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Monkey-based Research on Human Disease: The Implications of Genetic Differences

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Summary — Assertions that the use of monkeys to investigate human diseases is valid scientifically are frequently based on a reported 90–93% genetic similarity between the species. Critical analyses of the relevance of monkey studies to human biology, however, indicate that this genetic similarity does not result in sufficient physiological similarity for monkeys to constitute good models for research, and that monkey data do not translate well to progress in clinical practice for humans. Salient examples include the failure of new drugs in clinical trials, the highly different infectivity and pathology of SIV/HIV, and poor extrapolation of research on Alzheimer's disease, Parkinson's disease and stroke. The major molecular differences underlying these inter-species phenotypic disparities have been revealed by comparative genomics and molecular biology — there are key differences in all aspects of gene expression and protein function, from chromosome and chromatin structure to post-translational modification. The collective effects of these differences are striking, extensive and widespread, and they show that the superficial similarity between human and monkey genetic sequences is of little benefit for biomedical research. The extrapolation of biomedical data from monkeys to humans is therefore highly unreliable, and the use of monkeys must be considered of questionable value, particularly given the breadth and potential of alternative methods of enquiry that are currently available to scientists.

Key words: *animal model, epigenetics, gene expression, genetics, macaque, monkey.*

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Introduction

Justification for the use of non-human animals in biomedical experimentation rests on the assumption that there exists sufficient general similarity between each experimental species and humans, to enable the reliable extrapolation of data from the former to the latter. It is widely assumed, and asserted by advocates of animal research, that those similarities that exist are most pronounced in non-human primates (NHPs) compared to other non-human animals, given their relatively recent evolutionary divergence from, and high degree of genetic identity to, humans. Therefore, they argue, NHPs must serve as the best models for researching human biology, in cases where human subjects cannot be used. However, in view of their high cognitive and emotional capacities, and the greater cost of their use in terms of ethics and resources, NHPs are used in much lower numbers than, for example, rats and mice. Indeed, the species with the greatest similarity to humans of all — chimpanzees — will cease to be used worldwide in invasive research in the very near future, as a result of ethical concerns coupled with a consensus that chimpanzee use is not scientifically necessary (1).

Nonetheless, the number of other NHPs used is still considerable: in the USA, the latest published figures (2) show that 71,317 NHPs were used in research in the fiscal year 2010, with a further 54,435 NHPs housed in facilities, but not used experimentally (total = 125,752). More-recent figures for 2012, available on the government database (3), indicate that 64,067 NHPs were used in this fiscal year, with a further 43,149 housed, but not used (total = 107,216). Elsewhere, more than 6,000 were used across the EU Member States in 2011 (2), and 2,202 in the UK in 2013 (3). Where NHPs continue to be used, it is claimed that their high degree of biological similarity to humans means that, in certain circumstances, there is simply no alternative, and that they are a last, but important, resort that offers significant scientific advantages where no other approach will suffice (e.g. 4, 5). Such advantages are, it is argued, conferred by a genetic similarity between NHPs and humans that is extremely high. Chimpanzees are superficially 98–99% genetically similar to humans, though more-stringent and more-comprehensive evaluations put the figure closer to 93% (6). Two of the monkey species most commonly used in research, the rhesus monkey/macaque (*Macaca mulatta)* and the cynomolgus monkey/macaque (*Macaca fascicularis,* also known as the long-tailed or crab-eating macaque) are marginally more dissimilar to humans: some analyses suggest their similarity to humans is approximately 93%, though more rigorous comparisons put the fig-

ure at around 90% or even lower (7). While, at first sight, these similarity figures appear to be high, it is becoming increasingly evident that those genetic differences translate to profound biological differences that make these species unsuitable and poorly relevant models for humans, and/or which explain a number of observed empirical differences (see *Discussion*).

These differences seem obvious when one considers the extraordinary diversity of the order of primates, which comprises 78 genera and 478 species, including humans, and for which 66 new species were identified in the past decade alone (8). It is estimated that the primate lineage is approximately 63 million years old; that chimpanzees and humans diverged 6–7.6 million years ago; that New World monkeys (NWMs), such as marmosets, tamarins, woolly monkeys and squirrel monkeys, diverged around 31 million years ago; and that Old World monkeys (OWMs), such as macaques, baboons, green and vervet monkeys, diverged from their common ancestor at least 14 million, though possibly up to 35 million, years ago (9–13). Consequently, lineage-specific differences, both in gene sequences and in gene regulation, have had plenty of time to occur and accrue. This may be evidenced, for example, by distinct phenotypic differences between species of monkey, even though they are closely related (such as the rhesus and long-tailed macaques): in fact, genetic variability among regional populations of cynomolgus macaques surpasses that even of rhesus macaques, meaning that they can "…differ from each other as much as some species and are not always appropriate for use as the same animal model" (14). Indeed, the difference between Indonesian and Mauritian cynomolgus macaques, in particular, is considered "remarkable", leading them to "vary substantially" to the degree that they "…should not be included in the same experiments as models for heritable human diseases, because they may not be ideal for valid comparisons", and that "combining information on quantitative risk factors for disease from different populations of cynomolgus macaques could obscure risk factor–disease associations or create spurious or artificial associations that are biologically irrelevant" (14, 15). Even within species, there are significant differences: six rhesus macaque subspecies have been noted, displaying a variety of morphological, physiological, and behavioural characteristics (16). Geographical differences are also of major importance, affecting macaque populations worldwide, from Sumatra, Mauritius, Singapore, Cambodia and the Philippines (14), and impacting susceptibility to malarial parasites, SIV infection and pathology, and xenobiotic metabolism via cytochrome P450 (CYP) differences, for example (see *Results*).

It is these differences, and many others, that this review collates and explains in the context of genetics. Historically, genetic inter-species comparisons have been difficult, and therefore rare, due to the lack of knowledge of NHP genomes, and more recently they have been hampered by the poor quality of NHP genome assemblies (17). Nevertheless, knowledge of NHP genomes, in particular of the species used most commonly in biomedical research and testing — the rhesus and cynomolgus macaques (7) — has become sufficiently adequate to enable a number of studies, including the critical comparative analysis presented in this paper. These burgeoning data are increasingly underlining and substantiating King and Wilson's 1975 hypothesis (see 18) that variable gene regulation is the key to species differences, rather than variable gene sequences — a theory that becomes even more compelling when the effects of the inherent stress associated with laboratory life and experimentation on gene expression in those laboratory animals are considered.

We previously examined the 'genetic similarity' argument with a focus on chimpanzees, prior to the US Institute of Medicine's (IOM) inquiry and recommendation, and the consequent decision of the US National Institutes of Health (NIH) that invasive chimpanzee research in the USA should end (6), bringing the USA into line with other countries, such as EU Member States. This in-depth critical review underlined the genetic basis of generally accepted empirical failures of the chimpanzee model, such as its use in HIV/AIDS and hepatitis C research, alongside a comprehensive prospective view that the scale, breadth and consequences of the genetic differences known to date rendered the chimpanzee model unlikely, if not impossible, to be of significant human relevance in the future — in spite of the chimpanzee being more genetically similar to humans than any other non-human species. Similarly, this paper reviews the evidence to date on genetic differences between monkeys (mainly macaques) used in biomedical research and humans. Though it is not restricted to them, it focuses on the two species most commonly used globally: the rhesus macaque, most commonly used in biomedical research, notably in the fields of neuroscience, immunology and infectious diseases (especially AIDS research), and reproductive biology, stem cell biology, metabolism and obesity, diabetes, behavioural biology and addiction (9); and the cynomolgus macaque, most commonly (though not exclusively) used by the pharmaceutical industry and contract research organisations (CROs) for testing new drugs (19). It asks the same basic questions: can the empirical failings of monkey models in human-oriented biomedical research be explained by genetic differences, and what do these differences mean for the future use of monkeys in research? Can monkeys ever be regarded

as similar enough to humans to be essential, or even valuable, research models?

Methods

Papers describing important genetic differences between humans and macaques, which have significant or potentially significant functional biological consequences, were located via the GoPubMed database (20). Mainly, but not exclusively, they were identified via Medical Subject Headings (MeSH) searches of the 'Genetic Phenomena' MeSH descriptor and related sub-headings, filtered with the 'Macaca' MeSH descriptor. The major MeSH sub-headings used were Genetic Processes, Genetic Structures, Genetic Variation, Genotype, Phenotype, and Sequence Homology, which helped limit the scope of published papers from a total of more than 6,600 published between 2003–2013 inclusive. A total of 109 papers, mostly published in the past decade (2003–2013), but some dating from 1988, were selected from the results on the basis of their pertinence to this review, and were examined in detail. Their relevance, and suitability for inclusion, was determined empirically based on their description of some form of genetic difference(s) between an NHP species used habitually in biomedical research with a view to human medicine, and humans. The aim of this work is to illustrate, with a sound and comprehensive basis, that there are crucial inter-species differences with extensive and far-reaching effects. These differences permeate all aspects of gene expression and protein function, from chromosome and chromatin structure all the way through to post-translational modification.

Results

Cytogenetic and other major differences: Fusions, inversions and translocations

Major genomic alterations such as these are important, because they can significantly influence the expression of genes within, or in the vicinity of, the affected regions. This 'position effect' can influence gene expression via changes in the proximity and/or nature of *cis*-acting promoters and enhancers, via the local structural environment of chromatin, altering the accessibility of transcriptional proteins, and by way of gene-silencing effects, via the influence of nearby heterochromatic DNA (21). Consequently, chromosomal rearrangements are acknowledged as having been pivotal in evolution, reproductive isolation and speciation (e.g. 22–24).

There are many examples of major genomic differences between humans and other primates, which confer notable biological consequences. A study of the evolution of human chromosome 17 showed that a paracentric inversion occurred in the human/chimpanzee/gorilla ancestor, meaning that this chromosome differs in these three species compared to other NHPs such as rhesus macaques and marmosets, for example. Consequences in humans involve specific microdeletions and DNA duplications that are associated with various disorders, including: mental retardation; diabetes and renal disease; susceptibility to multiple sclerosis; several cancers; hereditary neuropathy with pressure palsies; Smith–Magenis syndrome; and Charcot–Marie–Tooth disease type 1A (24). A comparison of 9Mb of human chromosome 21 with that of the orang-utan, rhesus macaque, and woolly monkey genomes, showed that around 9% of chromosome 21 DNA is deleted in at least one NHP, and identified a total of 114 genomic rearrangements between humans and these NHPs, which were randomly distributed over genic and non-genic regions (22). These rearrangements are postulated to be involved in qualitative and quantitative gene expression differences between humans and NHPs; one of the deletions identified resulted in the inactivation of a gene in woolly monkeys that is involved in the synthesis of a cell-surface molecule used in the clinical diagnosis of cancer in humans (25).

An analysis through the human/chimpanzee/rhesus macaque lineages identified: human-specific inversions on chromosomes 1 and 18, a human-specific fusion creating chromosome 2, 43 microscopic breakpoints, and over 1,000 submicroscopic rearrangement-induced breakpoints, of which 820 occurred between the rhesus macaque and the human/chimpanzee ancestor (7). Notably, the X chromosome exhibited three times more rearrangements per megabase of DNA than the non-sex chromosomes; this might be of importance, due to the number of disorders related to X-linked genes (there are at least 126 such genes, for example, correlated with haemophilias and muscular dystrophies [26]).

By using an integrative 'genomic triangulation' approach, in which multiple independent sources of genetic information are brought together to reconstruct the ancestral genomic state and subsequent evolutionary processes, human-specific changes in genome structure were ascertained by comparisons with the chimpanzee and rhesus macaque genomes (17). This study identified a total of 288 human-specific genomic breakpoints. Discounting 158 breakpoints that may have been artefacts, 130 breakpoints were a result of 'intermediate' (10kb–4Mb) rearrangements, which included 64 insertions affecting 58 genes (of the insertions, 36 consisted of complete gene-copies),

and a further 22 genes were partially duplicated or contained an insertion.

Mobile DNA elements and copy number variation, etc.

The position effect described above, in which the altered location and/or local environment of a gene may have a profound effect on its expression, is an important aspect of the action of mobile or 'transposable' DNA elements — sequences of DNA that can move or transpose themselves to another location in the genome, sometimes also leaving a copy of that gene behind. The extent of the position effect's consequences can be appreciated, when one considers that repetitive sequences of DNA, most of which constitute these mobile elements, account for approximately 50% of the genome of primates, including humans, chimpanzees and rhesus macaques (27, 28). Even though the overall number of mobile elements is similar across many primate species, they have inserted into different genomic locations, and have therefore affected the evolution of those species via resultant differential gene expression (29). There are several types of mobile elements, which are characterised by different types of repetitive sequences, such as long and short interspersed nuclear elements (LINEs and SINEs, respectively; 30).

Alu *elements*

One especially abundant type of short interspersed nuclear element (SINE) is the *Alu* element, of which around one million copies have accumulated in primate genomes over the past 60 million years of evolution — a process that is ongoing (31). It is known, however, that the relatively-closely related chimpanzee genome has up to 100,000 fewer *Alu* elements than the human genome (32), and it has been conservatively estimated that around 110,000 *Alu* elements were specifically acquired in the OWM lineage (7, 27). Species including the lemur, marmoset, baboon, rhesus macaque, chimpanzee and human, all have different densities of *Alu* sequences in their genomes (the marmoset shows the highest and the lemur the lowest), all of which include many lineage-specific families of *Alu* elements (33). Their ubiquity and inter-species differences are compounded by the consequences of their activities: via their role in genomic rearrangements, they give rise to new exons, often in existing functional genes and with diverse splicing patterns (34); they affect gene function by physically disrupting coding sequences when they insert into new sites and/or vacate others, which might not be repaired adequately; they alter gene expression and/or function by disrupting promoter/enhancer regions and/or transcriptional splice-sites; and they often give rise to large duplicated regions of DNA ('segmental duplications'), when the mechanism of their transposition involves copying themselves, rather than a simple 'cut and paste' (see below). They are known to be involved in the tissue-specific regulation of gene expression and development, in nucleosome positioning, and in differential methylation (see 35). Due to their mutagenicity, *Alu* elements specifically are associated with various diseases, including: muscular dystrophy, several cancers (retinoblastoma, leukaemia, and breast and colon cancers), haemophilia, neurofibromatosis, type-2 diabetes, Alzheimer's disease, and Hunter and Sly syndromes (see 32, 33, 35, 36). Consequently, inter-species and intra-species differences in mobile elements, their locations and proximity to specific genes, etc., will differentially affect gene complement and expression, and also disease susceptibility and pathology (6). The power of *Alu* elements, which exert a greater influence on phenotype, species differences and disease susceptibility than previously thought (33), can be illustrated by the study of just four, all of a specific type, across seven primate species (human, chimpanzee, gorilla, orang-utan, baboon, rhesus macaque and lion-tailed macaque [*Macaca silenus*]); these *Alu* elements reside directly upstream of the genes they regulate and affect gene transcription by carrying *cis*-acting elements responsive to hormones, calcium, transcription factors and other effectors (37). These four elements, known to impact the expression of the parathyroid hormone (PTH) gene, the haematopoietic cell-specific FcεRI-γ receptor gene, the CNS-specific nicotinic acetylcholine receptor α3 gene, and the T-cell-specific CD8α gene, were found to be differentially distributed across these seven primate species, and in a way that "establishes a link between gene regulation and the divergence of primates". It had been previously noted that, "*Alu* insertions are now extremely attractive candidates for promoting differences in the developmental regulation of primate genes", on account of their "…genomic mobility, high CpG content, tissue-specific methylation, and their effect on chromatin structure and gene expression" (for references, see 37). One salient example of the power of just one differential *Alu* element is the *Alu*-mediated inactivation of the CMP-*N*-acetylneuraminic acid (*CMP-Neu5Ac*) hydroxylase gene in humans (38). Unlike the gene in the NHPs examined (chimpanzee, bonobo, gorilla, orang-utan, gibbon, baboon, and rhesus monkey), the human gene contains an *Alu*Y element that is different to the *Alu*Sq element found in the NHPs, and it is this *Alu*Y element that is believed to have deleted a 92bp exon in the human gene, ren-

dering it inactive. This results in a lack of a specific sialic acid residue on the surface of almost all cells in the human body, compared to the NHPs, which has significant consequences for susceptibility and resistance to microbial infection, as many pathogens initiate the infectious process via preferentially binding to particular sialic acid residues on cell surfaces, including influenza viruses and *Escherichia coli* (38, 39).

Long interspersed nuclear elements (LINEs)

With regard to LINEs, it has been conservatively estimated that approximately 20,000 of a certain type of LINE (L1) were specifically acquired in the OWM lineage (7, 27). It is also known that specific sub-types are known to differ between primates. For example, the L1PA5 element of the L1 LINE sub-family is present in 19,000 copies in the rhesus macaque genome, all of which are specific to the OWM lineage, which itself contains 32 OWM-specific L1 sub-families (27). The same report identified 80–100 active copies of potentially active L1 elements in the human genome, while just nine were found in the rhesus macaque genome. LINE-1 elements may induce 'gene breaking', splitting a host gene into two transcripts (40).

Endogenous retroviral mobile elements

Another type of mobile element, the endogenous retrovirus (ERV), is a so-called genomic 'footprint' of previous retroviral infection, and is inherited by successive generations (41). These ERVs are able to exert similar effects on gene complement and expression as other types of mobile elements, and the typical primate genome contains around half a million copies.

Consequences of insertional differences

A comparison of the human, chimpanzee and rhesus macaque genomes identified 112 examples of human-specific genomic insertions involving mobile elements (including LINEs, *Alu* elements and ERVs), which have given rise to gene transcripts that are specific to humans, of which 74 were associated with known genes (42). The consequences of this are much greater than the modest number may suggest. The insertion events had generated novel promoters and exons, novel intragenic and intergenic transcripts, novel functional RNAs (such as small interfering and micro RNAs), anti-sense transcripts, polyadenylation sites and splice sites, for example, and disrupted evolutionarily conserved (and therefore important) control elements, altering gene expression, activity and/or function.

DNA duplications

Segmental duplications resulting from the action of repetitive sequences, which have arisen over the past 35 million years of primate evolution, have been estimated to comprise up to 5–6% of primate genomes (23). They are frequently species-specific, and are known to have contributed greatly to evolution, speciation, gene innovation and therefore inter-species biological differences. With particular regard to the use of animals in biomedical research, they can affect disease aetiology/pathology and xenobiotic metabolism (e.g. 23, 43). They are known to underpin many genomic disorders, such as Smith–Magenis, Prader–Willi, velocardiofacial, DiGeorge, Angelman and cat-eye syndromes, neurofibromatosis type 1, and Charcot–Marie–Tooth disease type 1A, as well as a host of more-complex genetic traits (44), and have been implicated in the creation of regions of genomic instability that can affect predisposition to diseases. For example, a duplicated segment of human chromosome 5 is associated with the locus for, and may well affect predisposition to, the neurodegenerative disease, spinal muscular atrophy (23). It has also been estimated that duplication events may have created around 1,100 new transcripts over the last 35 million years of primate evolution (44), and that just 2.3% of the rhesus macaque genome consists of segmental duplications, compared to 5–6% for the human and chimpanzee genomes (7) — all of this significantly contributes to inter-species variability among primates. The biological significance of some duplications has been established (7); for example: susceptibility to HIV infection (CCL3L1-CCL4), toxicity response (cytochrome P450), and developmental regulation (KRAB-C2H2). There are others, including those which affect the immune response (via the major histocompatibility complex [MHC]/human leukocyte antigen [HLA] system; see below).

Consequences of DNA duplication variations

Crucially, many of these duplications are not simply 'lost' without consequences to the organism's genome, i.e. they do not just result in inactive gene copies and pseudogenes. An estimated 10% of lineage-specific gene duplications in primates provide new functions that are positively selected (45). Here are some examples to illustrate this point:

a) The KRAB-ZNF gene family encodes the largest class of mammalian transcription factors; more than 400 genes are present in the human genome, of which more than 136 are primate-specific (46). Seventy of these genes are present in segmental duplications, 24 of which are exclusive to hominids. In short, many KRAB-

ZNF genes undergo segmental duplication, followed by functional and regulatory diversification, often via sequence changes that alter DNA-binding and splicing specificities, as well as tissue-specific expression. Ultimately, in concert with the fact that each gene/gene product may affect the expression of hundreds of downstream target genes, this results in the altered structure and function of regulatory networks in different primate species.

b) Segmental duplication can result in copy number variation (CNV), in which different 'copy numbers' of the same gene, generated by duplication, give rise to significant differences in the expression of the affected genes. As a consequence, CNV is a major cause of genetic variation even among humans, i.e. within the same species, let alone between different species (47). CNV may affect, among other things, disease susceptibility, immune responses, and the formation of tumours (e.g. 48, 49). One study identified 51 genes with increased copy number in the rhesus macaque compared to humans (7). Notably, duplication and CNV have greatly influenced the evolution of a family of genes that is pivotal to the function of the immune system: MHC, or, in humans, the HLA system.

The HLA gene family comprises a large variety and number of polymorphic genes, which produce cell-surface molecules that facilitate recognition by — and in the case of 'foreign' molecules (for example, those derived from infectious agents), destruction by — immune cells. The HLA genes give rise to two classes of HLA molecules, which are involved in immune function in different ways: class I HLAs are involved in the binding and presentation of intracellularly-generated peptides to $CD8^+$ T-cells, whereas class II molecules present peptides of extracellular origin to $CD4^+$ T-cells. Both class I and class II HLAs are derived from three major classes of genes (HLA-A, B and C for class I; HLA-DP, DQ and DR for class II; 50).

Because of the important role of the MHC in several biological and medical areas, such as disease susceptibility and resistance, transplantation, reproduction and stress management, it has been studied extensively, both in humans and in the non-human species used in research, such as rhesus macaques (see 51). This degree of focus on MHC function has highlighted important inter-species and intra-species differences, including the creation, deletion and inactivation of genes resulting from extensive genomic duplications and other rearrangements, as well as differences in levels of allele expression.

The equivalent to HLA class I genes in rhesus macaques are known as Mamu-A and Mamu-B, and although an equivalent to the HLA-C locus is missing in rhesus and other macaque species (52), at least 37 extra class I genes exist in the rhesus macaque (11). In addition, macaque MHC gene copy numbers are greater than the copy numbers of the equivalents in humans and all four great-ape species, while the copy number of other immune-related genes (immunoglobulin lambda-like) is also higher in macaques. This substantiates claims that, "…although the macaque has been extensively used to model the human immune response, there may be substantial and previously unappreciated differences in HLA function between these species" (7). Furthermore, this may not be an inter-species problem of relevance and extrapolation: Mamu-A allele variations are also highly divergent and specific to populations of rhesus macaques, which mean that Indian, Burmese and Chinese monkeys differ markedly immunologically. The lack of relevance of immune-related data between different populations of macaques clearly has serious implications for the extrapolation to humans of data from macaque research, particularly with regard to responses to infectious agents. For example, it is known that different MHC class I alleles are associated with different responses to, and outcomes of, SIV/HIV infection (51). Furthermore, the MHC (comprising both class I and II alleles) is associated with more human diseases and immune-related disorders than any other region of the human genome, such as insulin-dependent diabetes, rheumatoid arthritis, ankylosing spondylitis, common variable immunodeficiency, and IgA deficiency (53, 54). Consequently, variability in MHC composition, processing and expression has significant impact on disease susceptibility and pathology.

c) A comparison of pooled genetic material from the human, chimpanzee, bonobo, gorilla, orangutan and Japanese macaque (*Macaca fuscata*), which covered approximately 30% of the human genome, identified 322 sites of large-scale inter-species copy number differences (55). Fourteen of these were human lineage-specific gains, most of which were in genomic regions previously identified as segmental duplications, and all of which represented copy number gains and contained known genes, including genes involved in immune (*VDUP1*) and oxidative stress (*FCGR1A*) responses. As expected, due to its greatest genetic divergence from humans, the Japanese macaque showed the highest number of lineage-specific deletions, but also the highest number of duplications of the species studied. In spite of current knowledge of macaque CNVs, it has been estimated that, "thousands of common macaque CNVs are yet to be identified" (56), although those that have been identified to date are of great interest. One study compared genomic regions associated

with CNVs in humans, chimpanzees and rhesus macaques, and found that many of these regions overlapped: more than 2,000 human CNVs overlapped with orthologous chimpanzee or rhesus macaque CNVs, and 170 of these overlapped with both (56). These CNVs were 'collapsed' into 34 'hotspots' for CNV formation, which were identified as being heavily associated with genes. Notably, CNVs in the rhesus macaque genome are much more likely to be associated with functional genes than are human CNVs. This is relevant to the use of macaques as a model species for human research, because CNVs alter the number and position of genes and/or elements that regulate gene expression. In addition, their effects are not limited to the exact CNV locus: studies have demonstrated that CNVs affect gene expression at other loci, such as where genes share the same promoter region (57), and where the presence or absence of related pseudogenes may affect the level of associated microRNA molecules (due to their degree of sequestration) with the consequent modulation of gene expression (58).

Many of these genes are involved in immune function, including genes comprising the Leukocyte Receptor Cluster (LRC), which itself includes killer-cell Ig-like receptor (KIR) genes, whose gene products interact with MHC class I molecules (see above). One example of an important gene affected by species-dependent CNV is the *CHEK2* gene, which is important for genome stability, and is known to be a multi-organ cancer susceptibility gene, including cancer of the breast, colon and prostate. It is present in just one copy in baboons, pig-tailed and rhesus macaques and orang-utans, but in 7–9 copies in chimpanzees and gorillas, and in 13–16 copies in humans (59).

d) Parallel duplications and losses of the *RHOXF2* gene in humans and 16 NHP species, alongside different patterns of expression, are thought to have important inter-species biological implications due to the role of the gene as a transcription factor and in developmental processes (60). Mediated by ERV activity, this has resulted in between one and six copies of the gene in each of these species; twice the quantity of *RHOXF2* DNA in rhesus and Southern pig-tailed macaques *(Macaca nemestrina)* compared to other types of macaque; very different patterns of expression in rhesus macaques, in which it is chiefly expressed in the lung rather than the testes; and a possible functional difference in rhesus macaques and leaf monkeys, due to sequence divergence between their two gene copies, in stark contrast to humans in which this has not occurred. Notably, *RHOXF2* is expressed differently in the brains of human newborns/embryos and adults, and it regulates the expression of at least three other genes involved in the function of the central nervous system (CNS). It is therefore thought to be involved in CNS function and brain development, with significant implications for inter-species differences. In summary, genes associated with CNVs are more likely to be differentially expressed between species, and CNVs are therefore hypothesised to be one of the major factors in inter-species variation due to differential gene expression (56).

Long inverted repeats

Another type of repeat is the 'long inverted repeat' (LIR), consisting of a sequence of DNA followed by its reverse complementary sequence. LIRs have been studied because of their roles in inducing genome instability, via gene amplification, recombination, DNA double-strand breaks and rearrangement, and also in gene expression regulation, via RNA interference, initiation of transcription and of DNA replication, and alternative splicing (see 61). Comparative studies of human, chimpanzee and rhesus macaque genomes identified, for example, different numbers of LIRs associated with orthologous genes in these species, including 546 in humans, of which 421 (77%) were human-specific, but only 130 in rhesus macaques, of which 107 (82%) were rhesus macaque-specific (61). Genes associated with the human-specific LIRs were involved in neural development and function, and cell communication.

Gene complement

The evolution of genomes over time, as well as causing changes in gene expression, regulation and gene products, also results in the creation of some new genes and the loss of others, largely due to the activity of mobile elements as described earlier. For example, even chimpanzees and humans, which have genomes more alike than any other human–non-human species pair, show large numbers of gene gains and losses over time. One study suggests that, since the evolutionary split between the species around six million years ago, chimpanzees have gained 26 genes and lost 729, while humans have gained 689 genes and lost 86 (62); another study claims (similarly) that humans have gained at least 678 genes, while chimpanzees have lost 740 (63). In primates, genome changes at the nucleotide level occur more slowly than in non-primates, but the gain and loss of entire genes are accelerated in primates compared to other mammals (see 63). This is thought to explain how humans and chimpanzees are so similar in terms of their shared gene sequences, yet display many

biochemical, physiological and behavioural dissimilarities. In fact, humans in particular have a rate of gene turnover 2.5× that of all other mammals, which includes several gene families, including transcription factors, immune response-related genes, and, notably, genes preferentially expressed in the brain (63). A computational analysis of primate genomes has predicted that 108 gene families have changed size during the evolution of primate species, and have changed at a much higher rate than overall primate rates of gene gain and loss — this includes 1,358 genes gained by the rhesus macaque lineage as a result of duplication events (7). One analysis of a relatively small proportion of the cynomolgus macaque genome compared 139 novel transcripts that contained at least 90bp open reading frames with the human genome, and showed that 64 of them (46%) could not be matched, suggesting that they were specific to the cynomolgus macaque (64). Many of the genes identified in this analysis were related to immune function, such as immunoglobulin genes, members of the tumour necrosis factor superfamily, and genes involved in B-cell function and the MHC, which are important regulators of inflammation, apoptosis, and the immune system in general.

Effects on immune responses

One family of genes intimately involved in immune function, which is greatly affected in terms of species-specific gene complement, is the MHC family; the absence of an orthologue for the HLA-C locus in macaques was an example discussed earlier among aspects of expression and CNV. Many new MHC genes and alleles have been generated via duplication and recombination processes, at the same time as others have been deleted or inactivated, leading to, for example, the Mamu-A locus in rhesus macaques becoming three times larger than its HLA-A human equivalent (51). While there are some broad similarities in terms of the configuration of the MHC loci across species, many gene lineages and alleles are specific to particular populations of monkeys. For example, rhesus macaques of Chinese origin have a higher general genetic variability than do those of Indian origin (which is not limited to, but includes, their MHC loci); in rhesus macaques of Burmese origin, half of their MHC class I alleles are novel (51). Indeed, the overall genetic backgrounds of rhesus macaques of different geographic origins are "remarkably divergent" (see 51), with serious consequences for their use in biomedical research with a human focus (see *Discussion*).

Effects on drug metabolism

In view of the extensive use of macaques in the testing of new drugs intended for human use, surprisingly little investigation has been conducted into the nature of their cytochrome P450 (CYP) enzyme orthologues — the class of enzymes responsible for around 90% of drug metabolism (65). However, one recent study identified 18 novel P450 sequences in the cynomolgus macaque, for which the protein identity with humans was 94–99% (19). This is notable, because it has been established that even minor variations in amino acid sequence (as little as a single conservative substitution) may cause significant differences in the activity and/or substrate specificity of P450 enzymes (66, 67), and that important differences in P450 activities exist with consequences for extrapolation between monkeys and humans (68). Comparative analyses of the genomes of key model organisms used in drug testing, such as two types of mini-pig, beagles, boxers, mice, rats, and cynomolgus and rhesus macaques, revealed "considerable variation in gene content", including "key genes in toxicology and metabolism" (69). One such example involved the UDP glucuronosyltransferase 2 genes (UGT2), the most important enzymes in Phase II metabolism, that have a critical role in the conjugation and elimination of toxic compounds, for which Vamathevan *et al.* (69) noted "the lack of conservation between human and macaques". This report also noted that both rhesus and cynomolgus macaques have around two-thirds of the ADMET genes (Phase I and Phase II enzymes, and transporter genes) that humans have, the most notable difference being that there are significantly fewer Phase II genes in macaques.

Coding sequence differences

An analysis of cDNA libraries from the rhesus monkey revealed that the coding sequences (CDS), as well as the 5′ untranslated regions (UTRs) of the cDNAs, were much less variable than the 3′-UTRs (70), in agreement with previous reports (71), which put the sequence identities of these regions with those of humans at around 98% (CDS) and 95% (3′-UTRs). This variability of UTRs is of interest, as inter-species differences between UTRs may affect gene regulatory regions, altering expression in several ways, via transcript stability, localisation, translation efficiency, etc. This same study also screened just over 1,800 rhesus monkey cDNAs, and found 61 sequences that had no human equivalent, which may therefore represent genes unique to the rhesus macaque. It also reported the existence of 214 human and monkey transcripts that showed different structures, i.e. contained insertions or deletions, 200 of which were in functional gene regions with potential functional consequences, and a number of rhesus monkey-specific splicing events that produced rhesus monkey-specific exons.

Gene expression differences

Even where genes may be identical or highly similar in different species, they may be expressed differently, at different levels, and/or in different tissues. Studies on differential gene expression have elucidated this area as the basis for widespread and varied species differences.

A comprehensive analysis of the genome of the cynomolgus macaque, focusing on the suitability of the species as a model for drug safety assessment, identified 6.5% of all expressed genes (n = 718) as being highly variably expressed in the livers of macaques of Philippine, Chinese and Mauritian origin (19). This illustrates that the results of drug studies may be heavily influenced by the type of macaque used, even of the same species. Many of these genes, as expected, had metabolic functions, though others were associated with immune functions. It also revealed much about comparative CYP expression in humans and cynomolgus macaques: while variation among individual macaques was low for the 50-plus cytochrome P450 genes, and overall inter-species expression correlated fairly well (r = 0.73), the expression of six of the enzymes varied greatly in the macaques, and nine of them had basal expression levels very different from the levels in humans; some CYPs were more active in macaques than in humans, and one third of them were differentially expressed, including the key enzyme, CYP1B1. These considerations led to the conclusion that, "…gene expression levels of certain cytochromes p450 can complicate the interpretation of primate drug metabolism experiments with respect to their translational relevance for humans" (19). Some of the other differentially expressed genes, such as various cytokines and chemokines, produce proteins that are assayed as part of pre-clinical and clinical drug trials, as increased levels indicate toxicity-mediated activation of the immune system, so they are of direct relevance to the suitability of the macaque for drug testing purposes. Notably, and in support of this finding, it has been previously concluded that immune responses in NHPs are "poor predictors of human defence reactions in clinical trials, which can lead to fatal outcomes" (19, 72).

Effects on immune responses

Studies of gene expression in stimulated primary monocytes from humans, chimpanzees and rhesus macaques have shown that immune responses to viral infections differ between primate lineages, supporting claims that lineage-specific immune responses are involved in species-specific differences in susceptibility to infectious diseases. Human responses, for example, are enriched for genes associated with apoptosis, cancer, and susceptibility to infectious and immune-related diseases, while chimpanzee responses are enriched for HIV-interacting genes (73). These investigations showed that 17.5% of genes demonstrated altered expression levels in at least one of the three species, and 25% of these genes (4.3% of the total investigated) had different expressions in all three species. These genes were involved in various immune-related processes and pathways, such as pro-inflammatory cytokines and chemokines. The authors conservatively estimated that 393 genes were unique to the rhesus macaque immune response. Interestingly, expression of the pro-apoptotic gene *CASP10* was greatly reduced, exclusively, in human monocytes. Mutations in, and reduced expression of, the *CASP10* gene have been associated with several human cancers (see 73), which have different prevalence across primate species.

Gene expression in the brain

With regard to the brain, one study reported that over 7% (893/12,473) and 6% (789/12,473) of genes in the cerebellum showed increased and decreased expression, respectively, in humans compared to rhesus macaques (74). Another noted that 91 genes were differentially expressed in human brains relative to those of rhesus macaques and chimpanzees (75). A 'whole blood' gene expression analysis in humans, cynomolgus and rhesus macaques, and African green monkeys, revealed each of the NHP species to be "dissimilar to humans" (the cynomolgus macaque was most dissimilar), and that 317 genes (including chemokines, and splicing and transcription factors) were differentially expressed in humans compared to the NHPs (76).

Gene expression in other organs

A genome-wide comparison of gene expression in the livers, kidneys and hearts of humans, chimpanzees and rhesus macaques, revealed many genes whose regulation has evolved under natural selection, supporting the theory that gene regulation contributes more to speciation than structural differences in the genes themselves. The comparison showed that the regulation of a large number of transcription factor genes and metabolic pathway genes had evolved under natural selection, particularly in the human lineage (18). While fewer genes were differentially expressed between species in the liver compared to the heart and kidney, the magnitude of expression differences in this organ was greater. Between humans and the rhesus macaque, there were 5,525, 6,250, and 5,545 genes differentially expressed in the liver, kidney and heart, respectively. The classes of dif-

ferentially expressed genes are again of interest, as they included transcription factors, which can affect the regulation of many hundreds of genes with significant phenotypic effects (77), as well as metabolism genes (particularly important for drug studies), and genes involved in neurodegenerative diseases and various cancers. These findings built on the results of a prior study from 2006, which similarly reported a relative and highly significant excess of transcription factors in the genes whose expression was specifically increased in humans compared to the chimpanzee, orang-utan and rhesus macaque, and which the authors believed influenced disease susceptibility and therefore had "implications for studies of human disease" (78). A study focusing on the heart identified 65 genes that were differentially expressed in humans, rhesus monkeys, rats, mice and dogs, with a variety of functions and involvements, including hormone-receptor binding, thyroid cancer, and proteasome function (79). Notably, more than 46% of these genes have been associated with cardiovascular disease. This finding may underline why, in cardiovascular disease research, "animal models fell short of the expected results, or even came out with opposite phenomena in many cases" (79).

Factors affecting gene expression

There are many molecular mechanisms and factors that can alter the expression of a gene, some of which have been investigated comparatively in multiple species.

Epigenetic factors

Epigenomic modifications can be considered to be chemical modifications to the genetic material *other than of the nucleotide sequence of the DNA itself*, which control and alter gene expression (80). Elicited by a multitude of internal and external factors, these modifications are of interest, because of their power (the ability to modulate the expression of many genes), range and complexity, intricate regulatory mechanisms, heritability, flexibility (permanence/reversibility), and, above all, the fact that they permit radically different temporal and spatial gene expression patterns, thereby promoting biological diversity from identical genetic sequences. Salient illustrative examples of their power include the food-directed alternative development of distinct queen and worker honeybees from identical genomes (81, 82), and the speciation of Darwin's finches in the Galapagos islands, highlighting epigenetics as a "major component of genome variation during evolutionary change" (83). With direct relevance to humans and animal models in biomedical research, epigenetic modifications help to explain how identical twins generally have different disease conditions, how hundreds of environmental toxicants associated with diseases do not induce DNA mutations, and how genome-wide association studies have revealed less than 1% of a specific disease population share a DNA sequence mutation. In short, many biological phenomena cannot be explained by 'classical' genetics, and the environment — via, to some degree, epigenetics — plays a major role (84). Epigenetics therefore has a significant influence on gene expression and associated biology, and is highly relevant to the consideration of animal models and their relevance to humans.

Epigenetic modifications are numerous (in their hundreds) and diverse (81, 85–87), affecting the DNA itself, or the histone-protein 'scaffold' around which the DNA is wound (88). They may exert potent modulatory effects on genes, both stimulatory and repressive, even to the degree of turning expression on or off. This is because they are mechanistically important in the 'on demand' de-condensation of chromatin, opening up the structure for the access of enzymes that transcribe, repair and copy the DNA in essential biological processes (86), and are therefore intimately associated with the modulation of gene expression, which, in turn, is finely tuned and tightly controlled, due to the complex combinations of modifications that exist (86, 87) — there might be as many as 2.2×10^{12} possible combinations (89).

Examples of environmental influences on epigenetics include many xenobiotics, such as various pollutants, cigarette smoke, changes in temperature and other stressors, and these influences have significant consequences during fetal development (90). Many xenobiotics, including several prescription drugs, pollutants, caffeine, and nicotine, as well as stress, affect normal fetal development via epigenetic mechanisms, and also modulate the perinatal programming of the hypothalamic–pituitary–adrenal (HPA) axis (91). HPA programming is crucial, because it interacts intimately with the immune system. Its dysregulation may lead to excessive inflammation via increases in the levels of circulatory inflammatory cytokines, concomitant decreases in anti-inflammatory cytokines, and alterations in the expression of genes involved in immune activation of peripheral blood cells, for example, along with general adverse effects on immune function and increased susceptibility to infectious and autoimmune diseases (see 92). Furthermore, while many epigenetic modifications induced by these factors are dynamic, transient and reversible, some are long-lasting, even semi-permanent, and may be trans-generational, inherited by an individual's offspring and persisting for several generations (93–95).

Epigenetic modifications are genome wide, though their effects on particular genes and

genetic pathways, and on eventual phenotypes, are especially notable when considering species differences and the use of animals as models for humans. It has been postulated, for example, that "the entire topology of a complex brain network can be reprogrammed by subtle adjustments of many genes that act additively to produce a given phenotype" (96). CYP genes, mentioned earlier, are regulated epigenetically, both transcriptionally and post-transcriptionally. The DNA methylation of CYP genes appears especially prevalent, including the CYP epoxygenase genes, which "could have significant consequences on drug and endogenous compound metabolism" (97). In fact, both DNA methylation and histone modifications contribute significantly to variability in function of many genes controlling the absorption, distribution, metabolism and excretion (ADME) of drugs, with just 20–30% of inter-individual variations in drug efficacy and toxicity due to genetic factors (98). Epigenetic factors are also strongly associated with susceptibility to cardiovascular diseases, ageing (97, 99), hypertension and preeclampsia (85), psychiatric disorders, including PTSD, depression, bipolar disorder and schizophrenia (95), systemic lupus erythematosus (100), and others.

Inter-species epigenetic differences have not been well investigated, given the nascent nature of the discipline, but, given the breadth and degree of intra-species variability that is already known to exist, these differences are likely to be numerous. However, current examples include DNA methylation's "surprising diversity in regulatory mechanisms and genome-wide profiles over various organisms", with "extremely diverse" distributions, regulatory mechanisms and potential functions across eukaryotic genomes (101); differences in lysine acetylation and methylation in histone H3 across different species (102); and an NHP gene-expression study that analysed a specific histone modification associated with transcriptional promotion, known as H3K4me3 (trimethylation of lysine 4 of histone 3), in lymphoblastoid cell lines from humans, chimpanzees and rhesus macaques. Inter-species differences were identified in the locations of H3K4me3, which correlated with genes known to be differentially expressed between the three species, and which were therefore conservatively estimated to be partly responsible for 7–10% of gene expression differences in primate lymphoblastoid cells (103). As part of this study, the authors also identified 5,420 genes as being differentially expressed between humans and rhesus macaques.

Transcription factors

Another salient example is transcription factors (as discussed above) — gene products that are able to modulate the expression of hundreds of genes that they specifically regulate, with notable effects on phenotype, and that appear to have been positively selected in humans compared to other primates (18, 77, 78). An analysis of the largest family of primate transcription factors, the Kruppel-type zinc finger (KZNF) family, revealed a host of lineage-specific duplications and deletions that had occurred over the evolutionary history of humans, chimpanzees, orang-utans and rhesus macaques, leading to 213 species-specific KZNF genes, including seven human-specific and 23 chimpanzee-specific genes (104). In addition, it appeared that the human lineage had lost ten such genes via pseudogenisation. The human genome was shown to have 609 KZNF genes *versus* 459 in the rhesus macaque (i.e. 150 more). The analysis also showed that humans had gained seven and lost ten KZNF genes, compared to the gain of 38 and loss of at least 40 such genes in the rhesus macaque. One transcription factor that displayed species-specific structural changes (ZNF80B) was found to have binding motifs on target genes that are important in neuronal function and development.

MicroRNAs and small interfering RNAs

MicroRNA molecules (miRNAs) are a relatively recent discovery, and have become a burgeoning area of research as they are expected to possess crucial regulatory functions and therefore to underlie many species differences, including those among primates. They are transcribed as precursor molecules (pre-miRNAs) of around 70 nucleotides (nt), which are processed into small (typically 20–24nt) RNA molecules, of which many thousands have been identified in all species to date. They operate in complex regulatory networks, repressing gene expression by binding to UTRs in messenger RNAs (mRNAs) as part of the RNA-induced silencing complex (RISC), to block their transcription and/or induce their degradation, among other functions (105). This process is known as RNA interference. miRNAs have been associated with varied physiological and pathological processes, including developmental patterning, cancer progression and neurological functions (106). One miRNA may be able to target the expression of hundreds of genes.

Significant differences exist in miRNA repertoire and function between species, due to their rapid evolutionary dynamics. In humans and chimpanzees, for instance, many precursor and mature miRNAs differ in sequence, expression and secondary structure, and/or are present in one of those species only (see 6); in humans and orang-utans, just 40% of more than 500 miRNAs show complete identity to their human orthologues; and in humans and rhesus macaques, just 42.5% of

pre-miRNA human homologues have similar structures to their human counterparts (107). The same study also identified 35 human miRNAs that were not present in the miRNA repertoire of at least two species of primate (chimpanzee, orang-utan and rhesus macaque), 12 of which were present only in humans, and noted that, even where miRNAs showed a high degree of sequence and/or structural similarity, significant differences in function could still be present via different expression levels, and target gene specificity as a result of the inherent fast rate of evolution of miRNA binding sites (108). Another study investigated miRNA expression and regulation in the brain, specifically in the prefrontal cortex and the cerebellum of humans, chimpanzees and rhesus macaques. It noted that up to 31% of the 325 miRNAs examined "diverged significantly" between humans and rhesus macaques, and that human-specific miRNAs were associated with neurons and target genes involved in neural functions, supporting the theory that miRNAs have contributed to the evolution of human cognitive functions (106). Of the 413 miRNAs expressed in the human brain, 11% were not detected in rhesus macaque brains, and almost one third (31%) of miRNAs common to the human and rhesus macaque prefrontal cortex were differentially expressed in those two species. Of these differentially-expressed prefrontal cortex genes, 77% were also differentially expressed in the human and rhesus macaque cerebellum. Such is the degree of change of miRNA expression and the repertoire of their target genes across NHP species — developmentally throughout NHP lifespan, and developmentally throughout the lifespan across NHP species — that miRNAs are thought to be the basis and major driving force of the evolution of the human brain (109). This was evidenced by a study of the prefrontal cortex and cerebellar cortex transcriptomes of humans, chimpanzees and rhesus macaques of different ages, which revealed significant variance of these types, in addition to sequence divergence in *cis*-regulatory regions (109). Notably, however, those genes whose expression varied both developmentally and species-dependently, had markedly high densities of predicted miRNA and transcription factor binding sites in their regulatory regions.

An analysis of miRNA expression in human and rhesus macaque embryonic stem cells (ESCs) revealed that, generally, expression was broadly similar — as expected, given their critical developmental roles. Crucially, however, the expression of some clusters of miRNAs differed significantly, including: the primate-specific chromosome 19 miRNA cluster (C19MC), containing more than 30 mature miRNAs, expressed in human ESCs, placenta and fetal brain, but almost absent in rhesus macaque ESCs; the miRNA cluster in the imprinted Dlk1-Dio3 region, enriched in rhesus macaque ESCs, yet rare in human ESCs; and the miR-467 cluster (110). A stringent analysis looked for orthologues of 1,733 annotated human miRNAs in 11 non-human species, to identify human-specific molecules (111). Ten human miRNAs were identified, for which there were no orthologues in any of the 11 non-human species, and a further 12 miRNAs that had human-specific sequence changes in the crucial seed region (the region of the miRNA that recognises and binds with target mRNAs). This study also identified one human-specific miRNA, miR-941, which was highly expressed in the brain and which has been implicated in neurotransmitter signalling via the roles of some of its target genes. Also of note was that the host gene of miR-941 (miR-941 is an intronic miRNA) — *DNAJC5* — encodes cysteine-string protein-α (CSPα), which has been linked to neurodegenerative diseases, including Huntington's disease and Parkinson's disease, and adult neuronal neroid-lipofuscinosis; and that miR-941 may be associated with Hedgehog and insulin signalling pathways, with associated roles in human longevity and some cancers (111).

Another type of RNA interference that modulates gene expression involves very similar, but not identical, types of small RNA molecules, known as small interfering RNAs (siRNAs). These may be generated via the transcription of pseudogenes — genes that are no longer able to produce a functional gene product (for example, due to changes in their coding sequence, such as frame-shifts or premature stop codons), but which are nonetheless still transcribed. These transcripts are then able to modulate the expression of other genes via direct antisense interference, or via subsequent siRNA generation. One investigation identified 1,750 transcribed pseudogenes in the human genome, of which only half were conserved in the rhesus macaque (112).

Ultraconserved elements

One investigation centred on ultraconserved elements (UCEs) — stretches of DNA (greater than 200bp) that have been conserved during evolution and that are perfectly identical between species, even between primates and rodents (113). Due to the degree of their conservation, and the fact that they are often located in the vicinity of genes involved in developmental regulation, they are considered likely to harbour critical biological functions. However, UCEs have been compromised during evolution, and the resulting UCE variance is concentrated in non-coding sequences, functioning as, and/or containing, *cis*-regulatory elements that control gene expression. Therefore, UCEs and variations in them chiefly influence gene regulation. It is therefore significant that UCEs are so

variable in primate species: 695, 653, and 635 UCEs exist in rhesus macaques, chimpanzees, and humans respectively, of which just 459 are shared in all three species (114). Conspicuously, UCEs have disappeared at a greater rate in humans. At least 26% of ancestral UCEs have diverged in hominoids, and a further 17% show comparative sequence changes in humans. Such variation, particularly as UCEs are often associated with transcription factor genes, developmental genes and genes of the CNS, is thought to have impacted spatial and temporal gene expression patterns of key gene regulatory and signalling networks, and therefore to have contributed to species-specific characteristics and variation in primates (114).

RNA editing

An additional factor which affects gene expression, is adenosine-to-inosine RNA editing, in which an enzyme converts adenosine (A) residues to inosines (I) in gene transcripts. The resulting inosines are read by the cell's translational and splicing machinery as guanosine (G) residues. This occurs in many loci in several thousand genes, resulting in altered gene expression via alternative splicing, mRNA stability, nuclear retention, and miRNA biogenesis and targeting, and altered properties and functions of gene products via amino acid sequence changes (115). The A–I editing rate and the resultant changes in gene function and expression are higher in humans than in NHPs (including rhesus macaques), due to primate-specific *Alu* sequences. Furthermore, this appears to particularly affect the human brain, via genes associated with neuronal functions and neurological diseases including bipolar disorder, motor neuron disease, Alzheimer's and Parkinson's diseases, schizophrenia, multiple sclerosis, and amyotrophic lateral sclerosis, as well as genes involved in immune function, inflammation, and cardiovascular diseases (115).

Differences in genetic sequences

Differences in gene coding sequence will, of course, directly impact gene function, and there are various examples of notable differences between humans and the model species used in biomedical research and testing. Loss of gene function — for instance, via mutation and exon deletion — has been linked to important human-specific phenotypes and human evolution (see 42). Similar mutations and deletions have also been linked to species-specific characteristics in the two main types of macaques used in biomedical research, namely the rhesus (*M. mulatta*) and cynomolgus (*M. fascicularis*) macaques. Overall, the general sequence identity between these two species is 99.21%, compared to a similarity between cynomolgus macaques and humans of 92.83% (19), and between rhesus macaques and humans of 93.54% (7). However, this simple sequence comparison is superficial and misleading. Including small genomic insertions and deletions, for instance, human–rhesus macaque identity decreases from around 93% to 90.76% — a figure that would be lower still, if regions that were difficult to align were also included (7).

More in-depth analyses

Ebeling *et al.* (19) went significantly further with their analysis, over and above a simple sequence comparison. In comparing protein-coding transcripts from all three species, they limited their scope to NHP transcripts that were related to human transcripts (i.e. were not NHP-specific). They found that (of around 11,000 mRNAs examined) the identity of transcripts between the two macaque species was high, typically ranging from 99.5–100%. Modal sequence identities for human–rhesus macaque transcripts were 94.6% (5′-UTRs), 97.9% (CDSs) and 93.4% (3′-UTRs); and for human-cynomolgus macaque, transcripts were 94.6% (5′-UTRs), 97.7% (CDSs), and 92.7% (3′-UTRs). Notably, these compared to human–chimpanzee identities of 98.7% (5′-UTRs), 99.3% (CDSs), and 97.9% (3′-UTRs) (116). As expected, the monkey identities are significantly lower, and the consequences of this are discussed later. In the same study, Ebeling *et al.* also investigated genes of pharmacological relevance in the same three species:

a) The solute carrier protein (SLC) gene family is large, and consists of 55 sub-families encoding at least 362 proteins that mediate the transport of organic solutes across cells and organs. Specifically, the sub-family 'solute carriers for organic ions' (SLCO) is pharmacologically relevant, as its products transport molecules to the liver for detoxification by cytochrome P450 and other metabolic enzyme systems. As a result, the human–*M. fascicularis* inter-species amino acid differences found in all SLCO family members, ranging from 0.75% to 9%, are likely to have functional consequences, as are the 1–6% amino acid differences identified in P450 variants in both species of macaque, which will adversely affect the translational value of macaque drug data for humans (19).
b) Inter-species differences were also noted in biomarkers of toxicity-mediated immune activation, which are used in drug safety studies to flag potential problems. Important genes showed amino acid differences of up to 10%; some, such as tumour necrosis factor (TNF) and interleukin-10 (IL-10), failed to cross-react in ELISA tests, despite amino acid identities of

97.3% and 95.9%, respectively. Also of concern was an inter-animal variability in biomarker expression of up to three $\log_2$ units, which led the authors to suggest that all monkeys used pre-clinically should be assessed for their expression of these biomarkers, so that outliers could be eliminated. Of course, this is not done in practice: far from making their use more human-relevant, this suggestion is simply illustrative of the difficulty in extrapolating any data from a variable population of monkeys to a variable population of humans.

Also of pharmacological relevance, inter-species differences in efflux transporters of hepatocytes have also been identified (see 117). These are important, due to the role of biliary excretion in the elimination of xenobiotics and related metabolites, in which differences may result in variations in systemic drug exposure and hepatotoxicity. In spite of knowledge of biliary clearance being central to the prediction of pharmacokinetics and the pharmacological and toxic effects of drugs, confounding species differences have been widely recognised for some time. For example, marked differences have been demonstrated in the activities of hepatocyte multi-drug resistance-associated proteins (MRP) and breast cancer resistance protein (BCRP) across species, including humans and monkeys, which are believed to contribute to species differences in *in vivo* hepatobiliary excretion. These results led to the conclusion that "…interspecies differences in BCRP/Bcrp functions need to be taken into consideration in the allometric prediction of hepatobiliary transport of its substrates", and that primary hepatocytes, either fresh or preserved, are a useful *in vitro* model for the prediction of human biliary transport.

Overall, human–rhesus macaque orthologous genes of 'high confidence' have a 97.5% identity at both nucleotide and amino acid levels (7). The same authors reported that a typical human gene differs from its rhesus macaque orthologue by 12 non-synonymous and 22 synonymous substitutions, compared to fewer than three and five, respectively, for the chimpanzee. In addition, 89% of rhesus macaque and 71% of chimpanzee orthologous proteins differ in amino acid sequence to some degree. The same study also identified 67 genes (many of which were associated with immune functions) as being positively selected across all branches of the phylogeny, specifically: two in humans, 14 in chimpanzees, and 131 in rhesus macaques. Finally, it was noted that several human-specific loci, when mutated, produced "profound clinical phenotypes", including severe mental retardation, and that "…the basic metabolic machinery of the macaque may exhibit functionally important differences with respect to our own" (7).

Differences in immune response

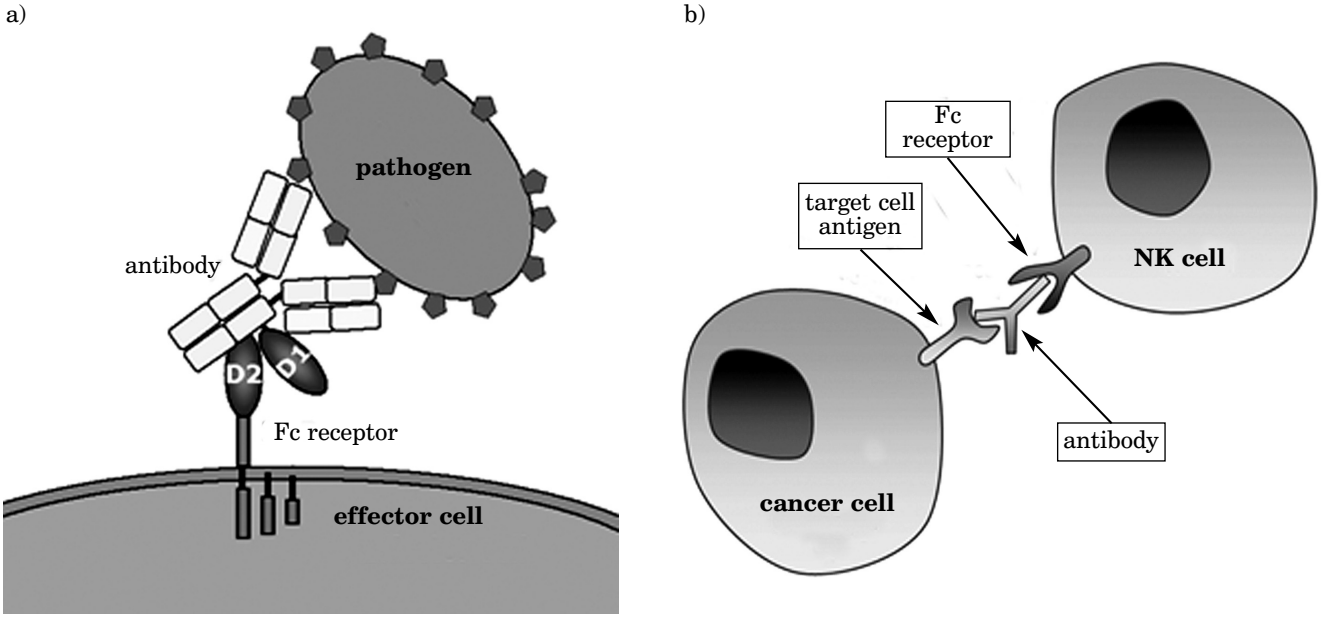
Furthering our understanding of the biomolecular complexities of the immune response, both in its protective role and also as the mediator of a vast range of autoimmune diseases, is confounded by the extrapolation to humans of results obtained in experimental animals. Some examples are outlined here:

— IL-8 has a crucial role in various immune response mechanisms, namely: chemoattraction of neutrophils, T-cells, basophils and NK cells; angiogenesis; and the modulation of expression of adhesion and MHC molecules. A comparative study in humans and four types of NHP identified differences in IL-8 receptors (IL8R). In humans, two genes encode IL8RA and IL8RB. The rhesus macaque and orang-utan IL8RA homologues, however, are pseudogenes, as they contain a 2bp insertion that has created multiple stop codons, while the IL8RB homologue is 3% different to human IL8RB at the amino acid level (118).

— A comparison of the MHC between humans and cynomolgus monkeys showed differences at both nucleotide and protein levels. MHC class I genes showed "weak amino acid similarity (< 90%)" to human sequences, with 109 amino acid substitutions identified, located in various regions of the proteins, including those involved in binding specificity (64). While some variation would be expected in these cases, due to the known intra-species polymorphisms in these molecules, there may still be some inter-species functional significance. Furthermore, while MHC class II genes and gene products showed general similarity to their human homologues, several amino acid differences were recorded, which "may represent basic differences in the immune responses between cynomolgus monkeys and humans" (64).

— Differences between Fc receptors in humans and Southern pig-tailed macaques have been identified, which have implications for the development and testing of new vaccines and therapeutic antibodies, including those for HIV/AIDS and some cancers (119). Fc receptors are cell surface molecules, primarily on effector leukocytes, which bind IgG antibodies to provide adaptive immunity (Figure 1). There are three classes of IgG FcRs in humans (FcγR I–III): the two major genes of the human FcγRII family encode FcγRIIa and FcγRIIb, and their splice variants, which have activating and inhibitory roles respectively in immune responses. Trist *et al.* (119) identified 26 conserved amino acid differences between human and pig-tailed macaque FcγRIIa, polymorphic variation of FcγRIIa between individual

Figure 1: Schematic representations of elements of antibody-mediated immunity



a) Illustrates how Fc receptors on the surface of effector white blood cells confer immunity by binding to antibodies associated with pathogens; b) illustrates how Fc receptors might be involved in cancer immunotherapy, i.e. by linking cancer cells to immune cells that can destroy them. As described in the main text, it is clear how Fc receptor variability, such as species differences, may result in differences in immune responses to pathogens, and in the activity of therapeutic monoclonal antibodies (cartoons obtained from Wikimedia.org).

macaques, and 23 inter-species amino acid differences in FcγRIIb.

These differences and polymorphisms result in a hierarchy of binding of human IgG1 and IgG2 antibodies, with consequences for IgG FcR interactions, not only in pig-tailed macaques, but also in rhesus and cynomolgus macaques, and other NHPs. They also demand caution in interpreting results of antibody (Ab)-based effects, as: "…Ab therapeutics, especially IgG2, may not behave *in M. nemestrina* or other macaque species as they may be expected to in humans"; "…the activities of mAbs designed to alter interactions between human Abs and huFcRs may not be faithfully recapitulated in preclinical studies in non-human primates, or at least in macaques"; "Similar caveats may apply to viral pathogenesis studies in macaques of human infections"; and "…interspecies and polymorphic differences…may translate to alterations of Ab-induced inflammatory outcomes *in vivo* in NHPs that are distinct from those in humans".

— Even within the same species, variability of experimental results is frequently a confounding factor, often due to genetic variability within the population studied. Due to these "highly variable" findings, thought to underlie acknowledged pharmacokinetic, toxicological and biochemical differences between individual monkeys, genetic polymorphisms were investigated in 49 genes of the immune system in cynomolgus macaques — the "standard species used pre-clinically for evaluating efficacy and toxicity of therapeutic drug candidates and vaccines" (120). A total of 580 polymorphisms were identified in these 49 genes: some were predicted to alter transcription factor binding; some to interfere with miRNA target sites; others caused frameshifts or generated premature stop codons. These polymorphisms suggested that the variable immune responses seen in different cynomolgus macaques have a genetic basis, and also may be associated with autoimmunity, metastasis, wound healing, cell adhesion, coagulation, cell differentiation, mycobacterial infections and other infectious diseases (see 120).

— It has been known for some time that polymorphisms in the promoter region of the TNF gene affect the susceptibility of humans to various diseases, including autoimmune disorders, some cancers and malaria (see 121). It is therefore surprising that macaques, which are exten-

sively used in malaria research, had not, until recently, had the promoter region of their TNF genes explored, especially because of the existence of several salient species differences between malaria pathogenesis in humans and macaques. An analysis of 40 rhesus and cynomolgus macaques, from different geographical regions, has shown that their susceptibility to different malaria parasites depends on these factors. For example, cynomolgus macaques from the Philippines develop mild, chronic infections, while infections in those from Mauritius are severe.

The *Plasmodium knowlesi* and *Plasmodium coatneyi* parasites cause acute, often fatal, infections in rhesus macaques, but only mild, chronic infections in cynomolgus macaques. Flynn *et al.* (121) showed that these differences are the result of the highly variable nature of the TNF promoter in both species of macaque, existing as 20 haplotypes defined by 14 SNPs, of which half might be functionally relevant. They also discovered that specific SNPs associated with susceptibility to malaria in humans are not shared with macaques, and noted that species differences in TNF regulation "warrant caution when macaques are employed as models to study disease pathogenesis and susceptibility". Interestingly, and highly pertinent to the use of *Plasmodium*-infected NHP models in research into human malaria, there is also marked genetic variability in the malaria parasites themselves. For example, most human deaths are due to *P. falciparum*, which is well adapted to human hosts. However, it is only "distantly related" to even other human *Plasmodium* species, such as *P. vivax*, and even more so to those better adapted to other apes, such as *P. reichenowi* and *P. gaboni* (122). Crucially, though the parasites share similar genetic backgrounds, there are important differences: hundreds of known *Plasmodium* proteins and putative genes are unique to individual species, and are believed to be responsible for differences in host specificity, pathogenicity, transmissibility and virulence (122, 123).

Neurodegenerative disease susceptibility

Humans and NHPs have been investigated for differences in their *Tau* genes, which are associated with several human neurodegenerative diseases ('tauopathies', such as Alzheimer's disease to which many NHPs are resistant. Given the high degree of similarity of *Tau* genes across primate species, it has been suggested that sequence differences may not be at the root of these phenotypic differences, and instead that nucleotide differences outside the coding sequences, perhaps with regulatory roles, must be the cause (124). Some notable differences have been identified, however, particularly in monkeys: for instance, different primate species carry different numbers of a 59–60bp repeat that exists in intron 9 of human *Tau* (e.g. humans have a tandem repeat, cynomolgus macaques just one), and an intronless gene called *Saitohin* (situated within *Tau* exon 9 in humans) is not present in the cynomolgus macaque (124).

Effects on enzyme activity and protein function

A study of acidic mammalian chitinases (AMCases) in humans and cynomolgus macaques revealed evolutionary and biochemical differences (125). Though the relevance of the differences is not yet known, they should be considered, because AMCases might be associated with asthma pathology, and because they are another example of how subtle changes in genetic sequence may result in significant changes in enzyme activity, with consequences for the evaluation of drugs and the use of monkeys to model human diseases. It was found that human and cynomolgus macaque AMCases had similar expression and pH profiles, but differed in genetic sequence and enzymatic properties, which were 50-times more catalytically efficient in the latter (125).

The study of the *MCPH1* gene — one of at least seven key genes known to be involved in the regulation of brain size during development — illustrates how specific mutations can result in functional changes, leading to altered regulatory effects in downstream genes, and ultimately significant species-specific phenotypes and evolution (126). The regulatory effects of human and rhesus macaque *MCPH1* were different in three out of eight downstream genes tested, and the human-specific mutations altered the regulatory effects on the downstream genes.

HIV/AIDS models

NHP models (usually rhesus and pig-tailed macaques) are used in HIV/AIDS research, and are typically infected with SIV, or an SIV-HIV hybrid known as SHIV. This is because the target cell of HIV ($CD4^+$ T-cell) is poorly receptive to entry by HIV-1 in macaques, with the exception of a few strains of the virus that have been adapted in the laboratory (see 127). A study of the CD4 receptor in both rhesus and pig-tailed macaques and in humans has shown that this difference in activity and specificity is due to a single amino acid residue at position 39, illustrating how such small differences can exert such significant effects. Moreover, differences between human and simian immunodeficiency viruses (HIV and SIV, respectively) are known to

be relevant to the interpretation, and confounding nature, of the SIV-infected macaque model of human HIV/AIDS. These significant differences, it is argued in some circles, seriously question the applicability of SIV/macaque models to human vaccine development, and are because HIV-1 evolved over a century in humans, while SIVs used in macaque research evolved in sooty mangabeys (SIVsmm) over millennia (see 128).

A study of 20 independent isolates of SIVsmm showed much lower diversity than HIV-1, probably due to mutations that have accumulated over time, as well as less selective pressure from immune responses that differ in terms of anti-SIV antibody titres, cytotoxic T-lymphocyte responses and immune activation (128, 129). In addition, inter-species genetic differences have been identified in host factors that restrict retroviral infection. The 'tripartite motif 5' (*TRIM5*) gene, for example, belongs to an approximately 70-strong gene family that encodes many factors with antiviral activity (see 130), which effectively restricts the host range of HIV-1: rhesus and cynomolgus macaque TRIM5 restrict HIV-1 infection, but not SIVmac infection, whereas human TRIM5 activity against these viruses is very weak. Such species differences are due to differences in amino acid sequences of a specific domain of the TRIM5 protein, which is illustrated by the finding that a single amino acid change at position 332 of human TRIM5 (from arginine to proline) confers potent restriction ability against HIV-1 and SIVmac viruses. In spite of these genetic differences, which lead to "differences in immunology, pathogenesis, and diversity" between SIV and HIV-1 (128), an acknowledgement that "…current SHIV models do not reflect HIV-1 variants circulating globally and thus do not fully recapitulate the viral factors that contribute to the infection dynamics being studied" (127), and an advisory caution that they "…should be considered when extrapolating from SIV/macaque experimental results to HIV/human vaccine applications" (127), some researchers maintain that NHPs remain an "invaluable tool" (128). With the recent passing of the HIV-infected chimpanzee as a usable model, due to its comprehensive failure and poor human relevance (1, 6, 131, 132), this is arguably a claim with little or no substance.

Differences in RNA splicing

Splicing factors (complexes of RNA and proteins) bind to specific sequences in pre-mRNAs during transcription to remove introns, resulting in mRNAs comprising exon sequences that are spliced together, which in turn serve as templates for translation. Alternative splicing also takes place in most genes, in which one or more exons may be removed from pre-mRNA molecules, in addition to introns. Such alternative exon usage enables multiple proteins, often with different functions, to be generated from the same gene, greatly increasing the scope of the genome and proteome with major consequences for the organism. Splicing differences result in variations in the stability, localisation and translation of the mRNA molecules, and in the specificity, efficiency, localisation and life cycle of the encoded proteins (see 133).

Although this may be considered a relatively poorly studied field, there is some evidence of species differences that affect splicing. For example, splicing factors are differentially expressed in humans and chimpanzees, including 43 in the testes and 20 in the brain (134). A study by Reyes *et al.* (133) examined the transcriptomes of five tissues (heart, liver, kidney, brain minus cerebellum and cerebellum) in six species (human, chimpanzee, bonobo, gorilla, orang-utan and rhesus macaque). They concluded that inter-species variability with regard to exon usage was dominant, and much more variable than inter-species gene expression, in agreement with prior findings showing that "splicing variation between species, even between equivalent tissues, exceeds the within-species variation across tissues" (135, 136). Of almost 120,000 exons studied in over 10,000 genes, just 3% of the exons in 16% of those genes showed conserved tissue-dependent usage between humans and rhesus macaques. In addition, splicing is affected via the action of mobile elements (that disrupt or create splice sites) and A–I editing, among others, as discussed earlier, and by crosstalk between DNA methylation and histone modifications (see the section on *Epigenetic factors* above, and, for example, 82).

The consequences of genetic differences: Translation of data from NHP research to humans

Given the extent of NHP use in research and testing, as well as its ethical and financial costs, surprisingly little critical analysis of its worth has been conducted to date. It must be concluded that the use of NHPs rests on an assumption, perhaps based on anecdotal evidence, that it is predictive of human biology and translates well. However, this can only be assessed, and established, by comprehensive and critical scientific inquiry. What investigation has been done, however, is far from supportive of the value of NHP use, or of its necessity in the future. Much of the following evidence is monkey specific, but more has been collated against the use of apes, primarily chimpanzees (131, 132, 137–139), and notably also considers the genetic basis of the failures of chimpanzee research in the same manner as this paper does for monkeys (6).

Toxicology and drug testing

With regard to NHP use in toxicology and drug testing, no data categorically demonstrate the predictive nature of NHP tests for human toxicity. The UK's National Centre for the 3Rs (NC3Rs) stated that there has "yet to be a comprehensive scientific review of the rationale for [primate toxicity testing]" (140), and the Toxicology Working Group of the UK Parliament's House of Lords Select Committee on Animals in Scientific Procedures opined that "the formulaic use of two species in safety testing is not a scientifically justifiable practice, but rather an acknowledgement of the problem of species differences in extrapolating the results of animal tests to predict effects in humans," and, "the reliability and relevance of all existing animal tests should be reviewed as a matter of urgency" (141). This means that NHPs are currently being used in toxicological testing with no scientific justification. Furthermore, single-dose toxicity tests, to which many non-human primates in regulatory safety tests are subjected, have been scientifically discredited (142): the cynomolgus macaque showed the drug TGN1412 (which almost killed six healthy clinical trial participants in 2006) to be safe, even at a dose 500 times higher than the human dose (143); the International Conference on Harmonisation (ICH), which harmonises medical testing requirements, notes that in regard to drug testing, primates "can differ from humans as much as other species" (144); in discussing whether primates or dogs were the more predictive for liver toxicity in humans, a researcher at AstraZeneca described "an inbuilt prejudice", and though there is an assumption that primates would "more closely mimic subsequent effects that might occur in man... there is little evidence to show that" (145); in a review of 25 cytotoxic cancer drugs, toxicity data from primate (and dog) studies "grossly overpredict[ed] hepatic and renal toxicity" in patients (146). In fact, evidence has shown that:

a) the primate tests for hepatic, renal and respiratory toxicities yielded high rates of false positive results when compared with subsequent human data (147);

b) results from NHPs in developmental toxicity testing correlate with known human teratogens only 50% of the time, less even than results from more evolutionarily distant species such as rats, hamsters and ferrets (148–150);

c) a recent statistical study of papers assessing the predictive nature of NHPs (among other species) in toxicology, noted that incorrect statistical definitions were often used that skewed the data in favour of the animal models. When the correct statistical definitions were used, it was discovered that there was no statistically credible evidence that NHP toxicology data "contributed any predictive value, either separately or in combination [with e.g. dog data]" (151);

d) an analysis of the prediction of drug-induced liver injury (DILI) by animal models revealed that NHPs were less predictive than rodents — which themselves failed to predict up to 51% of effects in humans (152); and

e) since their commercial introduction in the early 1980s, many non-steroidal anti-inflammatory drugs (NSAIDs) have been clinical failures. For example, having been found safe in year-long studies in rhesus monkeys, benoxaprofen caused thousands of serious adverse events and dozens of deaths within three months of its approval and marketing (153).

The lack of justification is not surprising, in view of the fact that the failure rate of new drugs in human trials is at record levels: 95% of drugs that enter clinical trials, having obtained Investigative New Drug approval based largely on animal test data, do not go on to obtain FDA New Drug Application approval for marketing (154). Furthermore, of the small percentage of drugs entering human clinical trials that do gain FDA approval, half may go on to be withdrawn or re-labelled post-marketing, due to severe or lethal adverse effects not detected in the animal tests. These approved drugs are directly responsible for a level of adverse drug reactions that constitutes one of the leading causes of death in the USA (155) and the UK (156).

Biomedical research

The evidence against NHP research is not confined to drug development and testing. In biomedical research, a wide range of evidence specific to monkey use has been published:

— In HIV/AIDS research, the use of macaques is widely considered to lead to failure and to be of questionable human relevance (157–164). Many, if not all, of some 100 different types of HIV vaccines were tested in monkeys with positive results, yet none provided protection or therapeutic benefit in humans, due to major differences in SIV-infected macaques compared to HIV-infected humans (157, 158, 162, 165–167).

— With regard to Alzheimer's disease (AD), many scientists have spent years trying to create an AD animal model with significant human relevance, but have failed (168–171), and have made very little progress in understanding its various pathologies. For example, plaques and tangles in the brain are the hallmark of AD in

humans, but not in NHPs (172). Humans and great apes possess a specific type of projection neuron in the anterior cingulate cortex, which is severely affected in the degenerative process of AD (173). Also, the neuropeptide galanin, that regulates cholinergic basal forebrain (CBF) function, differs in its chemoanatomic organisation across species: in monkeys, all CBF neurons co-express galanin, whereas in apes and humans, galanin is found within a separate population of interneurons that are in close apposition to the CBF perikarya (174). Because galaninergic fibres hyperinnervate CBF neurons in AD, inhibiting acetylcholine release in the hippocampus, this may exacerbate cholinergic cellular dysfunction in AD. This difference could be critical when attempting to create an NHP AD model. NHP use can also cause direct human harm, in addition to delaying progress and diverting research funds from more-relevant methods. The once much-vaunted AD 'vaccine', AN-1792, dramatically slowed brain damage in an AD mouse model, and "was well tolerated when tested in several animal species, including monkeys" in experiments prior to clinical trials (175, 176). Despite the encouraging NHP data, clinical trials were suspended following CNS-inflammation and ischaemic strokes in 15 participants (177).

— In stroke research, significant species-specific and even strain-specific differences in response to ischaemic injury exist (178). Decades of research have resulted in thousands of publications reporting more than 1,300 successful stroke interventions in animals (including NHPs), including more than 700 for acute ischaemic stroke, none of which has led to human benefit (179, 180). Some experts have labelled stroke animal models a failed paradigm: they have argued convincingly for human-based research (181, 182); lamented that animal models of stroke could not be translated to humans (183); and stated that "The repeated failures of laboratory-proven stroke therapies in humans can be due only to the inapplicability of animal models to human cerebral vascular disease" (184), "Ischaemic stroke is a case study in failed translation" (185), "After so many years of failure, it is appropriate to ask whether researchers should continue to pursue neuroprotective strategies for stroke", and "The stroke community needs to think long and hard about whether these animal models are financially and ethically viable" (186).

— Parkinson's disease (PD) has been studied using neurotoxic chemicals to induce superficial PD-like symptoms, predominantly in marmosets and macaques. Fundamental differences in the onset, type and persistence of symptoms exist in all the models, in addition to physiological differences such as the absence of Lewy bodies in NHPs. Species differences are known to play a role in the clinical expression, as well as in the cellular specificity of the lesions. For example, striatal degeneration in humans is frequently associated with dyskinesia, whereas striatal excitotoxic lesions alone are not sufficient to induce dyskinesia or chorea in NHPs. Also, the time-course of nerve cell degeneration, which normally evolves over several years in neurodegenerative diseases in humans, is for practical reasons replaced by a much shorter period of time in NHP models (187). Deep-brain stimulation of PD patients, often claimed to have been developed through NHP experiments, was actually discovered serendipitously in a human patient and arguably owes nothing to NHPs for its advancement (188, 189).

Other notable species differences

In addition to studying the genetic differences between humans and NHP model species, many of which are summarised in this review and which underlie the above-described empirical species differences, there are other, non-genetic studies of species differences — including differences between types of monkeys, as well as between monkeys and humans — worthy of note in any critique of using monkeys as models for humans. The outcomes of several of these studies are noted here, since, though they were not expressly genetic studies, the differences identified have a genetic basis.

Firstly, a review in 2003 outlined an important consideration that has since been underlined by genetic studies: "There is no such thing as a generic monkey" (190). Many species are used in research in many scientific areas, namely prosimians (e.g. galagos and lemurs), NWMs (e.g. marmosets, tamarins and squirrel monkeys), OWMs (e.g. macaques, baboons and African green monkeys), and, at least until recently, chimpanzees. Notable biological differences among species, even among closely related species such as rhesus and cynomolgus macaques, include: glucocorticoid resistance/circulating steroid levels, ovarian histology and steroid receptor biology, and disease susceptibilities and manifestations. Even considering that perhaps 4 or 5 species are commonly used in toxicology, and less than 20 genera in laboratories generally, this is scope for much confounding data. For instance, many monkey-specific diseases (including various viruses, parasites and cancers) are recorded as having confounded research studies (190). This review also notes caution over interspecies and geographical intra-species differences among macaques. Such caution is evident elsewhere in the literature. For example, it is noted

that rhesus macaques of Indian origin were used most often in HIV/AIDS research, simply because this was the species supplied to laboratories by breeding facilities in the USA (191). Then, a shortage of these animals prompted a search for an alternative, and attention was switched to the Chinese rhesus macaque, as it was "more readily obtainable". However, differences in susceptibility to SIV infection and disease were noted between the two, mirroring differences also seen among species of macaque (rhesus, cynomolgus and pigtailed). It was concluded that "…even subtle genetic differences between two subspecies (races) of primate may promote significant differences in the pathogenicity of the same virus" — a phenomenon that had also been seen in cynomolgus macaques infected with poliovirus, for example, where susceptibility could vary up to 10,000-fold depending on geographic origin; and in the course of disease following the infection of macaques with various malaria parasites (see 191). Other noteworthy differences have been found in the following areas of biomedical research:

a) In neuroscience, the mapping of anatomical brain networks and characterising neural connections (known as the 'connectome') via diffusion tractography helps the understanding of brain function, and as such it has been a focus of research in humans, though not to nearly the same degree in NHPs. One recent study, however, analysed, in parallel, the human, rhesus macaque and chimpanzee connectomes, and reported major species differences in the architecture of the inferior parietal cortex, polar and medial prefrontal cortices (192). These findings augmented previous studies demonstrating a greatly expanded, lightly myelinated region of prefrontal cortex in humans when compared with that in rhesus macaques and chimpanzees (193), and a more gyrified prefrontal cortex in humans compared to other primates, even controlling for differences in brain size (194). Functional consequences of these differences may involve sensory perception, visceral functions, higher order cognitive functions, and emotional and reward-related behaviours (see 192).

b) Several studies have noted a considerable number of genes associated with cancer, in terms of human–macaque inter-species differences in the occurrence and biology of tumours in these species. For instance, while colon neoplasia is common to both humans and rhesus macaques, the development of benign and precancerous polyps is not seen in the latter; and, unlike in humans, prostate and lung cancers are rare in rhesus macaques (195).

c) The reporting of gene differences involved in drug transport and metabolism is also prevalent, and again, these differences manifested in phenotypic differences. For example: some functional differences in CYP enzymes between species have been established, in spite of high sequence identities, such as marmoset and Japanese macaque CYP1A2; *CYP2C76* is present in cynomolgus and rhesus monkeys, but not in humans, and is known to be partly responsible for differences in drug metabolism in monkeys and humans; expression differences (and possibly transactivation differences) exist in *CYP1A1* and *CYP1A2*; substrate specificities of CYP2Cs may differ; quantities and metabolic activities of CYP2D and CYP3A4 differ; several CYPs are affected by CNVs; and null alleles of various CYPs exist in different species and within species arising from different geographical locations and even from different colonies, meaning "…the origin of the animals could be an important factor for successful drug metabolism studies using macaques" (196).

Discussion and Conclusions

Public opposition to animal experiments — particularly those involving NHPs — has been increasing for some time and is at a record level. A Europe-wide independent poll, conducted by YouGov in 2009, showed that 79% of respondents were opposed to experiments on animals not related to serious or life-threatening human conditions, and 81% believed new EU legislation should prohibit any experiments that would cause pain or suffering to primates (197). In spite of this level of opposition, experiments involving NHPs, and the number of NHPs used in them, continue to increase in parallel with it (see *Introduction*), with approximately 80,000 used in the last fiscal year for which figures are available in the USA and in the EU; in the UK, figures for 2013 (3) reveal a rise in the number of animals used to 2,202 (16 more), and in procedures to 3,236 (216 more). There has been a "steep increase" in the number of cynomolgus macaques being imported into the USA in recent years: 126,000 individuals were imported in 2000–2005, for example, from breeding farms in Indonesia, the Philippines, Mauritius and Indochina (198, 199), which breed them from wild-caught monkeys.

Two opposing viewpoints

While opposition to NHP experimentation may have a largely ethical and welfare-related basis, defence of the rise in NHP use rests on scientific necessity: i.e. that, because of the high level of genetic similarity of monkeys to humans (around

90%), they *must* be good models, without which it would be difficult — if not impossible — to develop safe and effective new drugs for humans, and to gain a deeper understanding of human diseases, in order to facilitate the design of treatments and cures.

Both sides of the argument have often essentially lacked a scientific perspective, as, based on the widespread assumption of human relevance of NHP use by those who commission, fund and conduct it, little critical analysis of its scientific worth and significance for human medicine had been attempted. Many of the critical studies that do exist have been included here, and in other reviews and reports published over the past decade (e.g. 161, 200–207). Notably, advocacy of NHP experiments based on their mere involvement in research, or on 'promising' results obtained in monkeys, is not sufficient. For them to be scientifically justified, they must be shown to be predictive of human biology, crucial to any biomedical breakthrough, and no alternative to them must exist.

The scientific argument against their use has intensified in recent years. Perhaps understandably, much of the criticism has come from scientists affiliated to animal welfare groups, as scientists who practise NHP research are reluctant to criticise it, even though the scientific method demands that they do so. Hypotheses must be tested; do claims that NHP experiments are predictive of human biology stand up to detailed scrutiny? Examples of their poorly predictive and confounding nature abound, which complement and augment the burgeoning ethical/welfare case against their use in research. Yet, superficial 'genetic similarity' arguments continue to be proffered in defence of their use.

The genetic argument

As long ago as 1975, it was first suggested — partly based on observations that, superficially, coding sequences of humans and chimpanzees were highly similar — that species differences could not be sufficiently explained by genomic sequence differences, but must be mostly due to differences in how genes were regulated, i.e. that regulatory elements were responsible for qualitative and quantitative gene expression differences, resulting in major phenotypic and physiological differences between primate lineages (208, 209). Gene expression alone, or at least to a large extent, is thought to be at the root of inter-species phenotypic differences, including, for instance, critical differences in liver function between NHP species, due to changes in transcription factor expression (78), and also human-specific features, such as highly developed language and complex tool-making (210).

The ethical argument

Though not the focus of this review, it would be remiss not to include some salient considerations of suffering, given that the scientific worth of NHP use in science cannot be used in isolation as an argument to defend it, and because the inherent stress and distress directly affect experimental results via modulation of gene expression. While it is obvious that NHPs are able to suffer, considerable empirical evidence of sentience and capacity to suffer supports this common-sense view. Rhesus macaques, for instance, can perform rudimentary arithmetic, think using symbols (211), possess an essential component of 'theory of mind' (the ability to deduce what others perceive on the basis of where they are looking; 212), refuse to take food when this means other individuals would receive electric shocks (213), have a social system with rules for specific relationships and social behaviour, often developing lifetime social bonds (214), and show highly innovative behaviour, which is only surpassed by *Pan*, *Pongo* and *Cebus* (215).

Experimentation itself causes harm. The Organisation for Economic Co-operation and Development (OECD; 216) and the Nuffield Council on Bioethics (217) list many conditions and clinical signs that may occur during chronic toxicity and carcinogenicity tests, which indicate an animal is experiencing pain and/or distress. Drugs to relieve pain and distress may be withheld, over concerns that they might alter the toxicity profile of the chemical being tested (218). Neurological and vision experiments often cause significant suffering, as they involve craniotomies, head stereotaxy via bars implanted into the skull and/or ears, coils implanted into the eyes to monitor eye movements, and often deprivation of food or water for many hours prior to the experiments, to motivate the animals to perform visual tasks. NHPs are known to have been kept, instrumented, in single caging for two years, while being used and re-used in vision research (219). In Parkinson's disease research, neurotoxins such as MPTP might be injected directly into monkeys' brains to damage them, causing them to experience severely restricted mobility, including an inability to feed or groom themselves (220, 221). In stroke research, head and neck arteries are blocked, which involves craniotomy, and sometimes removing an eye and cutting the optic nerve to access the brain via drilling through the eye socket (222, 223). Sometimes related procedures are carried out whilst the monkeys are awake and restrained in primate chairs to avoid the effects of anaesthesia (224).

Suffering may be psychological, as well as physical, evidenced by many macaques kept in standard laboratory cages exhibiting stereotypical behaviour (225). One study noted that 89% of

singly-housed rhesus macaques exhibited at least one abnormal behaviour (226), while another showed that self-injurious behaviour occurred in 10% of NHPs (227). The use of primate restraint chairs causes immense psychological stress and extreme distress, associated with physical problems such as inguinal hernia (a protrusion of abdominal-cavity contents through the inguinal canal), and rectal prolapse (228). NHPs also suffer due to their anticipation of painful procedures based on past experiences (229, 230), as well as due to laboratory confinement (231) and the resulting lack of agency and social interactions (232, 233). Furthermore, it is now well-known that even routine procedures that monkeys and other animals undergo in laboratories, such as simple handling, blood collection and drug administration, cause significant physiological stress (234). Transportation, change of environment and exposure to new laboratory staff and procedures induce changes in body weight, hormone levels, heart rate and blood pressure, all of which are indicators of stress (235, 236). Marmosets are well-known for avoidance behaviour during attempts at capture, and often become stressed and violent, even toward themselves; indeed, cage-capture of various NHP species is associated with signs of stress and distress (237).

These ethical considerations are directly linked to scientific concerns. Stress-related elevations of heart rate, blood pressure and a variety of hormone levels (including cortisol) influence the nervous and immune systems, and affect scientific data obtained from animals in laboratories (238–241) — far from helpful when researching new drugs and infectious agents (234). Handling has been shown to interfere with immunity (and therefore, for example, tumour growth and susceptibility to infectious disease; 242, 243), and this effect varies with regard to species, strain, age and sex. This all has important methodological implications (243). Indeed, warnings have been issued about the consequences of disregarding the effects of stress due to laboratory routines (239–241), yet this remains under-reported, or not reported at all, in scientific studies (244).

The effects of harm on gene expression

All the above attributes illustrate how monkeys can be harmed through psychological and emotional stress and distress, such as that caused by capture, confinement, transport, poor environments, and experimentation: harm that has been acknowledged in detail by the European Commission's own analysis (245). This harm is an important factor for the cost–benefit analysis of animal experimentation, which must swing the balance appreciably against the use of NHPs in research. However, this harm also affects experimental results via stress-induced changes in gene expression; changes that exacerbate inherent inter-species and intra-species differences in gene expression due to evolution, speciation and adaptation. These differences affect all levels of gene expression and protein function, as detailed earlier: genomic rearrangements, mobile DNA elements (e.g. LINEs and SINEs), gene duplications and deletions, copy number variation, differences in transcription factors and binding sites, DNA methylation, miRNAs and binding sites, gene editing and splicing, all contribute to human–monkey biological differences and have serious ramifications for the extrapolation of monkey experimental data to human medicine.

Empirically, we now appreciate more than ever the degree to which many diseases and disorders affect humans and non-humans differently (see above, and e.g. 73), and how difficult and unreliable is the extrapolation of drug toxicology and safety data from animals to humans (e.g. 246, 247). Not only is this phenotypic evidence mounting, but also the genetic underpinnings of it are becoming increasingly well-understood. The small degree of genetic difference suggested by crude and superficial alignments of shared sequences, especially between primates, are of little relevance to the central issue, which is the human relevance of NHP experimental data. As first suggested by King and Wilson in 1975 (208), evidence has now shown that differences in gene regulation, which result in widespread phenotypic species differences, are at the root of the lack of human relevance of animal models. Notably, the relatively few changes in genetic sequence appear to preferentially affect genes and genetic control elements with the power to modulate the expression, stability, and repertoire of products of hundreds, if not thousands, of other genes: transcription factors and their binding sites, splicing factors and their binding sites, miRNAs and siRNAs and their binding sites, and so on. Furthermore, it seems that the expression of genes principally involved in the function of the immune system is predominantly affected. This has clear consequences for the use of monkeys in scientific research, which is dominated by the study of diseases involving, at some stage, the immune system. Notable differences in gene expression in the liver — the major metaboliser of drugs — are also of serious concern in the use of monkeys in drug testing.

Lessons to be learned

The use of monkeys in science is increasing, in spite of the multi-faceted evidence against its efficacy: from 2000–2005, PubMed listed 3,713 papers involving rhesus macaques (7); a search for rhesus

macaque papers published in the five years up to July 2014 identified 5,367 publications, with a further 2,227 involving cynomolgus macaques (20). Evidence shows not only that monkeys are a poor model for human biomedical research, but also that they are so poor that monkey data rarely, if ever, directly translate to human benefit and that they can even impede medical progress. Moreover, there is now evidence that shows why they are such poor models: they are so fundamentally genetically and biochemically different, in so many crucial ways, that they can never constitute a good model for humans. They can never be 'human enough'. The argument that monkeys must constitute a good model for research on human diseases, based on their ostensible genetic similarity to humans, is unsound and should be dismissed. Arguments that the greater understanding of inter-species differences may improve the human translation of animal models (e.g. 19) is unconvincing and, ultimately, unhelpful. Only by moving away from monkey research, and by fully embracing and adopting superior, more-humane and human-specific alternative forms of scientific inquiry, can treatments or cures for the many diseases that blight the lives of hundreds of millions of people be realised as quickly and as safely as is possible.

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