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Advances in biotechnology: Genomics and genome editing

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Abstract

Genomics, the study of genes, their functions and related techniques has become a crucial science for developing understanding of life processes and how they evolve. Since the advent of the human genome project, huge strides have been made in developing understanding of DNA and RNA sequence information and how it can be put to good use in the biotechnology sector. Newly derived sequencing and bioinformatics tools have added to the torrent of new insights gained, so that 'sequence once and query often' type DNA apps are now becoming reality. Genome editing, using tools such as CRISPR/Cas9 nuclease or Cpf1 nuclease, provide rapid methods for inserting, deleting or modifying DNA sequences in highly precise ways, in virtually any animal, plant or microbial system. Recent international discussions have considered human germline gene editing, amongst other aspects of this technology. Whether or not gene edited plants will be considered as genetically modified remains an important question. This will determine the regulatory processes adopted by different groups of nations and applicability to feeding the world's ever growing population. Questions surrounding the intellectual property rights associated with gene editing must also be resolved. Mitochondrial replacement therapy leading to '3-Parent Babies' has been successfully carried out in Mexico, by an international team, to correct mother to child mitochondrial disease transmission. The UK has become the first country to legally allow 'cautious use' of mitochondrial donation in treatment. Genomics and genome editing will continue to advance what can be achieved technically, whilst society determines whether or not what can be done should be applied.

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Introduction

Biotechnology is arguably the original multidisciplinary science, utilising living systems or their products to provide goods and services. The burgeoning biotechnology sector has expanded to include new ways to accumulate and analyse information about the structure and function of the genomes that together with environment, determine all aspects of growth and development, through genomics. Major advances have also been made in genome editing, which allows novel and precise mechanisms to modify the structure of genomes. Most recently, the first examples of three-parent babies have added to the utility of biotechnology. These advances provide unprecedented opportunities to increase understanding of genome structure, epigenetics and gene transfer. Future applications of biotechnology will increasingly build upon genomics and genome editing progress, to span sub-sectors, requiring complex contributions from areas as diverse as microbial genetics, to bioinformatics, and from agricultural science to human reproductive physiology.

The burgeoning genomics revolution

Genomics sciences have become one of the biggest growth areas in biotechnology, by studying the nucleic acids (DNA and RNA) that drive gene expression for all organisms. Since the advent of the Human Genome Project in 1990, tasked with mapping and sequencing the 3 Bnbp that make up the haploid human chromosome set, a series of key genomics events have taken place (**Table 1**).

Table 1. Genomics revolution key events

Date	Event
1990	$2.7 Bn mapping and sequencing Human Genome Project launched
1995	1st Microbial genome sequenced: *Haemophilus influenza* (1)
1998	1st Multi-cellular organism genome sequenced: *Caenorhabditis elegans* (2)
1999	Human Chromosome 22 sequenced
2001	Draft version Human Genome published, jointly by Human Genome Project and Celera (3,4)
2004	'Finished' version Human Genome published (5)
2007	23andMe offer direct to consumer genetic testing
2014	$1000 Human genome sequencing commercially available, using Illumina HiSeq X Ten Sequencer for 30x coverage
2015	Personal Genome Project and Veritas Genetics launch $999 full-genome sequencing and interpretation service (6)
2016	'Consumer Genomics' offers DNA App style commercial services, e.g. Helix/National Geographic, AncestryDNA, 23andMe

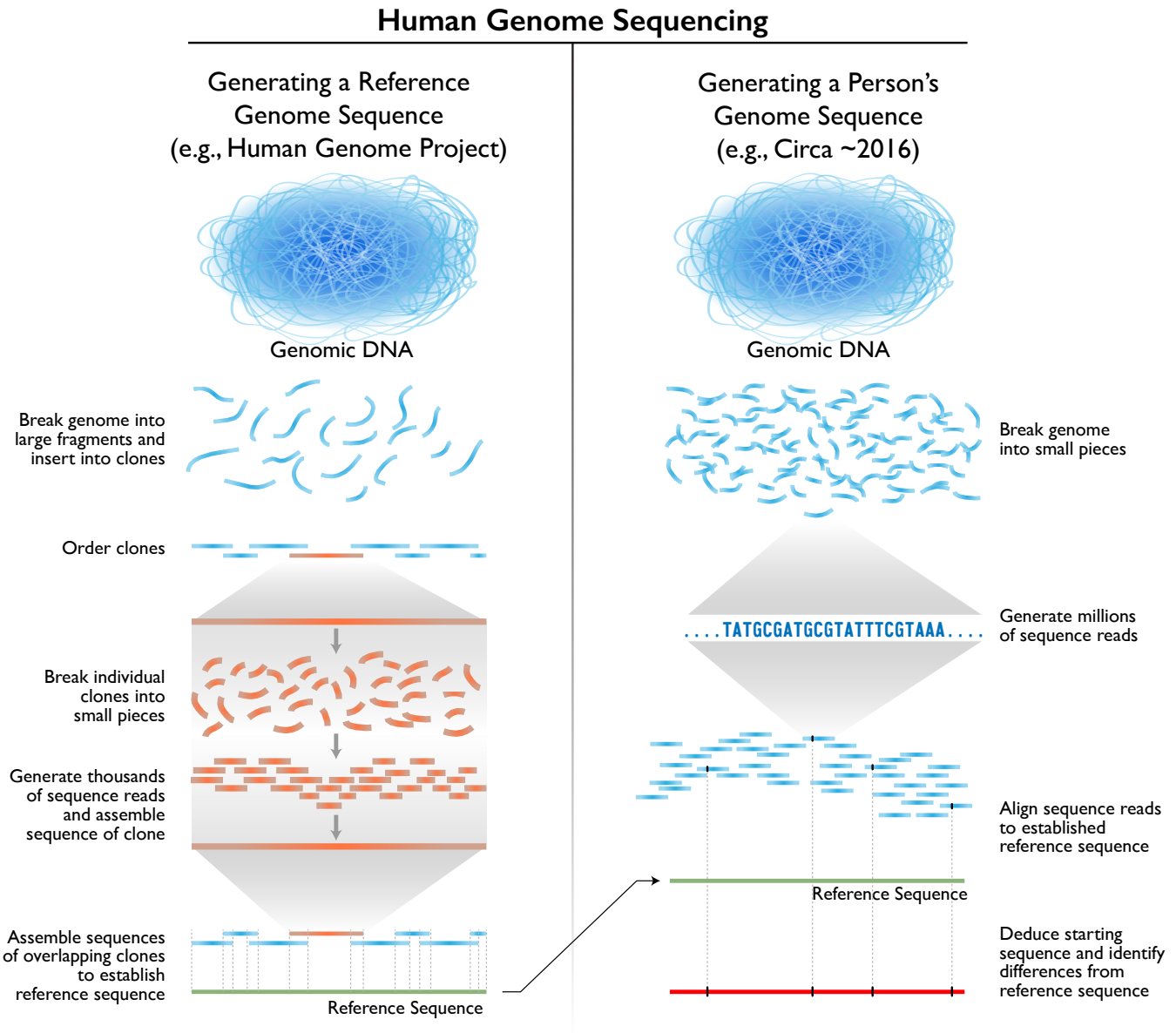

Figure 1. Approaches to human genome sequencing. Source: National Human Genome Research Institute.

Table 2. Genomic complexity

Organism	Genome size (n)	Genes
Escherichia coli	4.6 Mbp	4,377
Caenorhabditis elegans	100.3 Mbp	21,733
Homo sapiens	3.3 Bnbp	~21,000
Pinus taeda (Loblolly pine)	23.2 Bnbp	~50,000

The pace of technological advances in genomics, and the development of bioinformatics tools to analyse DNA sequences have substantially reduced costs, whilst increasing capacity and throughput. Next Generation Sequencing (NGS) platforms typically use massive parallel sequencing of clonally amplified or single DNA molecules spatially separated in a flow cell. Advances in speed, read length and throughput, and the availability of 'reference genome' sequences to which millions of reads can be aligned (**Fig. 1**) have contributed to dramatic reductions in the cost of genomic sequencing.

The vast majority of whole genome DNA sequences identified now are in 'draft' form, with >90% genome coverage and 99.9% accuracy. 'Finished' DNA sequences, with >95% coverage and 99.9% accuracy remain much more expensive to produce, because of the levels of analysis and curation involved, especially with complex genomes (**Table 2**).

Together, these advances have paved the way for the development of a large number of novel NGS applications in basic science as well as in translational research areas such as clinical diagnostics, forensic science and agrigenomics. The MinION from Oxford Nanopore Technologies (ONT) is the only handheld real-time DNA sequencer currently available. Unlike other platforms, nanopore sequencers do not monitor incorporations or hybridizations of nucleotides guided by a template DNA strand. Whereas other platforms use a secondary signal such as light, colour or pH, nanopore sequencers directly detect the DNA composition of a native single stranded DNA molecule. DNA is passed through a protein pore as current is passed through the pore. As the DNA translocates due to the action of a secondary motor protein, a voltage block occurs. This modulates the current passing through the pore (7). Tracing changes in these charges through time generates squiggle space, with voltage shifts characteristic of the particular DNA sequence in the pore, which can then be interpreted as a k-mer. Rather than having 1–4 possible signals, the instrument has more than 1,000, one for each possible k-mer, especially when modified bases present on native DNA are taken into account. The MK1 MinION flow cell structure is composed of an application-specific integrated circuit (ASIC) chip with 512 individual channels that are capable of sequencing at ~70 bp/second, with an expected increase to 500bp/second projected by 2017. The upcoming PromethION instrument is intended to provide an ultra-high-throughput platform reported to include 48 individual flow cells, each with 3,000 pores running at 500 bp/second. This equates to ~2–4Tb for a 2-day run on a fully loaded device, placing this instrument in potential competition with Illumina's HiSeq X-Ten system. Similar to the circular template used by Pacific Biosciences, which uses single molecule, real-time (SMRT) consensus sequencing (8), the ONT MinION uses a leader–hairpin library structure. This allows the forward DNA strand to pass through the pore, followed by a hairpin that links the two strands, and finally the reverse strand. This generates 1D and 2D reads in which both '1D' strands can be aligned to create a consensus sequence '2D' read. Enhanced nanopore sequencing approaches, such as the Roche Genia system, and the recently announced Illumina Firefly, with its one-channel complementary metal-oxide-semiconductor (CMOS) technology, point the way to further advances through superior performance, cost and time savings for large scale and clinical applications. NanoString Technologies' enzyme-free hybridization method, Bio-Rad's GnuBio using a fluorescence resonance energy transfer (FRET)-based approach, and the imaging based Electron Optica which uses an electron microscope-based system all aim to further enhance sequencing through unique technologies. These existing and forthcoming genomic sequencing tools have the potential to allow for revolutionary science, including direct sequencing of RNA or proteins, real-time genomic pathogen monitoring or precision medicine based on personal genome sequencing. Further cost savings are achievable by whole exome sequencing, concentrating only on coding regions, or exons, within genomes. Whereas draft human genomic sequencing can now be obtained for around $1000 including interpretation via FaceTime (6,9), whole exome sequencing, which is on a smaller scale, may soon cost as little as $100-500. Tumbling prices for sequencing services, allied to major imperatives to see genomic sequencing transferred to the clinical setting means that the age of personalised genomics is now upon us. These developments have also spawned 'Consumer Genomics' in which individuals can send off DNA from a single saliva swab for sequencing and access a range of analytical services, through the equivalent of 'DNA App' stores. The resulting information can be stored and accessed by any application throughout the life of an individual. Examples of 'Consumer Genomics' approaches include analysing the ancestry of your DNA, costing from as little as $100 using 700,000 genetic markers, mapping to 26 ethnic regions globally (10) and the series of health and nutrition related screens available from 23andMe (**Table 3**) for $165.

Providers of consumer genomics tests emphasise that results are informational, rather than predictive of diagnoses, or

Table 3: Aspects of 'Consumer Genomics' analysis

Genomic Component	Number Available	Example
Genetic risk factors	11	Hypertrophic cardiomyopathy (*MYBPC3* 25 bp deletion)
Inherited conditions	43	Glycogen storage disease 1a/b (*G6Pc* and *SLC37A4* variants)
Non-Health related traits	41	Alcohol flush reaction (*ALDH2* variant rs671)
Drug response	12	Clopidrogel (Plavix®) efficacy (*CYP2C19* variants)

Source: Welcome to You - 23andMe (2016). http://www.23andMe.co.uk

guarantees of ancestral lineages, despite being able to apportion components of DNA heritage to different racial and ethnic origins. A glimpse of the potential value of this approach comes from drug response analyses, of pharmacogenomic relevance, where for example, 15% of US citizens fail to respond to the widely prescribed clopidrogel (Plavix®) being unable to metabolise the pro-drug form into an active form used to prevent heart attacks and strokes. For a drug with global sales which reached a high of $9.9Bn in 2011, this represents a huge wasted resource, both in financial and risk terms, leading to increased numbers of heart attacks and strokes amongst unidentified non-responders (11), for which the Cytochrome P450 variant *CYP 2C19* is a reliable indicator (12).

'DNA App Store' type applications of consumer genomics will allow digitised genomic information to be accessed by any interested software developer, using a 'sequence once, query often' model (9). Low cost DNA Apps will undoubtedly emerge for both Apple and Android platforms. Two of the earliest developers of DNA App store prototypes are Helix, with substantial support from Illumina, and Veritas Genetics. Helix proposes to eventually drive down the cost of exome sequencing still further, to as little as $100, whilst warehousing genomic data for paid access by individuals or other app developers, such as Good Start Genetics, who offer EmbryVu pre-implantation and GeneVu (with Roche) gene carrier screening tests, supplemented by access to genetic counsellors (13). Other companies have invested in corporate genomic profiling, including Aetna/Newtopia who have combined limited genetic testing with psychosocial assessments to reduce risk of metabolic syndrome amongst employees (14), and Pathway Genomics, who have launched PathwayFit®, a $599 testing service generating personal nutrigenomic reports on >80 gene variants associated with aspects of diet, nutrition, exercise and metabolic health advice (15). Working with IBM Watson, Pathway have developed smartphone and web based apps in the United States, combining personal genomic data with artificial intelligence and deep learning tools to provide individualised strategies and recommendations towards optimal health. 'DNA App Store' type initiatives will undoubtedly drive down initial costs to individuals, making personalised genomics much more affordable, on a pay as you go basis for future screening (9).

Genomics projects are taking place on increasingly large scales, such as the Personal Genome Project, in collaboration with Veritas Genetics, and in the United Kingdom, through the 100,000 Genomes Project, led by National Health Service England. This aims to sequence 100,000 whole patient genomes from sufferers of 100 rare diseases and seven different types of cancer, within three years. >80% of rare diseases, defined as occurring in less than 1 in 2,000 of the population, have a genomic basis (16). By 5 December, 2016 16,171 whole genomes had been sequenced. This project involves 13 'Genomic Medical Centres' and 85 NHS Trusts, comprising 1,500 staff and is linked to 2,500 researchers worldwide. A key feature is the involvement of Genomics England Clinical Interpretation Partnerships, enabling clinicians, academics, and researchers to focus on a single disease or condition and analyse patient and familial genomic data through a series of dedicated 'Panel Apps'. The scale of this undertaking and the new technological solutions being developed within it have attracted several high profile commercial partners, such as Illumina for bioinformatics analysis including sequencing, data storage and interpretation. Twelve commercial pharma, biotech and diagnostics partners have come together through the Genomics Expert Network for Enterprises (GENE) Consortium to be able to use 5,000 whole genome sequences and participant health information on an annual subscription basis, costing in excess of £600,000 per company (17). This will lead to significant job creation opportunities, through drug development, new diagnostic tests, treatments, medical devices and ancillary services, alongside direct patient benefits. Genomics projects large and small give rise to a range of other 'omics science' investigations, from proteomics, to metabolomics and from transcriptomics to the study of interactomics, involving gene-gene, protein-protein or protein-ligand interactions, and how microbial populations interact within the gut (18,19). The can be no doubt, however, that the genomics age is here to stay, with an ever increasing range of tools, challenges and opportunities under development to benefit humankind.

The inexorable rise of genome editing

Precision editing of genomes using recognition sequences and nucleases, has rapidly become a method of choice for altering any gene, in almost any organism (20). Four principle methodologies are in use, Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENS), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas 9 Nuclease and the use of Cpf1 as an alternative to Cas 9 nuclease, described in **Table 4**. These systems have evolved in

Table 4. Genome Editing Tools

Editing Tool	Mechanism
Zinc Finger Nucleases	Zinc finger DNA-binding domain fused to DNA cleavage nuclease domain, recognising 9-18 bp sequence (22)
TALENS	Fusion specific TAL-effector DNA binding domain with DNA cleavage nuclease domain, e.g. Fok1 (23)
CRISPR/Cas9	Two-part guide RNA binds to target sequence and with Cas 9 nuclease cleaves double stranded DNA (20)
CRISPR/Cpf1	Single guide RNA combines with Cpf1 nuclease to target sequence for cutting and production of sticky ends (24)

bacteria to provide defence through adaptive immunity against bacteriophage attack (21), and were initially uncovered in *Escherichia coli*. Our understanding of them has since benefitted from studies in *Streptococcus thermophilus*, *S. pyogenes*, *Lacnospiraceae* and *Acidaminococcus*.

CRISPR technologies now overshadow the use of ZFNs or TALENS approaches, due to their relative simplicity and target specificity. CRISPR has been developed since 2005, when the importance of a protospacer adjacent motif (PAM) for target recognition became apparent (25). CRISPR was shown to be able to target DNA (26) through the transcription of small, spacer sequences, typically derived from previous viral attack into CRISPR RNAs (27). Together with Cas9 nuclease, these could create double stranded breaks in DNA at precise positions, 3 nucleotides upstream of the protospacer adjacent motif (28). Guide RNA (gRNA) consisting of CRISPR-RNAs (crRNA) and trans-activating CRISPR RNA (tracrRNA), pilots Cas 9 nuclease to targets (29) and functions in other species (30) extending the potential utility of CRISPR/Cas9. The true flexibility of this system became apparent in 2012 when the lab of Doudna and Charpentier demonstrated that the crRNA and tracrRNA could be combined to make a single synthetic guide to target Cas9 nuclease action on any specific sequence (20). Double stranded breaks induced by Cas9 are repaired by either non-homologous end joining events, or homologous recombination directed repair (20). This was the beginning of a huge expansion in the use of CRISPR/Cas9 for genome editing, allowing deletion, modification or addition of DNA sequences with a precision and accuracy previously unheard of (31). This approach was shown to be effective in mouse and human cells in 2013 (32).

Table 5. Genome Editing Publications 2012-2016

Year	Publications
2012	12,300
2013	14,700
2014	16,600
2015	19,700
2016	39,600

Source: Google Scholar, accessed 02.01.2017.

There has been a veritable explosion in genome editing research, with CRISPR/Cas9 regarded as the system with the most flexibility(33). Since 2012, articles on genome editing have increased from 12,300 to 39,600 (**Table 5**), demonstrating their applicability to any system, human, animal, or plant, alongside their bacterial origins (34,35). An alternative to the use of Cas9 nuclease is Cpf1, which requires only a single crRNA for targeting, uses smaller crRNAs, recognises a PAM 5' rather than 3' of the target site to generate sticky ends and may provide specificity enhancements over Cas9 (24). This may prove to be CRISPR/Cas9 nucleases biggest competitor (36,37).

Although not without difficulties, principally surrounding the frequency of off-target events, where unintended cutting at off-site targets may occur (24), precision genome manipulation with CRISPR/Cas9 is becoming commonplace in any organism in ways which were not previously feasible (38), and may be used to investigate control of any biological process (39). This includes designing effective gene drives, able to rapidly spread through insect populations in only a few generations (40,41) and Cas9 nuclease triggered chain ablation gene drive brakes, as a potential counterbalancing evolutionary force (42). Potential applications include curbing the spread of malaria, or of Zika virus. Applications in precision medicine have included reduced HIV loading or viral progenome elimination (43). Genetic repair of faulty retinitis pigmentosa genes in inducible pluripotent stem cells, affecting 1 in 4,000 people, demonstrates how genetically corrected stem cells can be used for transplantation purposes in retinal disease (44).

The most controversial developments in genome editing have surrounded the application of human genome editing. A recent International Summit on Human Gene Editing, organised by the US National Academy of Sciences, US National Academy of Medicine, the Royal Society and the Chinese Academy of Sciences (2015) discussed the potential and possible pitfalls of gene editing in humans (45). The Panel ultimately differentiated between four categories: basic and preclinical research, where modified cells should not be used to establish pregnancies; somatic cell clinical use, where proposed studies could affect only individuals they may be appropriately regulated within existing frameworks; and potential germline clinical uses, where alterations in gamete or embryos are possible. Germline clinical uses were considered higher risk and because of their potential to alter gene pools for future generations, should not be undertaken until relevant safety, efficacy, ethical and societal issues have been resolved (45). Chinese scientists have been engaged in two partially successful trials of CRISPR/Cas9 genome editing. Liang et al. (46) used CRISPR/Cas9 to edit beta-globin genes in tripronuclear (3PN) zygotes. The triploid nature of these embryos rendered them non-viable. The gene editing process was however, inefficient, and edited embryos were mosaic, with off-target cleavage effects also

apparent. An endogenous alternative globin gene (delta-globin, HBD) competed with injected oligos as the repair template and led to unanticipated mutations (47). Kang et al. (48) injected Cas9 mRNA, guide RNA and donor DNA for the naturally occurring CCR5Δ32 alleles into early, non-viable human 3PN embryos. The CCR5Δ32 allele renders cells resistant to HIV infection, by preventing HIV particles from entering T cell targets. Only 4 of 26 human embryos were modified successfully, from a total of 213 fertilized human eggs. Both of these studies emphasised the inefficiency of human embryo gene editing and that further understanding of how to limit off-target effects in particular, would be needed before proceeding to implantation or clinical applications (49). Further trials are underway in the United States and China. Animal science applications have also expanded rapidly, including modelling of veterinary diseases and the production of supermuscled pigs, by South Korean and Chinese scientists (38). Development of the supermuscled pigs used TALEN to introduce a mutation into the *MSTN* gene of pig foetal cells, disrupting myostatin inhibition of muscle growth. Thirty-two gene edited piglets with twice the lean meat content of unedited pigs were produced, but only 13/32 reached 8 months old, with only a single piglet considered healthy (50).

Developments in gene editing are not just limited to human and animal systems, but are coming to fruition in plant biotechnology too. Rapidly increasing world population, expected to reach 9.7 billion by 2050 (51) and increasingly unpredictable climate variation will place ever greater demands on global food production (52). Genome editing has a huge part to play in meeting this need, particularly if edited products containing only deletions or self-cloned additions or substitutions are classified as not requiring regulation, as in the USA (53), or stalled in the European Union regulatory impasse (54). This dichotomy rests on whether product or process based regulation is applied globally, and whether, in the case of the EU, crop plants without any additional DNA sequences are considered within the scope of the increasingly outdated EU Directive 2002/219. Plants with no additional DNA, or containing only native genome substitutions, are indistinguishable from parental lines at the DNA sequence level. They are likely to proceed through GMO regulations rapidly, once EU and possibly member-nation policies, have been determined. In contrast, an increasing range of genome edited plants have been considered as not requiring US Dept. of Agriculture oversight (not requiring regulation).

At least five genome edited crop plants have been produced, using Zinc Finger Nuclease, TALENS and CRISPR/Cas 9 nuclease technologies. USDA determined that Dow AgroScience's ZFN-12 phytate reduced maize was not a regulated article, being produced by ZFN induced partial deletions of the IPK 1 gene (55). TALEN has been used to simultaneously edit three homeoalleles in hexaploid bread wheat for broad spectrum resistance to powdery mildew (56), whilst Calyxt's mildew resistant 'MLO-KO' wheat, produced using TALEN has been determined to be outwith USDA oversight (57). CRISPR/

Figure 2: Sulphonyl-Urea herbicide tolerant canola 5715 seed packaging. Source: Cibus.com

Cas9 has provided the majority of crop plant genome editing applications(58). Penn State's Yinong Yang has developed mildew resistant white button mushrooms (*Agaricus bisporus*) in which CRISPR/Cas9 has been used to delete several 1-14 nucleotide regions of the polyphenol oxidase gene, responsible for browning, has led not only to non-browning but also to enhanced shelf life. These mushrooms were judged to be 'without need for USDA oversight' and are thus available for commercial scale release (59,60). DuPont Pioneer have obtained a similar positive determination for their ARGOS8 waxy maize, gene edited using the native maize GOS2 promoter. ARGOS8 is a negative regulator of ethylene responses. DuPont Pioneer's waxy maize provides 5 bushels/acre grain yield advantage under flowering stress conditions and no yield penalty under well watered conditions (61). Du Pont Pioneer 'Waxy Maize' should reach the marketplace within five years (62,63).

Perhaps the most advanced example of gene edited crop plants comes from Cibus' sulfonyl urea herbicide tolerant canola 5715 (oil seed rape, *Brassica napus*) which does not require USDA oversight. By using CRISPR/Cas9 nuclease, a wide range of broadleaf weed control has been achieved. 50,000 acres of Canola 5715 were planted in the USA in 2014, with further scale up for USA commercial release in 2016 and planned release for Canada in 2017(**Fig. 2**; 64). Canada adopts a product based regulatory approach similar to that of the USA. Cibus is also developing similar products in flax, for 2022 release.

There is no doubt that gene editing, led by CRISPR/Cas9 and probably CRISPR/Cpf1, will have a fundamental role to play in future human somatic and germline cell manipulations (38). Insights into the editing process, reducing off-target effects and unintended mutations, gene regulation, imaging and epigenetic marking via sequence specific modifications will contribute to our future understanding in biomedicine, and agriculture (22). Ownership of the intellectual property rights surrounding CRISPR, Cas 9 nuclease and other gene editing tools are

currently ill-defined, with at least three groups claiming ownership. This may, at least in the short term, limit commercial and clinical applications (65).

Mitochondrial Replacement Therapy and '3-Parent' Babies

Although not currently regarded as either genome or gene editing, the potential to explore mitochondrial replacement to overcome mitochondrial disease risk though maternal inheritance is being studied intensively. Mitochondrial DNA, which harbours only 37 genes and makes up 0.1% of total genomic DNA, is extensively involved in cell energy production. mtDNA mutations can cause severe debilitation in children, or prove fatal due to energy system defects or faulty neural system development. Mitochondrial replacement, for example through transferring nuclei from defective maternal egg cells to donor egg cells lacking known mitochondrial mutations could allow parents with maternal rare mitochondrial diseases to produce healthy babies. Zhang et al. announced the world's first '3-Parent' baby, (66) with the successful birth followed spindle nuclear transfer, in which the nucleus was removed from a maternal egg and inserted into an enucleated donor egg, which was then fertilised in vitro. This avoided the need to waste any human embryos (67). Five embryos were created, one of which developed normally and proceded to implantation and live birth of a boy. Mitochondrial analysis revealed that < 1% of mitochondria carried the Leigh Syndrome defective gene, compared with the 13% considered necessary for likely phenotypic expression.

Kang et al. (68) have replaced mitochondria in human oocytes carrying pathogenic mitochondrial DNA mutations, to investigate how mitochondrial replacement therapies can potentially prevent mother to child mtDNA disease transmission. After mitochondrial replacement, the eggs were fertilised, and healthy embryos taken to the blastocyst stage (69). As yet, '3-Parent' Babies are only legally permitted in the UK, as the Human Fertilisation & Embryology will permit cautious use of mitochondrial donation in treatment (70). Following the birth of the Mexican baby boy, it is likely that other countries will advance consideration of the appropriateness of mitochondrial treatments being allowed to proceed to implantation and term birth. This approach is however, not without controversy, including whether or not the egg donor will have any legal rights or obligations. In the UK Parliamentary approved situation, for example, egg donors have no such rights whatsoever.

Controversy therefore surrounds aspects of genome editing and the application of mitochondrial replacement therapy through three parent babies. The years to come will see resolution of questions such as whether genome editing is classified as genetic modification in different regions of the world, together with ethical, moral and legal questions surrounding three parent babies. What is clear, however, is that further advances in genomics and genome editing will continue to advance the art of what is technically possible, whilst leaving society to decide whether what can be achieved should be applied.

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