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ARTICLE

Live birth after PGD with confirmation by a comprehensive approach (karyomapping) for simultaneous detection of monogenic and chromosomal disorders

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Dr Senthil Natesan graduated with a first class honours degree in agriculture at the Tamil Nadu Agricultural University (TNAU), Coimbatore, India and following an MSc in plant physiology at the Indian Agricultural Research Institute, New Delhi, India obtained his PhD in the Department of Plant Sciences, University of Cambridge, UK for his work on the molecular basis of communication between plastids. Since joining Bluegnome (now Illumina), Cambridge as a senior scientist in 2011, he has played a leading role in the development of karyomapping and in particular developed a rapid protocol for genotyping single nucleotide polymorphisms (SNPs) using bead arrays and more recently validating karyomapping for clinical application to diagnose single gene defects.

Abstract Preimplantation genetic diagnosis (PGD) for monogenic disorders has the drawback of time and cost associated with tailoring a specific test for each couple, disorder, or both. The inability of any single assay to detect the monogenic disorder in question and simultaneously the chromosomal complement of the embryo also limits its application as separate tests may need to be carried out on the amplified material. The first clinical use of a novel approach ('karyomapping') was designed to circumvent this problem. In this example, karyomapping was used to confirm the results of an existing PGD case detecting both chromosomal abnormalities and a monogenic disorder (Smith–Lemli–Opitz [SLO] syndrome) simultaneously. The family underwent IVF, ICSI and PGD, and both polar body and cleavage stage biopsy were carried out. Following whole genome amplification, array comparative genomic hybridisation of the polar bodies and minisequencing and STR analysis of single blastomeres were used to diagnose maternal aneuploidies and SLO status, respectively. This was confirmed, by karyomapping. Unlike standard PGD, karyomapping required no a-priori test development. A singleton pregnancy and live birth, unaffected with SLO syndrome and with no chromosome abnormality, ensued. Karyomapping is potentially capable of detecting a wide spectrum of monogenic and chromosome disorders and, in this context, can be considered a comprehensive approach to PGD.

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Introduction

Preimplantation genetic diagnosis (PGD) is in its 25th year of clinical application. Historically, PGD was used to prevent transmission of inherited monogenic disorders (Handyside et al., 1992). Direct mutation detection was later augmented by the use of linked markers and the use of minisequencing to carry out accurate diagnosis (Fiorentino, 2012a). It was soon expanded to include testing for chromosome disorders to reduce adverse reproductive outcomes associated with advanced maternal age, such as recurrent pregnancy loss and repeat implantation failure. Initially fluorescence in situ hybridisation (FISH) was used (Munné et al., 1993), but array comparative genomic hybridization (aCGH) for cytogenetic analysis has more recently been employed (Fiorentino, 2012b). Advancement of PGD can also be attributed to the development of state-of-the-art technologies. Both monogenic diagnosis and aneuploidy detection (plus HLA haplotyping) can now be determined from seperate aliquots of the same whole genome amplified product (Handyside and Xu, 2012). A test that combines chromosomal and monogenic disorder detection in a single assay, however, has hitherto been elusive.

Significant drawbacks are associated with current technologies because the diagnostic approach needs to be inherently tailored to the disorder and the individual family under investigation. Diagnostic strategies also need to be optimized for single or small numbers of cells. In practice, this can take days or weeks, with the corresponding delay, stress and reduction in fertility potential to couples as they wait for test validation. An approach that does not require individual-specific validation is, therefore, a priority for all families seeking PGD. Moreover, the ability to combine monogenic disorder detection with high-resolution chromosome analysis in a single test would potentially find widespread clinical application (Handyside and Xu, 2012).

Karyomapping (Handyside et al., 2010) uses the principles of linkage analysis to detect any monogenic disorder plus a spectrum of molecular cytogenetic abnormalities in a single assay. The process involves genome-wide single nucleotide polymorphism (SNP) analysis of the parents and an appropriate family member (e.g. an affected child), thereby identifying 'informative' loci for each of the four parental haplotypes and their inheritance pattern in the resultant IVF embryos. Karyomapping can also diagnose aneuploidy, triploidy, parthenogenetic activation and uniparental heterodisomy (Handyside et al., 2010). Here, we extend karyomapping into a clinical setting by reporting its successful use for PGD in confirming (in real time) current standards of mini-sequencing, linked markers and aCGH in a family at risk of transmitting Smith–Lemli–Opitz (SLO) syndrome.

Materials and methods

The couple who underwent PGD and karyomapping were at risk of transmitting SLO syndrome, an autosomal recessive condition leading to multiple congenital abnormalities and mental retardation. Both parents were unaffected carriers, with mutations in the 7-dehydrocholesterol reductase gene (*DHCR7*) on chromosome 11q12-q13. The maternal parent was of advanced maternal age with evidence of decreased ovarian reserve. The couple reported six natural pregnancies: a full term healthy daughter; two terminations for fetal SLO syndrome; two first-trimester spontaneous abortions of unknown cause; and one second-trimester pregnancy loss diagnosed with SLO syndrome. They previously completed, without success, two treatment cycles of IVF, ICSI and PGD using standard approaches, and gave informed consent for treatment by PGD and karyomapping. Ethical approval was granted by the treatment license awarded to the Bridge Centre, London (reference number L0070-19-c, approved 26/03/20130) and the University of Kent Local Research and Ethics Committee (approved 09/04/2012). The family received counselling for ICSI and PGD at the Bridge Centre; Reprogenetics (Oxford, UK) carried out molecular diagnostics. A short cycle flare protocol was used for ovarian stimulation. An HCG trigger was administered after 11 days of stimulation, and transvaginal ultrasound-guided oocyte retrieval was carried out 35 h later.

For PGD, the primary diagnosis of chromosome constitution was carried out on first and second polar bodies using aCGH. The diagnosis of SLO status was carried out on cleavage-stage biopsied single cells using mini-sequencing and linked markers on whole-genome amplified material. The above was confirmed by karyomapping on the same whole genome amplified material. From the outset, the decision was made to disclose all results, with the exception of the transferred embryos.

Following ICSI, biopsy of the first polar body in each oocyte was conducted on day 0. Biopsy of the second polar body was conducted after fertilization. Polar bodies were subjected to whole genome amplification (Sureplex; Bluegnome Ltd, Cambridge, UK) and aCGH (24sure; Bluegnome Ltd) to determine aneuploidy status. Cleavage stage biopsy proceeded at day 3 after fertilization and, to perform whole-genome amplification, cells were amplified using multiple displacement amplification (Repli-g Midi kit, Qiagen, Germany, with modifications). All blastomeres were tested directly for mutations in the *DHCR7* gene. Short tandem repeat (STR) primers for three loci linked to the *DHCR7* gene (D11S4139, D11S4143, D11S4207) were amplified in separate single-plex polymerase chain reactions using Hot Master Taq DNA polymerase (5PRIME, Hilden, Germany), which were detected by capillary electrophoresis (3130 Genetic Analyzer (Applied Biosystems, USA) and analysed using GeneMapper software v4.0 (Applied Biosystems, USA). Minisequencing involved the SNaPshot Multiplex Kit (Applied Biosystems, USA).

To allow rapid diagnosis with fresh embryo transfer, the protocol for genome-wide SNP genotyping using bead arrays (Human CytoSNP 12; Illumina Inc, San Diego, CA, USA) was shortened from 72 to 24h as previously described (Natesan et al., 2014). Genomic DNA from father, mother, a previously affected fetus and MDA product from the embryo samples were genotyped. Karyomapping of the SNP data was achieved by a dedicated module in the bead array software (Bluefuse

Multi v4; Illumina Inc). The genotype data was also exported as an Excel compatible file for Karyomapping analysis using a Visual Basic for Applications macro as described previously (Handyside et al., 2010) in Microsoft Excel. Karyomapping results were compared with the original diagnosis of markers linked to the *DHCR7* gene and mini-sequencing of the mutation directly. Cytogenetic data were compared with the aCGH data derived from polar bodies.

Results

Eight oocytes were retrieved, of which six were mature metaphase II oocytes suitable for ICSI. The first polar body (PB1) was biopsied from each of the six oocytes after ICSI. Six normally fertilized two pronuclei zygotes were produced, and the second polar body (PB2) was removed. Array CGH analysis of PB1 and PB2 revealed a normal pattern in zygotes 1 and 4 with chromosome 22 gain (PB1) and loss (PB2) in embryo 2; in embryos 3 and 5 a normal pattern in PB1 contrasted with gain of chromosome 15 and 22, respectively, in PB2 (**Table 1**). Five of six 2PN zygotes formed embryos, which were biopsied. Mutation analysis revealed a normal pattern in embryos 1, 3 and 5 (**Table 1**). Embryo 1 was thus transferred. Karyomapping confirmed the *DHCR7* status in all embryos examined (**Figure 1** and **Table 2**). The aneuploidy results were concordant with results after aCGH of the polar bodies (**Table 1**). A pregnancy ensued leading to the live birth of a healthy male infant free of Smith–Lemli–Opitz syndrome, and with no apparent chromosome abnormality.

Discussion

In this PGD case, we combined aneuploidy testing by array CGH on polarbodies with mutation and linkage based testing for Smith–Lemli–Opitz (SLO) on single blastomeres following whole genome amplification. Because of a history of miscarriage the patient requested testing for aneuploidy in addition to the disease status for SLO. Karyomapping thus enabled us to confirm both sets of results in a single assay and 'in real time', providing proof of principle of its potential for application in a clinical setting. Concordance with established gold standards confirmed the accuracy of karyomapping for linkage based detection of monogenic diseases. Analysis of polar bodies by aCGH and blastomeres by karyomapping permitted the comparison of aneuploidy results from oocytes and the subsequent embryos. One advantage of karyomapping over aCGH is that it distinguishes the parent and phase of origin of meiotic chromosome error. For example, as shown in **Table 1**, embryo 3, the gain of chromosome 15 in the PB2 suggested a meiosis II error, which was confirmed as a reciprocal loss of chromosome 15 in the embryo. This was also mirrored in embryo 5 for chromosome 22 and the normal diagnosis of embryo 4 matched in both polar bodies and blastomere. In embryos 3 and 5, karyomapping confirmed that the further abnormalities were paternal in origin, and would therefore not have been detected in the polar bodies. In embryo 2, a reciprocal gain and loss of chromosome 22 in PB1 and PB2, respectively was not reflected as an abnormality in the embryo, thereby invoking the widely reported mechanism of precocious separation of sister chromatids in the first meiotic division, in this case, balanced in the second meiotic division resulting in a euploid conceptus (Angell, 1991; Gabriel et al., 2011; Handyside et al., 2012; Kuliev et al., 2011).

The use of PGD aneuploidy screening (PGD-AS) for the purposes of improving IVF success rates has met with widespread controversy (Mastenbroek et al., 2007; Summers and Foland, 2009; Thornhill and Handyside, 2009). One criticism, that PGD-AS using FISH screens only a small proportion of the total chromosome complement (Summers and Foland, 2009; Thornhill and Handyside, 2009) can also be addressed through aCGH, and clinical validation of this

Table 1 Comparison of analyses by mutation, linkage, array comparative genomic hybridization and karyomapping.[a]

Embryo ID	*SLO status by mini-sequencing and linkage analysis*	*SLO status by karyomapping*[b]	*PB1 by aCGH*	*PB2 by aCGH*	*Karyotype by karyomapping*	*Comment*
1	Unaffected	Not tested	Normal	Normal	Not tested	Transferred
2	Paternal carrier	Paternal carrier	Chr 22 gain	Chr 22 loss	Normal	Not transferred
3	Unaffected	Unaffected	Normal	Chr 15 gain	Maternal monosomy 15; paternal monosomy 7	Not transferred
4	Affected	Affected	Normal	Normal	Normal	Not transferred
5	Normal	Normal	Normal	Chr 22 gain	Maternal monosomy 22; paternal deletion 15q	Not transferred
6	No result	No result	Normal	Chrs 12 and 14 gain	Arrested, no result	Not transferred

aCGH = array comparative genomic hybridization; SLO = Smith–Lemli–Opitz.

[a]Each embryo had one blastomere biopsied and a combination of mini-sequencing plus analysis of linked markers made the initial diagnosis of SLO status. Array comparative genomic hybridization on both polar bodies was used to carry out maternal aneuploidy analysis. Karyomapping diagnosed both SLO status and chromosome copy number simultaneously.

[b]See **Figure 1** and **Table 2**.

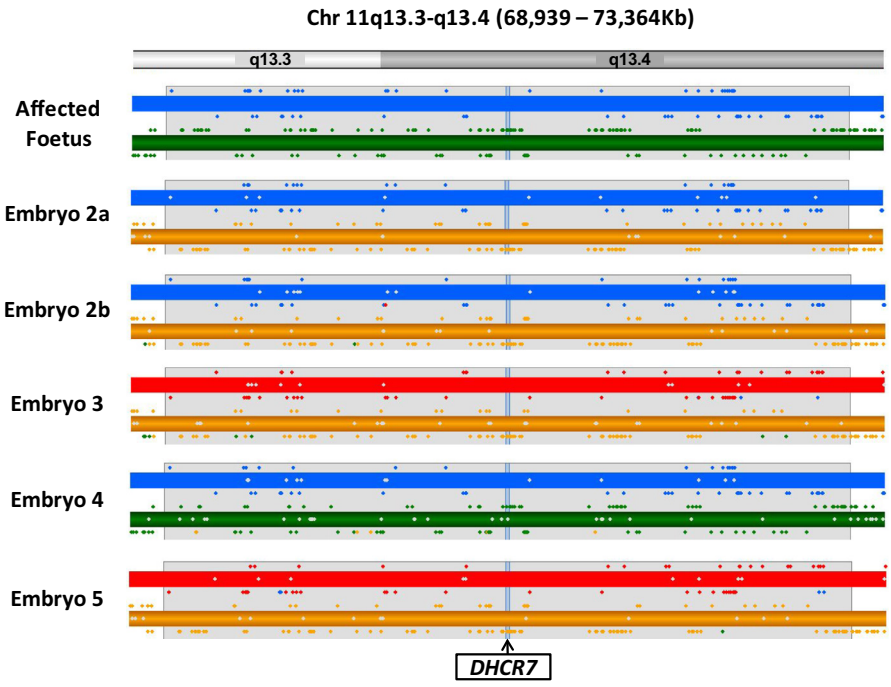

Figure 1 Karyomaps of the Smith–Lemli–Opitz gene (*DHCR7*) region on chromosome 11, for the affected fetus and five embryo samples. The *DHCR7* gene is located between the two dark blue lines (arrow) and the proximal and distal 2 Mb flanking regions are highlighted (grey). For each sample, the paternal chromosome haplotypes (top) are represented in blue/red, and the maternal haplotypes (below) in orange/green. Haplotypes inherited by the reference sample are set as the blue/orange haplotypes. (For a more detailed description of karyomaps, see Natesan et al., 2014). In this case, the reference is an existing child known to be an unaffected carrier of the paternal mutation. Therefore the blue (paternal) and green (maternal) haplotypes have the mutant alleles as confirmed by the karyomap of the affected fetus. The results for the embryos are summarized in Table 2.

Table 2 Interpretation of results from Figure 1.

Description	*Pattern observed around SLO locus(Paterna/Maternal)*	*Diagnosis*
Affected foetus	Blue/Green	Affected
Embryo 2a	Blue/Yellow	Paternal carrier
Embryo 2b	Blue/Yellow	Paternal carrier
Embryo 3	Red/Yellow	Unaffected
Embryo 4	Blue/Green	Affected
Embryo 5	Red/Yellow	Unaffected

SLO = Smith–Lemli–Opitz.

procedure is now established (Fiorentino, 2012b). Treff et al. (2010) reported the use of SNP arrays for the detection of chromosome imbalance both for PGD-AS and for the screening of translocation carriers.

Karyomapping uses a similar approach (i.e. SNP arrays) to Treff et al. (2010) and, combined potentially with quantitative analysis, would have the ability to distinguish any numerical or structural chromosome abnormality as well as the parent and phase of origin of the abnormality. The latter has clinical implications when considering obstetric outcomes, for example, mosaic pregnancies resulting from trisomies of meiotic origin often lead to specific phenotypes, such as pregnancy loss, intrauterine growth retardation or excessive birth weight, whereas those of post-zygotic origin generally proceed to term without clinical consequence (Griffin, 1996). Karyomapping can also detect uniparental heterodisomy, which can lead to Prader–Willi or Angelman syndromes, as well as abnormal patterns of genome duplication seen with, for example, molar pregnancies.

Karyomapping has a number of limitations. For instance, lack of DNA from a family member of known disease status, can limit the use of karyomapping; this is nonetheless the same as any PGD in which linkage analysis is involved. The higher density of markers when SNP arrays are used compared with the 'standard' PCR-based strategies means that there is a greater likelihood of the family member being 'informative', even if there is a recombination at or near the locus of interest. Nonetheless, if a recombination in either parent, reference individual, or embryo is directly next to the locus

of interest, this may (rarely) render individual diagnoses unreadable by this method. As with all PGD, karyomapping does not a-priori detect new mutations (Rechitsky et al., 2011) nor the confounding effect of pseudogenes. Moreover, karyomapping per se does not detect post-zygotic chromosome duplication nor copy number variation. Nonetheless, in our view, use of karyomapping, for the most part, represents an advancement on the current state of the art for PGD.

Several investigators have reported the simultaneous detection of monogenic and chromosomal disorders. To the best of our knowledge, the first of these was a case report by Brezina et al. (2011). More recently, however, Rechitsky et al. (2013) described detection of cytogenetic disorders and cystic fibrosis simultaneously. These, ultimately used different approaches to detect the monogenic and chromosomal disorder, and the issue of having to tailor the test to the disease in question thus remains. Much research has been conducted on the use of haplotyping for the detection of monogenic and chromosomal disorders (Rechitsky et al., 2006; Renwick et al., 2006, 2010). Karyomapping is essentially an extension on this pioneering work except that, rather than using tailored, linked markers, application of a whole genomic platform permits the use of a single test for each and every case. Karyomapping is, moreover, platform independent. Here, we used SNP arrays; the binary nature of the output, however, means that any platform could potentially be used. This includes single cell whole-genome sequencing where we propose that, even with the most robust whole-genome amplification sequence, gaps would inevitably arise in the assembly. These could be overcome by adaptations of the karyomapping algorithm. In a procedure in which diagnostic speed is of the essence, it seems reasonable to suggest that a rudimentary whole-genome sequence (which would take the shortest time to generate) followed by karyomapping would be the most accurate and expedient means of achieving a diagnosis. Karyomapping, therefore, has inherent 'future-proofing' in its design, and adaptations of the algorithm could, we believe, form the basis of most PGD worldwide.

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References

Angell, R.R., 1991. Predivision in human oocytes at meiosis I: a mechanism for trisomy formation in man. Hum. Genet. 86, 383–387.

Brezina, P.R., Benner, A., Rechitsky, S., Kuliev, A., Pomerantseva, E., Pauling, D., Kearns, W.G., 2011. Single-gene testing combined with single nucleotide polymorphism microarray preimplantation genetic diagnosis for aneuploidy: a novel approach in optimizing pregnancy outcome. Fert Ster 95, 1786.e5–1786.e8.

Fiorentino, F., 2012a. Molecular genetic analysis of single cells. Semin. Reprod. Med. 30, 267–282.

Fiorentino, F., 2012b. Array comparative genomic hybridization: its role in preimplantation genetic diagnosis. Curr. Opin. Obstet. Gynecol. 24, 203–209.

Gabriel, A.S., Thornhill, A.R., Ottolini, C.S., Gordon, A., Brown, A.P.C., Taylor, J., Bennett, K., Handyside, A.H., Griffin, D.K., 2011. Array comparative genome hybridization on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans. J. Med. Genet. 48, 433–437.

Griffin, D.K., 1996. The incidence, origin and etiology of aneuploidy. Int. Rev. Cytol. 167, 263–296.

Handyside, A.H., Xu, K., 2012. Preimplantation genetic diagnosis comes of age. Semin. Reprod. Med. 30, 255–258.

Handyside, A.H., Lesko, J.G., Tarín, J.J., Winston, R.M., Hughes, M.R., 1992. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. [see comments]. N. Engl. J. Med. 327, 905–909.

Handyside, A.H., Thornhill, A.R., Harton, G.L., Mariani, B., Shaw, M.A., Affara, N., Griffin, D.K., 2010. Karyomapping: a novel molecular karyotyping method based on mapping crossovers between parental haplotypes with broad applications for preimplantation genetic diagnosis of inherited disease. J. Med. Genet. 47, 651–658.

Handyside, A.H., Montag, M., Magli, M.C., Repping, S., Harper, J.C., Schmutzler, A., Vesela, K., Gianaroli, L., Geraedts, J., 2012. Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilization. Eur. J. Hum. Genet. 20, 742–747.

Kuliev, A., Zlatopolsky, Z., Kirillova, I., Spivakova, J., Cieslak Janzen, J., 2011. Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing. Reprod. Biomed. Online 22, 2–8.

Mastenbroek, S., Twisk, M., van Echten-Arends, J., Sikkema-Raddatz, B., Korevaar, J.C., Verhoeve, H.R., Vogel, N.E., Arts, E.G., de Vries, J.W., Bossuyt, P.M., Buys, C.H., Heineman, M.J., Repping, S., van der Veen, F., 2007. In vitro fertilization with preimplantation genetic screening. N. Engl. J. Med. 357, 9–17.

Munné, S., Lee, A., Rosenwaks, Z., Grifo, J., Cohen, J., 1993. Diagnosis of major chromosome aneuploidies in human preimplantation embryos. Hum. Reprod. 8, 2185–2191.

Natesan, S.A., Bladon, A.J., Coskun, S., Qubbaj, W., Prates, R., Munne, S., Coonen, E., Dreesen, J.C., Stevens, S.J., Paulussen, A.D., Stock-Myer, S.E., Wilton, L.J., Jaroudi, S., Wells, D., Brown, A.P., 2014. Handyside AH. Genome-wide karyomapping accurately identifies the inheritance of single-gene defects in human preimplantation embryos in vitro. Genet Med. 2014. doi: 10.1038/gim.2014.45. [Epub ahead of print].

Rechitsky, S., Kuliev, A., Sharapova, T., Laziuk, K., Ozen, S., Barsky, I., Verlinsky, O., Tur-Kaspa, I., Verlinsky, Y., 2006. Preimplantation HLA typing with aneuploidy testing. Reprod. Biomed. Online 12, 89–100.

Rechitsky, S., Pomerantseva, E., Pakhalchuk, T., Pauling, D., Verlinsky, O., Kuliev, A., 2011. First systematic experience of preimplantation genetic diagnosis for de-novo mutations. Reprod. Biomed. Online 22, 350–361.

Rechitsky, S., Verlinsky, O., Kuliev, A., 2013. PGD for cystic fibrosis patients and couples at risk of an additional genetic disorder combined with 24-chromosome aneuploidy testing. Reprod. Biomed. Online 26, 420–430.

Renwick, P.J., Trussler, J., Ostad-Saffari, E., Fassihi, H., Black, C., Braude, P., Ogilvie, C.M., Abbs, S., 2006. Proof of principle and

first cases using preimplantation genetic haplotyping–a paradigm shift for embryo diagnosis. Reprod. Biomed. Online 13, 110–119.
Renwick, P., Trussler, J., Lashwood, A., Braude, P., Ogilvie, C.M., 2010. Preimplantation genetic haplotyping: 127 diagnostic cycles demonstrating a robust, efficient alternative to direct mutation testing on single cells. Reprod. Biomed. Online 20, 470–476.
Summers, M.C., Foland, A.D., 2009. Quantitative decision-making in preimplantation genetic (aneuploidy) screening (PGS). J. Assist. Reprod. Genet. 26, 487–502.
Thornhill, A.R., Handyside, A.H., 2009. The role of aneuploidy screening in human preimplantation embryos. Biennial Rev Infert 276–288. Springer.
Treff, N.R., Su, J., Tao, X., Levy, B., Scott, R.T., Jr., 2010. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. Fertil. Steril. 94, 2017–2021.

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