

2015 William Allan Award<sup>1</sup>Kay E. Davies<sup>2,\*</sup>

I would like to begin by thanking Gene Fisch for his very generous introduction. This award is a great honor and is testament to the many talented students and research fellows who have worked with me over the years. I would also like to acknowledge my mentors, who introduced me to genetics for the first time 35 years ago. I am very grateful to the many friends and collaborators who have debated with me long into the night on the best ways forward at many genetics meetings. The ASHG meetings have always been special to me as I watched the society keep pace with the ever-changing field of genetics both in research and in delivery to the patient. Finally, I would like to thank my family, who have supported me at all points in my career, even when they were not sure where the next steps were taking me!

Over the last 30 years, I have participated in an extraordinary journey from the isolation of fragments of the X chromosome for the diagnosis and carrier detection of X-linked disorders to the development of therapies for Duchenne muscular dystrophy (DMD), but first let me give a bit of personal history.

I never thought for one moment that I would have the opportunities I have had in genetics. I studied chemistry at Oxford and had never studied biology at school because the timetable did not allow it. I needed Latin to get into Oxford, and therefore that took precedent. At Oxford I was very much inspired by Dorothy Hodgkin, who was a fellow at my college, Somerville. She was still active in the 1970s and attended one of my early lectures. Somerville has many famous alumni: Margaret Thatcher also studied chemistry there, which shows that such a background is a good training for anything! (Incidentally,

both the German leader Angela Merkel and Pope Francis studied chemistry, so maybe this is good training for leadership.)

My studies for my Ph.D. focused on chromatin structure in the era before nucleosomes had been described, and this gave me a good grounding in protein biochemistry. This led to the French laboratory of André Sentenac, who was cloning RNA polymerase genes from yeast. This was my first encounter with genetics, and I was immediately hooked. Once the paper from Y.W. Kan's laboratory on the diagnosis of sickle cell anemia came out,<sup>1</sup> the application of restriction-fragment-length polymorphisms (RFLPs) to the localization of disease genes was proposed by Botstein and colleagues.<sup>2</sup> The potential of this new genetics for the diagnosis of disease as well as the identification of disease genes was obvious. I was invited to join the laboratory of Bob Williamson in London; he was already ahead in his thinking on the application of this new genetics to human disease because of his interest in cystic fibrosis. However, he recognized that it was better to focus on a disease where the chromosomal localization of the gene was known, such as X-linked DMD, because we were not sure that there would be sufficient variation in the genome for mapping all diseases. DMD is a devastating X-linked recessive disorder characterized by progressive muscle wasting and weakness. Patients are typically wheelchair bound by age 12 and die from respiratory failure or cardiomyopathy in their 20s.<sup>3,4</sup> When we began this work, no prenatal diagnosis was available, the carrier test was unreliable, and there was no effective treatment.<sup>5</sup> The only clue to the localization of the gene was the existence of females with balanced X-autosome translocations with breakpoints in Xp21, and there was a need for X-linked markers for mapping X-linked diseases. Importantly, the Cystic Fibrosis Trust, which had funded the group to collect blood samples from all cystic fibrosis (CF) patients in the UK and funded my fellowship, also showed great foresight and backed this scientific rationale.

Our first objective was to make a library of highly enriched sequences for the X chromosome as a potential source of DNA probes that we could develop as RFLP probes. This required the sorting of chromosomes from a 48XXXX cell line in Glasgow with Bryan Young, who was setting up this technique for chromosome 22.<sup>6</sup>

<sup>1</sup>This article is based on the address given by the author at the meeting of The American Society of Human Genetics (ASHG) on October 9, 2015, in Baltimore, MD, USA. The audio of the original address can be found at the ASHG website.

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It was an arduous task in those days to focus fluorescence-activated cell sorting (FACS) to deflect the chromosomes that we wanted, and very often this required adjusting the cathode oscilloscope by hand and working into the early hours. The small amounts of DNA were extracted from the chromosomes and then cloned into phage libraries for further study. The localization of the sequences was confirmed with somatic cell hybrid cell lines that contained different fragments of the X chromosome. Such lines were derived from work in Hilger Roper's laboratory, which supplied the DNA. We eventually managed to identify enough sequences distributed randomly along the chromosome to generate the first genetic map of the human chromosome at 10 cM intervals with Ray White and Dennis Drayna and colleagues.<sup>7</sup> We were not alone in this work: Louis Kunkel in Sam Latt's laboratory had also cloned an X-chromosome-enriched library, and Peter Pearson together with Gert van Ommen had produced other key markers for the localization of the DMD gene (see Guiraud et al.<sup>5</sup> for a review).

It was at this point that I met DMD patients and their families for the first time as we began to work closely with them. I was inspired by their courage and confidence in us, and we were persuaded by the muscular dystrophy charities in the UK and US (the Muscular Dystrophy Campaign and Muscular Dystrophy Association, respectively) to take on the challenge of finding the disease gene. I realized the enormous unmet clinical need for an accurate test and an effective treatment. I was to dedicate my academic career to meet this need, and we are almost there.

For the mapping of the DMD locus, we focused on markers around Xp21 because of the female translocations suggesting that the gene might lie in this region. We were very fortunate to collaborate with Peter Harper's group because they had collected clinically well-characterized DMD-affected families. In 1981, we produced the first markers that localized DMD to Xp21 and used flanking markers for prenatal diagnosis of the disease.<sup>8</sup> Peter Harper and colleagues used these markers to show that Becker muscular dystrophy (BMD), which has a much milder clinical course, was likely to be allelic.<sup>9</sup> It is interesting to note that it could take up to 3 weeks to complete these diagnostic tests by Southern blots, whereas now the same tests can be completed within hours via PCR. It was also the time when chorion villus sampling was being developed for prenatal diagnosis. Bob arranged collaboration with Charles Roedeck, who pioneered this technique, and one of our first diagnoses was of a twin pregnancy. Fortunately, this turned out to be a boy and a girl, so we could easily confirm the diagnosis that this apparently normal pregnancy was not the result of sampling the same twin twice!

At this time, Bob Williamson returned to the identification of the CF gene, and I pursued the DMD gene at the Institute of Molecular Medicine at Oxford, where I was fortunate to be mentored by David Weatherall, a leader in the study of thalassaemia. It was very inspiring to

work alongside such an eminent academic clinician who attracted so many other young scientists such as myself at the interface of basic science and medicine.

The DMD gene was eventually identified by two independent strategies. Louis Kunkel identified the gene by taking advantage of a deletion seen in a patient suffering from DMD,<sup>10</sup> chronic granulomatous disease, and retinitis pigmentosa in Xp21, first reported by Uta Franke.<sup>11</sup> Ron Worton's group identified the gene through the cloning of the breakpoint in a female suffering from DMD with a breakpoint in Xp21 and the rRNA genes on chromosome 21.<sup>12</sup> This was an exciting time for diagnosis, and these groups made probes to facilitate prenatal diagnosis and carrier testing as soon as they became available. Using these markers, my group showed that although the DMD gene showed a high rate of deletion, there were two hotspots for deletion.<sup>13</sup> These hotspots are now well characterized and have enabled many mutations in the gene to be diagnosed with a relatively small number of probes.<sup>14</sup> Monaco et al.<sup>15</sup> also demonstrated that BMD patients are mildly affected because the deletions do not destroy the reading frame of the mRNA, and therefore a truncated, partially functional protein is produced.

The DMD gene turned out to be remarkable: not only is it encoded by a large 14 kb mRNA, but the coding region is also spread over more than 2 Mb of DNA. The large extent of the gene was established by my group<sup>16</sup> and by workers in Holland and Germany<sup>17,18</sup> with the use of pulsed-field electrophoresis, which showed that very large deletions (1–2 Mb) could be seen in patients who suffered from only DMD. Further refined mapping of the gene showed that it consists of 79 exons spread over more than 2 Mb of DNA and that some introns cover more than 200 kb. Thus, the high mutation rate for DMD can be explained by its large size. However, as of yet there is no explanation for why there is such a high rate of deletion or why these occur predominantly in two regions within the gene.

Elegant work from the Kunkel laboratory showed that the gene encodes a 427 kD structural protein (named dystrophin because its loss results in muscular dystrophy) localized at the sarcolemma.<sup>19,20</sup> The groups of Campbell and Ozawa showed that dystrophin is bound to a complex of proteins that link the extracellular matrix via laminin to the internal cytoskeleton via actin.<sup>21–23</sup> In the absence of dystrophin, the sarcolemma becomes more susceptible to contraction-induced injury, resulting in continual muscle regeneration and necrosis, leading to myofiber loss.

Through the characterization of deletions in patients, we demonstrated that a mild phenotype is compatible with large deletions of the central rod domain of the DMD protein, dystrophin.<sup>24</sup> The late Sarah Bunday sent us a sample from one of her patients who presented with a particularly mild form of BMD. He had served in the army and not shown any symptoms until his 40s. Characterization of his deletion showed that, surprisingly, 46% of the region coding for the central rod domain was deleted. Our hypothesis was that the mild presentation could be the result

of some compensatory gene in his genome or that this minigene was partially functional. One of the patient's relatives had the same deletion and was also mildly affected, arguing for the latter scenario. We tested this hypothesis by reconstructing his gene as a transgene under the control of the skeletal actin promoter and expressing it in the *mdx* mouse model of the disease.<sup>25</sup> The minigene was able to prevent the pathology in this mouse model. Thus, in spite of some initial skepticism, it was shown that minigenes, modeled on these large deletions, function as a replacement for the missing gene.<sup>25</sup> This dystrophin minigene has been distributed worldwide and is used as the basis for numerous gene-therapy protocols, and further development of microdystrophin genes is being introduced into current gene-therapy paradigms.<sup>26,27</sup>

As part of our work on the identification of gene deletions, we screened our fetal-muscle cDNA library with fragments of the gene to isolate further coding fragments. One of these cDNA fragments gave a strong signal in the screen, but it did not map to any of the X chromosome fragments in our somatic cell hybrid lines. Here, we were fortunate to have Diane Hill, who ran the sequencing facility in Dunedin, New Zealand, visit the laboratory. We were very excited when we obtained the sequence: the cDNA was indeed different from dystrophin cDNA, but it was also remarkably similar. We had identified an autosomal paralog of dystrophin.<sup>28</sup> We went on to show that this gene is localized on human chromosome 6 and encodes a 13 kb mRNA, which is also spread over many exons in an arrangement very similar to that of the exons in the dystrophin gene (approximately 1 Mb).<sup>29</sup> We called the protein utrophin because unlike dystrophin, whose expression is confined to muscle and the brain, utrophin is expressed in most tissues.<sup>30,31</sup> Like dystrophin, utrophin is a large cytoskeletal protein (394 kDa)<sup>32</sup> but is only expressed in small amounts in adult muscle.<sup>33</sup> Their genomic structures are similar,<sup>34</sup> which suggests that dystrophin and utrophin are related by an ancestral duplication event, although which gene came first is unknown. Our laboratory was the first to clearly suppose that utrophin might be capable of performing the same cellular functions as dystrophin and be used as a surrogate for DMD.<sup>35</sup>

Our studies of the localization of utrophin in muscle showed that it is found at the sarcolemma in utero and is progressively replaced by dystrophin during development.<sup>36–38</sup> In adults, utrophin is enriched in skeletal muscle at the neuromuscular and myotendinous junctions<sup>31</sup> and at the sarcolemma in regenerating myofibers.<sup>39</sup> In DMD patients, utrophin spreads to the sarcolemma, but we demonstrated that this was likely to be stabilization of the protein rather than transcriptional upregulation of the gene.<sup>40</sup>

In view of the high homology between the genomic sequences, we characterized the promoter regions of the two genes. Dystrophin has five promoters that drive its expression in skeletal muscle, the heart, and the brain.<sup>41</sup> The dystrophin locus also expresses smaller isoforms that encode the C-terminal region. Utrophin has smaller iso-

forms encoding the C-terminal regions in a similar way, but the 5' controlling region of the full-length gene is quite different. Significantly, the utrophin gene has a CpG island in the 5' region, and an N-box is responsible for the expression of utrophin at the synapse. The N-box is a 6 bp motif first characterized by Changeux and colleagues and shown to be very important for the expression of the acetylcholine receptor at the synapse.<sup>42</sup> Mutation of this N-Box motif at the 5' end of the utrophin gene results in the extra-synaptic expression of the protein.<sup>43</sup> Thus, the reason for the different localizations of dystrophin and utrophin lies partly in their transcriptional control.

We reasoned that since the main difference between dystrophin and utrophin lies in their regulation, utrophin should be able to compensate for the lack of dystrophin in DMD if it is expressed at high enough levels in the muscles of patients. This is supported by the fact that dystrophin and utrophin are found at the sarcolemma together in early human fetal life, as mentioned earlier.<sup>36</sup> We tested this hypothesis in the *mdx* mouse model of DMD by generating transgenic mice expressing utrophin under the control of the skeletal-muscle actin promoter. The utrophin was able to prevent the pathology of the *mdx* mouse in a dose-dependent manner.<sup>44</sup> We went on to suggest that the upregulation of the expression of the utrophin gene would produce a clinical effect.

At this stage, it was important to elucidate the function of utrophin in muscle. If we were to increase the levels of utrophin, would this disturb other functions? Our laboratory also enhanced the understanding of utrophin function through gene-knockout technology. The utrophin-deficient mouse shows mild reduction at the neuromuscular junction, demonstrating that utrophin functions not in the formation of acetylcholine receptor clusters but rather in the stabilization of such clusters.<sup>45</sup> This mouse showed no other symptoms, suggesting that it does not play a major role in muscle or other tissues. We also suggested that the *mdx* and utrophin-deficient mice might be relatively mildly affected given that dystrophin and utrophin could compensate functionally. We validated this by generating a double mutant lacking both dystrophin and utrophin.<sup>46</sup> This double-knockout mouse suffers a severe progressive muscular dystrophy much more like the severe clinical course seen in DMD patients and dies at 20 weeks. This model has been used as a more stringent model for preclinical studies. For example, we have shown that the severe phenotype can be prevented by administration of snRNAAV constructs containing sequences that promote the skipping of the stop codon in exon 23 of the gene in the *mdx* mouse.<sup>47</sup>

Recent studies have shown that although it is similar to dystrophin, utrophin cannot anchor nNOS to the sarcolemma.<sup>48</sup> Nevertheless, it is important to note that BMD patients who lack the nNOS binding site are often mildly affected, and therefore the lack of nNOS binding might not be very significant.<sup>24</sup> This could also be compensated by treatment with PDE5 inhibitors, such as tadalafil.<sup>49,50</sup>

Utrophin also does not interact with microtubules in the same way that dystrophin does<sup>51</sup> and therefore might not prevent microtubule-lattice derangement, potentially leading to aberrant calcium regulation and increased reactive oxygen species.<sup>52,53</sup> Despite these subtle differences, as described by our lab and others, utrophin can act as a surrogate for dystrophin in dystrophic muscles.<sup>54–56</sup> In the *mdx* mouse, the utrophin A isoform, found at the neuromuscular junction, is naturally increased (1.8-fold) as a part of the natural repair process in the absence of dystrophin. This repair mechanism also occurs in DMD patients.<sup>57–59</sup> Increasing utrophin A expression up to 2- to 4-fold more than the wild-type level prevents the development of pathology in the *mdx* mouse.<sup>44,60</sup> Importantly, this increased level of utrophin is significantly less than the normal levels found in the kidney and liver<sup>44</sup> and is not toxic in a broad range of murine tissues.<sup>61</sup> Nevertheless, the actual levels of dystrophin or utrophin needed to provide functional clinical benefits are still unknown whatever the approach used. Previous work has indicated that approximately 20% of wild-type dystrophin levels are required for a significant correction of the muscle pathology.<sup>62</sup> However, an increase in dystrophin to 4% of wild-type levels in the *mdx* mouse is sufficient to increase survival, suggesting that lower levels might be beneficial in humans.<sup>63</sup> We would therefore anticipate that similar sub-normal levels of utrophin would show a benefit.

There are several advantages of utrophin-modulation therapy: increased utrophin levels will be applicable to all DMD patients regardless of the dystrophin mutation and do not appear to be toxic.<sup>61</sup> Furthermore, a systemic strategy designed to increase the endogenous utrophin level to treat all types of muscle, including the heart and diaphragm, will not result in an immune response. Strategies to increase utrophin levels include direct delivery of the protein<sup>64</sup> or stabilization of the protein or RNA,<sup>65,66</sup> viral approaches,<sup>67,68</sup> and non-viral approaches such as biglycan.<sup>69</sup> We have shown that oral administration of small molecules designed to modulate utrophin expression at the transcriptional level can decrease the progression of the disease in the *mdx* mouse and represents a promising therapeutic avenue for DMD.<sup>70</sup> An effective small molecule to modulate utrophin expression should target skeletal and cardiac muscle and result in an increase in the localization of utrophin throughout the myofibers.

Over the last 3 years, we have made considerable progress toward the goal of utrophin modulation by using this small-molecule approach in collaboration with Summit Therapeutics. We set up a screen in an *mdx* cell line for molecules that would increase the transcription of utrophin.<sup>71</sup> This screen has led to the development of a first-in-class drug (SMTC1100) for the modulation of utrophin; this drug has just completed a phase 1 trial in DMD patients and is now moving into phase 2 trials.<sup>72</sup> More recently, from this screen we characterized another molecule that increases utrophin levels more than SMTC1100 and has better pharmacokinetics. Pre-clinical data from an

*mdx* trial of one of these molecules, SMT022357, show that daily treatment with the drug results in higher utrophin and dystroglycan levels in skeletal muscle. We have shown significant improvement in the ability of *mdx* mice to recover from eccentric contractions, a marker of muscle function.<sup>73</sup> Consistent with this, we observed a significant reduction in fiber regeneration, particularly in the diaphragm. These results highlight the efficient distribution of the drug and the positive systemic effect of this utrophin-modulation strategy (for a review, see Guiraud et al.<sup>5</sup>). We have also developed new cell-line screens from which we are developing other lead candidates that act differently than SMTC1100 and SMT022357. Thus, we have a very promising molecule in phase 2 trials and are following up on other lead compounds for DMD therapy using utrophin modulation.

Several other approaches for DMD therapy that target the primary cause of the disease are making significant progress. Readthrough of stop codons has produced the paradigm for clinical trials,<sup>74</sup> and the first drug using this approach, ataluren, has recently received a conditional marketing authorization in Europe. Exon 51 skipping looks promising, and clinical trials are ongoing. Both Prosensa NV/BioMarin and Sarepta Therapeutics have filed nondisclosure agreements recently for their exon-51-skipping therapies drisapersen<sup>75</sup> and eteplirsen, respectively.<sup>76</sup> However, these drugs only tackle the problem in a small fraction of the 13% of patients who have mutations treatable by exon 51 skipping because patients need to be ambulant for entry into any trial. Current constructs for exon skipping do not enter the heart, and other exons need to be and are being targeted. Their efficacy also needs to be improved. Multi-exon-skipping strategies might be more widely applicable but are in their infancy.<sup>77</sup> As this review goes to press, the FDA is considering approval of submissions from companies involved in exon skipping. The effects reported thus far are small, and further trials are needed to demonstrate that enough dystrophin is produced to provide a clinically meaningful effect. Nevertheless, proof of principle has been shown, and next-generation exon-skipping chemistry, such as the tricycloDNA chemistry,<sup>78</sup> should provide better efficacy. Gene therapy delivering minigenes is progressing well and has shown promising data in dogs without immunosuppression.<sup>79</sup> Current challenges include delivery, obtaining high titers of virus, and safety of systemic delivery. Stem cell strategies could have long-term value, and approaches using CRISPR/Cas9 are rapidly being explored but ultimately will require viral delivery.<sup>80</sup>

These genetic approaches can be used in conjunction with pharmacological approaches that slow the progression of the disease; combination therapy could ultimately provide the best strategy for treating patients.<sup>70,81</sup>

Progress in the development of the diagnosis and treatment of DMD has very much depended on close collaboration among scientists, clinicians, and patients. The continued support of major Duchenne charities (the UK Muscular Dystrophy and US Muscular Dystrophy

Association) and the regular interaction with patients and their families have played a very important role in my career and the progress that has been made. In the future, it will be even more important to involve patients in the design and development of therapies to ensure that patients can benefit maximally from any approach. DMD is a complex disease with a lot of variation in its progression between patients. It will be important to develop patient-focused therapies depending on the stage of the disease progression and the age of the patient.

Much of the progress in DMD has depended on technical innovation (PCR, development of gene-therapy vectors, exon-skipping chemistry), and further developments in biomarkers are needed. There will also be innovation in monitoring patients' responses and adverse events. This is an exciting time in the translation of genetics and genomics into effective treatments. What I thought was impossible 20 years ago is now very close to reality. The dream of an effective treatment of DMD, and with it treatments for other rare diseases, will soon be realized.

### Acknowledgments

I am particularly grateful to Simon Guiraud for assistance in the finalization of this summary of my William Allan Award lecture. I dedicate it to all boys affected by Duchenne muscular dystrophy and their families, who live with the disease every day. K.E.D. is a shareholder of Summit Therapeutics.

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