one chromosome) in which aneuploidy is corrected by the loss of the additional chromosome during cell division. Owing to the random loss of the extra chromosome, the resulting daughter cell might contain two copies of a chromosome from the same parent (uniparental disomy).

Trophectoderm
Cells of the outer layer of a blastocyst, which provide nutrients to the embryo and develop into the fetal part of the placenta.

Uniparental disomy
(UPD). The presence of two copies of a chromosome, or part of a chromosome, from one parent and no copy from the other parent.

Whole-exome sequencing
(WES). The isolation and subsequent sequencing of the fraction of the genome that consists of protein-coding sequences (the so-called exonic sequences). The isolation is performed by capturing the exonic segments using complementary oligonucleotides as bait.



screening to assist embryo selection[129]. If proof-of-concept studies are confirmed, genome-wide aneuploidy screening may well become standard practice for all IVF embryo transfers.

The potential of an embryo to grow successfully into a healthy individual is not determined only by its nuclear genomic composition. An intriguing correlation between altered levels of mitochondrial DNA and reduced embryonic viability has been reported[130], which can possibly be used as a biomarker to improve IVF. Time-lapse microscopy has enabled associations to be drawn between morphokinetic parameters and embryonic developmental potential[131]. Understanding the molecular causes that underlie the morphokinetic parameters may further improve embryonic selection. By combining time-lapse imaging with chromosomal analysis, significant differences in the duration of the first cleavages were observed that relate to euploidy or aneuploidy in developing embryos[132,133]. New genomic technologies enabling combined genomic and transcriptomic analysis[134], as well as genomic and epigenomic analyses[135], in combination with time-lapse microscopy will be some of the tools that will provide a systems-biology view of embryonic development, further disclosing the causes of embryonic demise and the physiology of a healthy embryo.

Ethical considerations

Whole-genome analysis of pre-implantation embryos provides information about not only the disorder tested for, but the whole genomic make-up of the embryo. This not only allows for improved selection, but also provides information on genetic variants that are associated with several non-health-related traits. These prospects raise difficult ethical questions. Some people may see this as the slippery slope towards the 'designer child' (REF. 136), whereas a different perspective is that it enables prospective parents and professionals to take into account the welfare of the future child. Following the principle of procreative beneficence, it is common practice to rank embryos and select the embryo with the highest chance of resulting in a healthy individual[137]. This raises questions as to whether prospective parents have the right to select for the best embryo and how to define 'best', especially in the context of genome-wide analysis. If broader testing is introduced, genetic counselling and informed decision-making will become increasingly difficult. Moreover, in current PGD clinical practice, the treating team has a moral co-responsibility regarding the well-being of the future child.

Similarly, the rapid evolution from narrow-range prenatal genetic screening (targeted mainly at identifying severe chromosomal imbalances) towards non-invasive, comprehensive genome-wide analysis of the fetus requires novel ethical frameworks[138,139]. Ethical concerns have long been hampering the routine introduction of chromosomal microarrays for prenatal diagnosis[140]. There was (and still is) concern about how to deal with unsought-for findings, late-onset disorders, CNVs with variable expressivity and/or penetrance, and variants with only mild phenotypic anomalies. Variants of uncertain significance encompass a range of findings that can in most cases be subclassified as pathogenic, benign or truly of unknown clinical significance based upon factors such as size, gene content, inheritance status and the presence of overlapping CNVs in low numbers of patients or controls[141]. Current estimates suggest that the number of cases in which difficulties in counselling arise due to the detection of variants of unknown significance and risk factors are limited to 1–2%[15,17]. Nevertheless, more research and consensus guidelines in this area would help both clinicians and families.

It is possible to calculate a population-based penetrance risk of recurrent CNVs based on the frequencies in patients and controls[142], but it remains impossible to predict the phenotypic outcome in the future individual. Thus, in the prenatal setting and in the absence of phenotypic anomalies, the classification of a CNV as pathogenic when there is evidence for incomplete penetrance is questionable. Two main approaches are followed, the first being that all CNVs

Figure 4 | **Principles for pre-implantation genetic diagnosis using single-cell haplotyping.** Single-cell haplotyping helps to infer inherited disease variants genome-wide within cells biopsied from human *in vitro* fertilization (IVF)-derived embryos. Here, we explain two main principles for haplotyping single cells using either discrete single-nucleotide polymorphism (SNP) genotype calls (AA, BB or AB) or SNP B allele frequency (BAF) values of the cell. Both methods require parental SNP genotypes to be phased with the disease variant. **a** | An example of a family with an autosomal dominant disorder (the father and the child are affected in the example). DNA from the parents and the affected child is first genotyped genome-wide, as are the whole-genome amplification (WGA) products of single blastomeres biopsied from IVF-derived embryos (pink and blue coloured cells). On the basis of the affected child's genotype, the parental SNPs can be phased with the mutant allele. For simplicity, only paternal-informative SNPs — defined as heterozygous in the father but homozygous in the mother — are shown with applicable phasing rules (step 1). The paternal homologue that is transmitted to the affected child must contain the causative mutation and is denoted homologue 1 (H1) (step 2), whereas paternal H2 carries the normal allele. **b** | Subsequently, the parental haplotypes inherited by the embryo can be inferred from the discrete SNP genotype of a blastomere. Adjusted phasing rules allowing for Mendelian inconsistencies may be used, as allele drop out (ADO) occurs during single-cell WGA and genotyping (indicated with an asterisk) (step 3). In addition, SNPs without Mendelian inconsistencies (indicated with a double dagger) may have undergone ADO resulting in erroneous paternal haplotype assignment. Such WGA artefacts are largely random and can be resolved by using segmentation algorithms. Following segmentation, and hence single-cell haplotype inference, the inheritance of the mutant or normal allele at the disease locus can be imputed in the embryo (step 4). **c** | SNP–BAF values can be used instead of discrete SNP genotypes for single-cell haplotyping. BAF values preserve the signal of both alleles of a heterozygous SNP subjected to WGA bias (whereas discrete diploid genotypes (as in panel **b**) are enforced by a genotyping algorithm and may lose valuable allelic signal) and, importantly, also enable haplotyping across copy number variants (CNVs) in the cell[118]. Based on defined phased parental SNP genotype combinations, as illustrated in alternative step 3, the SNP–BAF values of a blastomere are binned into paternal-informative SNP subcategories P1 (red box) and P2 (blue box); a similar approach is used for, maternal-informative SNPs (not shown). Note that P1 and P2 are conceptually different from H1 and H2. The phased parental genotypes defining P1 and P2 have been selected such that when the cell inherits H1 from the father, and either H1 or H2 from the mother, the P1 SNP–BAFs have values of either 0 or 1 (corresponding to homozygous AA and BB genotypes in the cell, respectively) and the P2 SNP–BAFs have a value of 0.5 (corresponding to heterozygous genotypes in the cell). By contrast, when the cell inherits H2 from the father the P1 SNP–BAFs have a value of 0.5 and the P2 SNP–BAFs have a value of either 0 or 1. Subsequently, for a defined subset of SNPs (that is, parental 'BA' SNP calls; marked in bold) the single-cell BAF values are mirrored around the 0.5 axis (alternative step 4) allowing segmenting single-cell P1 and P2 BAF values for consecutive SNPs in the genome (step 5). The resulting P1 and P2 BAF segments (depicted in blue and red, respectively) now define the haplotype blocks inherited from paternal H1 and H2. For H1 loci, P1 and P2 SNP–BAF segments have values of 0 and 0.5, respectively, whereas H2 P1 and P2 have values of 0.5 and 1, respectively. Using the inferred single-cell H1 and H2 architecture, the mutant or normal allele at the disease locus can be imputed in the embryo (step 6).

are reported back to the patients. However, the disclosure of such data may in fact be more harmful to them as the information given does not contribute to making informed decisions. In the second approach, CNVs are not reported back to the patient; for example, CNVs that are known to be rare or are novel inherited CNVs of questionable pathogenicity, and imbalances for known 'risk loci' for which the future penetrance is uncertain and with low odds[143]. Examples of such recurrent CNVs include the 15q11.2 microdeletion (penetrance risk of 10.4%)[142], which contains the *NIPA1* gene and is associated with developmental delay, behavioural problems and/or schizophrenia, and the 22q11.2 duplication (penetrance risk 21.9%[142]), which affects the *TBX1* gene and is associated with cardiac anomalies. In the absence of cardiac anomalies on initial clinical referral, this would be reported and followed up with detailed ultrasound examination for the presence of cardiac defects. Likewise, information overload threatens pre-test counselling, and therefore it has been argued that novel, more generic forms of informed consent are needed[144].

NIPT is an example in which the implementation has been primarily technology-driven rather than a carefully planned introduction of a mature technology into existing public health prenatal diagnosis programmes. Concerns about routinization of NIPT are being expressed, despite the fact that the first professional guidelines have just been drafted, all indicating the need for further studies on ethical and societal aspects[138,145]. The total cost of comprehensive non-invasive whole-genome analysis, including pre- and post-test counselling and follow-up investigations might exceed what a public healthcare system is able, or willing, to spend. Society will need to make choices such as restricting screening offers only to severe childhood conditions or treatable fetal disorders, whereas a broader scope of disorder screening may be offered privately[146]. This raises serious issues — such as how to provide equal access, how to define severe conditions, the provision of counselling in both settings and providing parents with information to make informed choices — that will need to be addressed in the future.

Conclusions

With further technological improvements and increasing success rates, prenatal and pre-implantation diagnosis of genetic disorders will become commonplace, and with increasing public acceptance a continued growth in their implementation can be anticipated. This implementation, in turn, will reduce the frequency of rare severe inherited genetic diseases. Increasingly, more common genetic variants causing late-onset disorders (for example, *BRCA1* and *BRCA2*) or recessive disorders (for example, cystic fibrosis) could also be selected against and will eventually become rare. In the future, new diagnostic technologies will not only provide a tool to give parents the option of an informed choice, but they will also lead towards fetal personalized medicine: prenatal diagnosis may well serve a dual role of prenatal screening for severe conditions as well as an opportunity to detect conditions treatable during prenatal or even postnatal life[147]. It is important to engage in a public debate about the use and the potential advantages, as well as the challenges, of prenatal genetic testing to pave the way towards general acceptance and integration of these technologies into standard patient care.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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