Spinocerebellar ataxia type 7 in southern Africa: An epidemiological, molecular and cellular study

By

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Declaration

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Abstract
Spinocerebellar ataxia type 7 (SCA7) is an inherited neurodegenerative disease caused by a pathogenic expansion of a CAG repeat within the ataxin 7 gene, resulting in an expanded polyglutamine tract in the ATXN7 protein. SCA7 patients suffer from selective degeneration of cerebellar Purkinje neurons and retinal photoreceptors, which leads to the development of various neurological symptoms, and blindness. SCA7 is considered to be a relatively rare disease, but South Africa has an increased prevalence of the SCA7 due to a founder effect within the black African population. In this study, three distinct but complementary approaches were taken to investigate SCA7 in South Africa, with the aim of estimating the prevalence of the disease, developing improved approaches for molecular diagnostic testing, and establishing a model for in vitro studies of pathogenesis.

Firstly, interrogation of patient-based records spanning 26 years revealed that 88 individuals had been molecularly diagnosed with SCA7 in South Africa, which resulted in one of the largest patient cohorts reported globally. This group also included four non-South African families. Haplotype analysis showed that two Zambian families and a Namibian family share the South African SCA7 founder haplotype, indicating that the founder effect extends beyond South African borders. The fourth family, from Nigeria, did not have the same haplotype.

The second aspect explored the utility of an additional confirmatory test for SCA7. Many South African individuals show apparent homozygosity during molecular testing for SCA7 using a standard multiplex PCR assay. Re-testing of 111 samples using an alternative singleplex PCR reaction revealed pathogenic expansions in six samples which were previously considered to be homozygous. A triplet repeat primed PCR assay was subsequently optimised and implemented into the routine molecular testing protocol, in an attempt to provide all tested individuals with a definitive molecular diagnosis.

Finally, induced pluripotent stem cell (iPSC) technology was used to determine whether an in vitro model of the retinal degeneration associated with SCA7 could be developed. iPSCs were created from two SCA7 patients and an unaffected control individual, and were subjected to a differentiation protocol to derive retinal cells. Since ATXN7 forms part of two transcriptional activator complexes, the expression profiles of 23 genes were monitored in the retinal cells, in order to identify transcripts which were consistently dysregulated in patient-derived cells. Various genes, including ataxin 7, showed expression changes, but the high levels of heterogeneity in morphology and gene expression indicated that the model required refinement before the results could be pursued. Nonetheless, this study is the first to describe an in vitro investigation of SCA7 patient-derived retinal cells.

Together, these results have laid the groundwork for further investigations into the prevalence of SCA7, as well as the development of more sophisticated models for in vitro studies. These may ultimately lead to better management, care and therapeutic interventions for SCA7 patients on the African continent.
Acknowledgements

The financial assistance of the National Research Foundation (NRF) and the Deutscher Akademischer Austausch Dienst (DAAD) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to these agencies. A sincere thanks to the National Research Foundation, the Medical Research Council of South Africa, and the University of Cape Town for funding various aspects of this project.

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On a personal note, I'd like to thank my friends and family for their endless love, support and encouragement.

To my incredible mom - I'm not sure how to express my gratitude for your emotional and financial support. Thank you for helping me to pursue my high school dream of becoming a geneticist, and for being my biggest fan.

To Graeme – there are no words. Thank you for learning to say "Spinocerebellar ataxia", asking questions, making tea and having an endless supply of hugs. Here's to many more exciting chapters.

Proverbs 25:2
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<th>Description</th>
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<tbody>
<tr>
<td>ADCA</td>
<td>Autosomal dominant cerebellar ataxia</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>anti-NP</td>
<td>Anti-nucleocapsid protein</td>
</tr>
<tr>
<td>ARR3</td>
<td>Arrestin 3</td>
</tr>
<tr>
<td>ATXN7</td>
<td>Ataxin 7</td>
</tr>
<tr>
<td>BEX1</td>
<td>Brain expressed, X-linked 1</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>CD30</td>
<td>Cluster of differentiation 30</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>c-MYC</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRX</td>
<td>Cone-rod homeobox</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf-1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNAJA1</td>
<td>DnaJ (Hsp40) homolog, subfamily A, member 1</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DRPLA</td>
<td>Dentatorubral-pallidoluysian atrophy</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EMQN</td>
<td>European Molecular Genetics Quality Network</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Forkhead box A2</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GNA1T1</td>
<td>Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1</td>
</tr>
<tr>
<td>GRIA2</td>
<td>Glutamate receptor, ionotropic, AMPA 2</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington disease</td>
</tr>
<tr>
<td>HSP105</td>
<td>Heat shock protein 105</td>
</tr>
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<td>HTT</td>
<td>Huntingtin</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>IRBP</td>
<td>Interphotoreceptor Retinoid-Binding Protein</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblast cell line</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>LEFTY2</td>
<td>Left-right determination factor 2</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>NHLS</td>
<td>National Health Laboratory Services</td>
</tr>
<tr>
<td>NOG</td>
<td>Noggin</td>
</tr>
<tr>
<td>NR2E3</td>
<td>Nuclear Receptor Subfamily 2, Group E, Member 3</td>
</tr>
<tr>
<td>NRL</td>
<td>Neural retina leucine zipper</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>POU domain class 5 transcription factor 1</td>
</tr>
<tr>
<td>OLG1</td>
<td>Oligodendrocyte transcription factor 1</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OPN1LW</td>
<td>Opsin 1 (cone pigments), long-wave-sensitive</td>
</tr>
<tr>
<td>OPN1MW</td>
<td>Opsin 1 (cone pigments), medium-wave-sensitive</td>
</tr>
<tr>
<td>OPN1SW</td>
<td>Opsin 1 (cone pigments), short-wave-sensitive</td>
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<tr>
<td>OTX2</td>
<td>Orthodenticle homeobox 2</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired box 6</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Poly-HEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
</tr>
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<td>pri-miRNA</td>
<td>Primary microRNA</td>
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<td>PRPH2</td>
<td>Peripherin 2</td>
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<td>Quantitative PCR</td>
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<td>RCVRN</td>
<td>Recoverin</td>
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<td>Repeat expansion detection</td>
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<td>Rhodopsin</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>RORα</td>
<td>RAR-related orphan receptor alpha</td>
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<td>RAR-related orphan receptor beta</td>
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<td>RP</td>
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<td>RPE</td>
<td>Retinal pigment epithelium</td>
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<td>Retinal pigment epithelium-specific protein 65kDa</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SA</td>
<td>South Africa</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt/Ada/Gcn5 acetylase</td>
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<tr>
<td>SBMA</td>
<td>Spinal and bulbar muscular atrophy</td>
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<tr>
<td>SCA</td>
<td>Spinocerebellar ataxia</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>SCAANT1</td>
<td>Spinocerebellar ataxia-7 antisense noncoding transcript 1</td>
</tr>
<tr>
<td>SeVdp</td>
<td>Replication-defective and persistent Sendai virus vector</td>
</tr>
<tr>
<td>SFEB</td>
<td>Serum-free embryoid body-like</td>
</tr>
<tr>
<td>Sgf73</td>
<td>SaGa associated Factor, 73 kDa</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
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<td>SMA</td>
<td>Smooth muscle actin</td>
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<td>Single nucleotide polymorphism</td>
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<td>SSEA4</td>
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<td>SPT3/TAF9/GCN5 acetyl transferase complex</td>
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<td>TFTC</td>
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</tr>
<tr>
<td>TRβ3</td>
<td>Thyroid hormone receptor β2</td>
</tr>
<tr>
<td>TUBB3</td>
<td>Beta 3 class III tubulin</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Ubiquitin carboxyl-terminal esterase L1</td>
</tr>
<tr>
<td>UCT</td>
<td>University of Cape Town</td>
</tr>
<tr>
<td>β-INF</td>
<td>Interferon beta</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromole per litre</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1 The Autosomal Dominant Cerebellar Ataxias

In 1982, a seminal paper by Anita Harding proposed a new classification system for the late onset autosomal dominant cerebellar ataxias (ADCAs) (Harding, 1982). This group of disorders had been described in multiple families since the 19th century, however the vast clinical heterogeneity evident in these patients warranted a new system of classification. Harding’s classification was subsequently refined in 1993, and grouped the ADCAs into three main categories based on clinical presentation (Table 1) (Harding, 1993). Since the discovery and development of molecular genetics, many of the genetic loci responsible for these disorders have been elucidated, and the group is more commonly referred to as the Spinocerebellar ataxias (SCAs).

Table 1: Modified Harding’s classification of ADCAs (adapted from Duenas et al., 2006)

<table>
<thead>
<tr>
<th></th>
<th>ADCA I</th>
<th>ADCA II</th>
<th>ADCA III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical presentation</td>
<td>Cerebellar syndrome with ophthalmplegia/pyramidal/extrapyramidal signs/cognitive impairment/peripheral neuropathy</td>
<td>Cerebellar syndrome with pigmentary retinopathy</td>
<td>Pure cerebellar syndrome</td>
</tr>
<tr>
<td>Neuropathology</td>
<td>Degeneration of the cerebellum, and of the basal ganglia/cerebral cortex/ optic nerve/pontomedullary systems/spinal tracts/peripheral nerves</td>
<td>Cerebellar and pigmentary retinal degeneration</td>
<td>Cerebellar degeneration</td>
</tr>
<tr>
<td>SCAs</td>
<td>1, 2, 3, 4, 8, 10, 12, 13, 17, 18, 19/22, 20, 21, 23, 24, 25, 27, 28, 32, 33, 34, 35, 36</td>
<td>7</td>
<td>5, 6, 11, 14, 15, 16, 26, 29, 30, 31</td>
</tr>
</tbody>
</table>

The SCAs are broadly characterised by progressive cerebellar ataxia (the inability to co-ordinate movement), however additional neurological symptoms are variably associated (Table 1). ADCA type I contains the broadest group of SCAs, where neuropathology is often spread across multiple sites, and includes the most common SCA types (1, 2 and 3). SCA7 is the only SCA to be classified as ADCA type II, due to the unique involvement of pigmentary retinopathy, whilst the SCAs with degeneration limited to the cerebellum are classified as ADCA type III.
1.2 Polyglutamine repeat disorders

The SCAs are a group of disorders that are classified based on shared or similar clinical presentations. A subset of these disorders are caused by the same type of genetic mutation. SCA types 1, 2, 3, 6, 7 and 17 are additionally classified as polyglutamine repeat disorders, since they are caused by the expansion of a CAG repeat past a pathogenic threshold, which is translated into an extended polyglutamine tract within the corresponding protein. Three additional non-SCA neurodegenerative disorders are classified as polyglutamine diseases, namely Dentatorubral-pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA) and Huntington disease (HD) (Table 2).

In the polyglutamine diseases there is a well documented inverse correlation between the CAG repeat size and the age of onset of disease symptoms, such that individuals with a larger repeats develop symptoms at an earlier age than those with smaller repeats (David et al., 1998). Juvenile onset is associated with large repeats, but onset is usually during the third or fourth decade of life, with the diseases progressing to death within 10 to 20 years (Durr, 2010). Genetic anticipation is another feature of the polyglutamine diseases, whereby the size of the CAG repeat often increases with each successive generation, resulting in offspring with an earlier age of onset and a more rapid progression of disease symptoms (Durr, 2010).
Table 2: Known polyglutamine diseases, their associated proteins and function, and CAG repeat thresholds (adapted from Smith et al., 2013)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Associated protein</th>
<th>Protein function</th>
<th>((\text{CAG})n) disease threshold (full penetrance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>Huntingtin</td>
<td>Microtubule-mediated transport, vesicle function, transcriptional regulation, anti-apoptotic</td>
<td>&gt;40</td>
</tr>
<tr>
<td>DRPLA</td>
<td>Atrophin-1</td>
<td>Transcriptional co-repressor</td>
<td>&gt;49</td>
</tr>
<tr>
<td>SBMA</td>
<td>Androgen receptor</td>
<td>Regulation of gene expression</td>
<td>&gt;38</td>
</tr>
<tr>
<td>SCA1</td>
<td>Ataxin 1</td>
<td>Transcriptional co-repressor</td>
<td>&gt;39</td>
</tr>
<tr>
<td>SCA2</td>
<td>Ataxin 2</td>
<td>Epidermal growth factor trafficking</td>
<td>&gt;35</td>
</tr>
<tr>
<td>SCA3</td>
<td>Ataxin 3</td>
<td>Deubiquitination, transcriptional regulation</td>
<td>&gt;61</td>
</tr>
<tr>
<td>SCA6</td>
<td>Ca(^{++}) channel subunit</td>
<td>Mediation of calcium ion entry</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SCA7</td>
<td>Ataxin 7</td>
<td>Co-activator of transcription, cytoskeleton stabilisation</td>
<td>&gt;36</td>
</tr>
<tr>
<td>SCA17</td>
<td>TATA binding protein</td>
<td>Transcriptional activation</td>
<td>&gt;49</td>
</tr>
</tbody>
</table>

1.2.1 Epidemiology of the polyglutamine SCAs

The polyglutamine SCAs show a highly variable frequency and distribution across the globe. SCA3, also known as Machado-Joseph disease, is frequently reported as the most prevalent type of polyglutamine SCA when compared to SCA1, 2, 6 and 7. However, SCA1 and SCA2 have a similarly high representations in some countries such as Italy and India (Basu et al., 2000; Brusco et al., 2004). SCA17 is considered to be very rare, with fewer than 100 families reported. In several cases the change in distribution of the polyglutamine SCAs in a particular area is due to a founder effect, leading to a disease allele being present in an unusually high frequency in a distinct population. Multiple founder effects for the polyglutamine SCAs have been reported in different geographical regions, including South Africa (SA) (Table 3).
### Table 3: Polyglutamine SCA founder effects (adapted from www.scabase.eu, accessed May 2014)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Founder effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCA1</td>
<td>Siberia, India, South Africa</td>
<td>(Goldfarb et al., 1996; Mittal et al., 2005; Ramesar et al., 1997)</td>
</tr>
<tr>
<td>SCA2</td>
<td>Cuba, India</td>
<td>(Allotey et al., 1995; Choudhry et al., 2001)</td>
</tr>
<tr>
<td>SCA3</td>
<td>Portugal, Netherlands</td>
<td>(Gaspar et al., 2001; Verbeek et al., 2004)</td>
</tr>
<tr>
<td>SCA6</td>
<td>Germany, Japan, Netherlands</td>
<td>(Dichgans et al., 1999; Mori et al., 2001; Verbeek et al., 2004)</td>
</tr>
<tr>
<td>SCA7</td>
<td>Mexico, South Africa, Scandinavia</td>
<td>(García-Velázquez et al., 2013; Greenberg et al., 2006; Jonasson et al., 2000)</td>
</tr>
<tr>
<td>SCA17</td>
<td>Not determined</td>
<td></td>
</tr>
</tbody>
</table>

#### 1.2.2 The polyglutamine SCAs in South Africa

In 1994 Bryer and colleagues embarked on the first clinical and molecular investigation of the polyglutamine SCAs in SA. Although the clinical presentations of the South African patients were similar to those seen globally, SA showed a unique distribution of the polyglutamine SCAs amongst the 44 described families. Whilst there was a high proportion of SCA1 and SCA2 families (50% and 16% respectively), there was also a high proportion of SCA7 families, who were all of black African ethnic origin (27% of families). Only two patients tested positive for the SCA3 expansion, and neither were from South Africa (one individual was from a Portuguese family, the other from Somalia). A single individual of European descent was found to have SCA6. These results were published in 2003 (Bryer et al., 2003), and no additional studies have reported the spectrum and prevalence of the polyglutamine SCAs in southern Africa.

In order to investigate the origin of the SCA7 mutation in SA, five SCA7 families were genotyped for four microsatellite markers and a single nucleotide polymorphism (SNP) across a region flanking the ataxin 7 gene on chromosome 3 (Greenberg et al., 2006). The analysis revealed a common haplotype in all patients tested, indicative of a founder effect within black African SCA7 patients in SA. Similar
studies in Scandinavia and Mexico have revealed evidence for SCA7 founder effects in families from those regions (García-Velázquez et al., 2013; Jonasson et al., 2000), but investigations into the origins of the SCA7 mutation in other countries have not been reported.

1.2.3 Polyglutamine disease pathology

Although the polyglutamine disease genetic loci are spread across multiple genes with differing protein functions, and a different pathogenic threshold exists for each disease (Table 2), the commonalities in disease symptoms and progression, as well as the common type of mutation, suggest that similar pathogenic mechanisms may be involved across the group (Figure 1). Disease pathology is more commonly thought to be as a result of a toxic-gain-of-function conferred to the mutant protein, resulting in multiple downstream deleterious effects (Figure 1). Proteins with extended polyglutamine stretches form β-sheet rich structures and collect to form insoluble aggregates, often termed nuclear inclusions (Chen et al., 2002). These inclusions contain the mutated protein, along with ubiquitin, components of the proteosome, and heat shock proteins (Havel et al., 2009). The remaining proteosomes are unable to fully degrade the polyglutamine-containing proteins, which are released back into the cell (Venkatraman et al., 2004). Together, impaired degradation by the cell’s ubiquitin-proteosome and heat shock response systems is thought to contribute to disease pathology. The majority of the polyglutamine ataxia proteins are involved either directly or indirectly in transcriptional regulation, by acting as components as transcriptional regulatory complexes or through interactions with transcription factors (Matilla-Dueñas et al., 2010). Downstream aberrant functioning of transcriptional regulation as a result of the mutant protein may contribute to existing pathogenic pathways (such as down-regulation of heat shock protein expression), in addition to accounting for cell-type specificity seen in the polyglutamine SCAs. Degeneration may be further exacerbated through the activation of the mitochondrial apoptotic pathway (Chou et al., 2006; Wang et al., 2010) or inhibited respiration in mitochondria as a result of increased reactive oxygen species (Puranam et al., 2006). RNA toxicity has also been recently implicated in polyglutamine pathogenesis. Toxic effects may be due to the
presence of the CAG repeat within mRNA molecules, leading to alternative splicing, aberrant interactions with cellular proteins or nucleolar stress (Fiszer and Krzyzosiak, 2013). Whilst these pathogenic pathways may provide multiple potential therapeutic targets and approaches, many of the key factors and specific processes involved in the polyglutamine diseases remain to be elucidated.

Figure 1: Pathways of polyglutamine disease pathogenesis. Pathogenesis has largely been attributed to the effects of the mutant protein, although a role for RNA toxicity in the development of disease has also been proposed. The mechanism by which polyglutamine proteins exert their toxic effects varies according to the protein, and may include proteolytic cleavage (leading to the production of toxic fragments), impairment of the ubiquitin–proteasome pathway, formation of aggregates of mutant protein (involving the sequestration of wild-type polyglutamine protein and other important cellular components such as transcription factors), dysregulation of transcription, either directly or as a result of aggregate formation, and mitochondrial dysfunction, all of which result in deleterious downstream consequences. Reproduced from Watson and Wood (2012) by permission from Cambridge University Press.
1.3 Spinocerebellar ataxia type 7

1.3.1 Discovery and cloning of the gene

SCA7 patients suffer from selective degeneration of cerebellar Purkinje neurons and inferior olivary nuclei, regions of the brain that are involved in the control and coordination of movement (Gouw et al., 1994). Blindness occurs as a result of degeneration of the neural retina (photoreceptors and ganglion cells), which may be accompanied by deterioration of the optic tract and visual cortex (Gouw et al., 1994; Mohan et al., 2014a). Research efforts to identify the underlying genetic cause of SCA7 began in the mid 1990's. Since SCA7 was classified in a distinct category (ADCA type II) due to the presence of retinal degeneration, it was hypothesised that the underlying mutation would be within a novel genetic locus, rather than within candidate loci responsible for other ADCA subtypes, such as SCA1 or SCA2. Linkage analysis among four large families mapped the candidate region to chromosome 3, which was further reduced to an 8 cM interval region around the marker D3S1285 (Benomar et al., 1995). Due to the presence of anticipation in age of onset in SCA7 families (a hallmark associated with known polyglutamine disorders such as SCA1, 2, 3 and HD), an additional study utilised repeat expansion detection (RED) to screen for CAG repeat expansions in eight SCA7 families (Lindblad et al., 1996). Positive RED products were found in genomic DNA from all affected individuals, with a calculated average expansion size of 64 CAG repeats. In 1997 the ataxin 7 gene (ATXN7) was identified by positional cloning, and SCA7 became the eighth disease to be classified as a polyglutamine repeat disorder (David et al., 1997). The sequence of ATXN7 revealed no functional insights into the role of the corresponding protein. However, it was postulated that the Ataxin 7 (ATXN7) protein may be a transcription factor, since it was present in the nuclear fraction of lymphoblasts and contained a nuclear localisation signal (David et al., 1997).

1.3.2 Molecular Diagnosis of SCA7

Although the function of the ATXN7 protein remained unknown, a routine molecular diagnosis could be offered to SCA7 families. Alleles in unaffected individuals contained 7 to 17 CAG repeats, whereas SCA7 patients carried alleles
ranging from 38 to 130 repeats. In 1998 Stevanin and colleagues explored the role of so-called intermediate alleles in SCA7 families. Individuals with 28 to 35 CAG repeats were asymptomatic, but were found to have offspring with alleles beyond the pathogenic threshold, possibly explaining the persistence of SCA7 within the population in spite of marked disease anticipation. The repeat reference ranges for SCA7 have been defined and changed over time, with the current accepted ranges outlined by the European Molecular Genetics Quality Network (EMQN) on the SCAbase website (www.scabase.eu), as listed in Table 4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal alleles</th>
<th>Uncertain alleles</th>
<th>Reduced penetrance alleles</th>
<th>Full penetrance alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATXN7</td>
<td>4-19</td>
<td>28-33</td>
<td>34-35</td>
<td>36-460</td>
</tr>
</tbody>
</table>

Patients with a suspected SCA7 mutation are typically referred to a molecular diagnostic laboratory for a confirmation of a clinical diagnosis. Standard molecular diagnosis methods utilise a polymerase chain reaction (PCR)-based method whereby two sequence-specific primers (one with a fluorescent tag) flank the CAG repeat region. PCR amplification of the region results in two distinct products, representing each CAG repeat allele. Subsequent capillary electrophoresis allows for accurate sizing of each fragment, whereafter the size of the CAG repeat can be calculated. In cases where a single wild-type fragment is detected (apparent homoallelism), the patient can not be given a definitive diagnosis, since the result may be due to allele drop-out in the reaction. In these cases a confirmatory assay such as Southern blot or triplet repeat primed PCR (TP PCR) may be employed (Sequeiros et al., 2010a). Due to the overlapping symptoms shared by some of the polyglutamine disorders, some laboratories may use a multiplex-based assay that tests for pathogenic alleles at multiple loci, such as SCA1, 2, 3, 6 and 7 (Bauer et al., 2005; Dorschner et al., 2002). The National Health Laboratory Services (NHLS) at Groote Schuur Hospital in Cape Town is the only centre in Africa offering a molecular diagnostic service for the polyglutamine SCAs. The laboratory uses two multiplex PCR assays to test for SCA1, 2, 3, 6, 7, 12 and 17, but there are no confirmatory tests in place to confirm or disprove cases of apparent homozygosity.
1.3.3 Treatment of SCA7

There is currently no treatment or therapy for SCA7. Patient management includes the introduction of canes and walkers and other home modifications to aid mobility. Patients suffering from dysarthria may benefit from speech therapy, and low-vision aids are introduced for patients with visual problems. Although there are no current pharmaceutical interventions suitable for individuals with SCA7, studies have shown that patients may benefit from interferon beta (β-INF) treatment. The introduction of β-INF into COS-7 cells transfected with mutant ATXN7 resulted in the selective degradation of the mutant protein, reducing the number of aggregates within the cell (Janer et al., 2006). In an in vivo study, Chort and colleagues found that β-INF treatment in SCA7 knock-in mice resulted in improved cerebellar function and increased Purkinje cell survival (Chort et al., 2013). β-INF has been used as an approved drug for the treatment of multiple sclerosis for more than 15 years, and exhibits few side effects. The success of the above experiments in the mouse model suggests that it is likely to be introduced into patient clinical trials in the near future.

A prospective molecular therapy approach for SCA7 has employed the use of RNA interference (RNAi). Researchers within the Division of Human Genetics at the University of Cape Town have investigated and developed a potential allele-specific silencing approach that has thus far proven successful in vitro, in both transfected cells and SCA7 patient fibroblasts (Scholefield et al., 2014; Scholefield et al., 2009). The RNAi-based approach may be a promising avenue for future therapies; however, scientists are currently burdened with challenges regarding the dosage and delivery of the effectors (Boudreau et al., 2011; Gao and Huang, 2013). It is also likely that new techniques such CRISPR-based genome editing will provide alternative therapeutic approaches for the treatment of SCA7 and other neurodegenerative diseases.

1.4 SCA7 and the eye

1.4.1 Structure of the human retina

Human eye development is a tightly regulated and conserved developmental process. In humans, ocular development is first evident around 22 days gestation,
where lateral bulges known as optic grooves start to enlarge to form optic vesicles (Carlson, 2004). Through the co-ordinated effects of multiple signaling molecules and induction processes, the optic vesicles invaginate to form the optic cup, and the lens vesicle appears. The inner layer of the optic cup thickens and begins to form the neural retina. The neural retina matures to form a multilayered structure containing highly specialised light-sensing cells and other support cells (Carlson, 2004). The light sensing neural pathway contains a chain of three neurons (Figure 2). Rod and cone photoreceptors are light sensing cells that exist in the outer layer of the retina, with their nuclei in the outer nuclear layer. Cone photoreceptors are responsible for colour vision and perception in bright light, and are concentrated in the central macular region of the retina, decreasing in number towards the periphery of the eye. In humans, three different types of cones are specialised for detection of long (L-cones), medium (M-cones) or short (S-cones) wavelengths of light (corresponding to red, green and blue light respectively). Rod photoreceptors aid in perception in low light conditions, and outnumber cone cells 18-20:1 in humans (Swaroop et al., 2010). The processes from the rods and cones extend into the outer plexiform layer, where they synapse with bipolar cells in the inner nuclear layer. The bipolar cells send processes into the inner plexiform layer, where they synapse with ganglion cells contained in the ganglion cell layer. The ganglion cells send long processes that enter the nerve fibre layer to exit the eye and join the optic nerve to reach the brain (Carlson, 2004).

The retinal pigment epithelium (RPE) layer lies outside the rod and cone layer, and assists in support and maintenance of the neural retina, by transporting nutrients and recycling photopigments generated during the visual cycle. The RPE also phagocytoses shed photoreceptor membranes to assist in renewal of outer segments (Strauss, 2005). The inner and outer plexiform layers contain horizontal and amacrine cells, which are responsible for the horizontal distribution of the light perception signal (Figure 2). Muller cells are glial cells that act as architectural support as well as aiding in metabolism and waste removal.
1.4.2 The SCA7 retinal phenotype

SCA7 was classified as a distinct type of ADCA by Harding due to the presence of pigmentary retinal degeneration. Her report of "Family 3" in 1982 described affected individuals with poor vision and difficulty in walking, with optic atrophy, poor perception of light, marked retinal pigmentary degeneration and slow saccadic eye movements (Harding, 1982). The general description of the SCA7 retinal phenotype is that of macular degeneration, with initial deterioration of central vision declining toward complete blindness (indicative of cone-rod dystrophy) (Michalik et al., 2004). Histological examinations show severe ganglion cell loss, photoreceptor degeneration and thinning of the nuclear and outer plexiform layers (Figure 3). SCA7 patients may present with visual symptoms either before or after the onset of ataxia (Miller et al., 2009), although retinal degeneration often precedes neurodegeneration in patients with larger CAG repeats. There are some clinical reports of post-mortem retinal tissue examinations and imaging studies from SCA7 patients, however knowledge regarding the temporal and spatial degeneration in the SCA7 patient retina, particularly at the cellular level, is limited.

Figure 2: Cellular structure of the human retina. Adapted from Wilkenson-Berka (2004), reproduced with permission from Cambridge University Press.
Figure 3: Pathological hallmarks of SCA7 retinal degeneration. (a) Macula of an unaffected individual, showing a normal macula (black arrow), optic disc (black arrowhead) and well-developed vasculature (white arrow). (b) The retina of a SCA7 patient shows a pale optic disc (arrowhead) and atrophy of the RPE and choroid layer (arrow). (c) The fundoscopy of a South African SCA7 patient retina shows atrophy of the macular region (white arrow). (d), (e) Histology of the retina of an unaffected individual (d) and a SCA7 patient (e). The control individual shows organisation of the retinal pigment epithelium (P), outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (G). The retina of the SCA7 patient shows degeneration of the photoreceptor nuclei (usually in the ONL), disorganisation of the INL (black arrowhead) and migration of melanin pigment into the retina (arrows). (a, b, d, e adapted from Michalik et al., 2004, reproduced with permission by Nature Publishing Group). Image (c) courtesy of the Department of Ophthalmology, University of Cape Town.
1.4.3 Molecular pathogenesis of the SCA7 retinal phenotype

1.4.3.1 Expression and localisation of ATXN7

Early studies investigating the spatial expression of the ATXN7 protein revealed a widespread distribution across multiple cell and tissue types, including striated muscle, lung, liver, kidney, testis and the thyroid gland (Cancel et al., 2000; David et al., 1997). Subsequent studies have extended the expression profile of the protein (Figure 4), confirming that ATXN7 expression is not limited to regions of SCA7 pathology.

At the cellular level, ATXN7 has been found to be localised within both the cytoplasm and the nucleus of transfected cells and post-mortem tissues (Einum et al., 2001; Jonasson et al., 2002; Kaytor et al., 1999). Analysis of brain tissue from unaffected individuals and SCA7 patients demonstrated intense nuclear staining in cerebellar Purkinje cells, with more diffuse expression in the cytoplasm (Einum et al., 2001). Within the retina, ATXN7 was found in cell bodies within the INL and ONL (bipolar and photoreceptor cells), and ganglion cells. High expression levels of ATXN7 were found within cone cell inner and outer segments, with less intense staining in cone body cytoplasm and rod cell dendrites (Einum et al., 2001).

The presence of nuclear inclusions is considered a hallmark of polyglutamine repeat diseases, however their direct role in pathogenesis has been questioned (reviewed in Rudnicki and Margolis, 2003). Immunohistochemical analysis of SCA7 patient
brain and retinal tissue revealed the presence of nuclear inclusions within affected cell types, which were absent in an unaffected control individual (Mauger et al., 1999). The inclusions contained both wild-type and mutant ATXN7 protein, along with ubiquitin. Subsequent studies have revealed multiple additional proteins within these inclusions, including heat shock proteins, transcription factors and components of the proteasome (Zander et al., 2001), although more thorough analyses of additional neuronal cells demonstrated that inclusions were not limited to areas of the brain affected by neuronal loss (Holmberg et al., 1998; Jonasson et al., 2002). Together, these studies have revealed multiple potential roles of the inclusions, both in protection and pathogenesis. Sequestration of the mutant proteins within the inclusions may lessen their harmful effects, but additional recruitment of protective proteins and key transcription factors may ultimately contribute to pathology.

1.4.3.2 ATXN7 and retinal degeneration

The reason for the selective degeneration of specific retinal cell populations was unclear until 2001, when La Spada and colleagues produced transgenic mice expressing ATXN7 in the retina and central nervous system (CNS), with either 24 or 92 glutamines (La Spada et al., 2001). Mice expressing the expanded ATXN7 construct developed cone-rod dystrophy and lost visual function over time, implicating a degenerative rather than developmental process. Yeast two-hybrid assays revealed an interaction between ATXN7 and the cone-rod homeobox protein (CRX) (Mohan et al., 2014b). CRX is a nuclear transcription factor that plays a essential role in the differentiation of vertebrate photoreceptors, and controls a transcriptional network including photoreceptor-specific genes such as rhodopsin and the colour opsins (Chen et al., 1997; Livesey et al., 2000). La Spada demonstrated that expanded ATXN7 suppressed transcriptional transactivation by CRX, resulting in reduced expression of four tested CRX target genes (blue cone opsin, cone arrestin, rod-α-transducin and rhodopsin). Together, these results suggested that retinal pathogenesis in SCA7 patients may be due to an aberrant interaction between expanded ATXN7 and CRX, resulting in transcriptional dysfunction in photoreceptors.
Following various yeast studies, Helmlinger and colleagues showed that ATXN7 formed part of two complexes, the TATA-binding protein-free TAF-containing complex (TFTC) and the SPT3/TAF9/GCN5 acetyl transferase complex (STAGA) (Helmlinger et al., 2004b). Both complexes contain the histone acetyltransferase (HAT) called GCN5 (also known as K(lysine) acetyltransferase 2A, or KAT2A), and act as transcriptional activators by acetylating histone H3 (Helmlinger et al., 2006a) (Figure 5). In transgenic mice expressing mutant ATXN7 in rod photoreceptors (90 glutamines), H3 histones were hyperacetylated. Surprisingly, this chromatin decondensation in rod photoreceptors corresponded with down-regulation of associated genes, including rhodopsin (RHO) and CRX (Helmlinger et al., 2006a). These expression results were confirmed in an additional microarray-based study (Abou-Sleymane et al., 2006). Although the composition and HAT activity of the TFTC/STAGA complexes was not affected by the presence of mutant ATXN7, it was found that their recruitment to rod-specific promoters was dysregulated. The reason for downregulation of genes was unclear, leading to the hypothesis that additional downstream activities may be also dysregulated, such as the formation of pre-initiation complexes or disruption of RNA Pol II activity. The exact effects of mutant ATXN7 on TFTC/STAGA composition and activity remain to be elucidated, particularly in affected patient-derived cells. However, it has been acknowledged that transcriptional dysregulation is a key feature associated with the disease (Chou et al., 2010; Ström et al., 2005).
18

Figure 5: The involvement of ATXN7 in transcription of retinal genes. ATXN7 forms part of the STAGA transcriptional activator complex, which possesses HAT activity through GCN5. Interaction with transcription factors such as CRX results in acetylation of histone H3, resulting in transcription of retinal genes. Adapted from Palhan et al., (2005), with permission by the National Academy of Sciences of the United States of America.

1.5  In vitro and in vivo modelling of SCA7

1.5.1  Cell-based models

Investigations into the cellular and molecular pathology of SCA7 have been challenging. This is mostly due to the lack of relevant patient-derived tissue for in vitro studies, including affected cell types such as neurons and photoreceptors. It is possible to culture adult retinal neurons from post-mortem tissue (Gaudin et al., 1996; Romano and Hicks, 2007), however the logistics of establishing these cultures within 48 hours after death is problematic. Furthermore, the post-mitotic nature of the mature neurons results in a limited amount of biological material from each patient, since the cells can not be expanded in culture.

Some efforts have focused on utilising other cell types from SCA7 patients, such as dermal fibroblasts or Epstein-Barr virus (EBV)- transformed lymphoblast cell lines (LCLs) (Helmlinger et al., 2004b; Sopher et al., 2011; Tsai et al., 2005). While the use of these patient-derived cells types is advantageous since they contain SCA7 patient genomic material, these cells are unaffected by SCA7 pathology, therefore they may not be suitable for understanding the diseased state. This is further complicated by the fact that primary cells such as fibroblasts have a
limited culture lifespan \textit{in vitro}, and gene expression profiles in LCLs may be altered by the EBV transformation process (Çalışkan et al., 2011).

The more common methods of \textit{in vitro} study have used established primate- or human-derived established cell lines, either transfected with the ATXN7 protein (full length or truncated) or manipulated to express the mutant or wild-type transcript (Table 5). Animal-derived cell lines such as COS-1 and -7 (African green monkey kidney fibroblasts), NIH-3T3 (Swiss mouse embryo fibroblasts) or PC12 (derived from rat adrenal medulla) lack a human genetic background, and are likely to express a species-specific form of ATXN7. Other human cell lines are derived from cancer cells (such as HeLa cervical cancer cells or SK-N-SH neuroblastoma cells) and may exhibit associated genomic alterations such as aneuploidy. Whilst these studies have made valuable contributions to the knowledge of wild-type and mutant ATXN7 function (Table 5), one can not ignore the potentially compounding effects of the underlying genomic composition of the cell.
Table 5: Cell models of SCA7.

<table>
<thead>
<tr>
<th>Cell line symbol</th>
<th>Cell line full name/description</th>
<th>Details</th>
<th>Experiments/findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS-1</td>
<td>African green monkey kidney fibroblast-like cell line</td>
<td>Transfected with truncated ATXN7 cDNA constructs with 10 or 60 CAG repeats.</td>
<td>Are TFC/STAGA subunits recruited into nuclear inclusions by mutant ATXN7?</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td>COS-7</td>
<td>African green monkey kidney fibroblast-like cell line</td>
<td>Transiently transfected with ATXN7 (10Q or 72Q).</td>
<td>SUMOylation attenuates the aggregation propensity and cellular toxicity of the polyglutamine expanded ATXN7</td>
<td>(Janer et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transfected with tagged full-length ATXN7 with 10Q or 100Q.</td>
<td>ATXN7 interacts with a Cbl-associated protein that it recruits into neuronal intranuclear inclusions</td>
<td>(Lebre et al., 2001)</td>
</tr>
<tr>
<td>FIB</td>
<td>Patient- and control-derived human dermal fibroblasts</td>
<td>Derived from individuals with 15 or 55Q.</td>
<td>Analysis of ATXN7 expression</td>
<td>(Sopher et al., 2011)</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney 293 cells</td>
<td>FLAG-tagged full length ATXN7 with 10 or 60 glutamines.</td>
<td>Normal and mutant ATXN7 interacts with TRRAP, GCN5 and TAF10</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transfected with truncated ATXN7 cDNA constructs with 10 or 60 CAG repeats.</td>
<td>Are TFC/STAGA subunits recruited into nuclear inclusions by mutant ATXN7?</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transfected with expression plasmids containing full length ATXN7 cDNA with 10Q or 100Q.</td>
<td>Design of RNAi Hairpins for Mutation-Specific Silencing of ATXN7</td>
<td>(Scholefield et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressing normal (10Q) and mutant (100Q) full-length &amp; truncated forms of ATXN7 with tags or GFP.</td>
<td>In SCA7 cells proteins are recruited in inclusions &amp; caspase-3 is activated.</td>
<td>(Zander et al., 2001)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cervical cancer cell line</td>
<td>Transfected with truncated ATXN7 cDNA constructs with 10 or 60 CAG repeats.</td>
<td>Are TFC/STAGA subunits recruited into nuclear inclusions by mutant ATXN7?</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transiently transfected with ATXN7 (10Q or 72Q).</td>
<td>SUMOylation attenuates the aggregation propensity and cellular toxicity of the polyglutamine expanded ATXN7</td>
<td>(Janer et al., 2010)</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Description</td>
<td>Treatment and Expression</td>
<td>Results and Findings</td>
<td>References</td>
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<tr>
<td>LCL</td>
<td>Epstein-Barr virus transformed lymphoblastoid cells</td>
<td>Derived from SCA7 patient (51Q) and unaffected control (10Q).</td>
<td>Normal and mutant ATXN7 interacts with TRRAP, GCN5 and TAF10</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td></td>
<td>Isolated from patients (41Q and 100Q) and unaffected controls.</td>
<td>Decreased expression of HSP27 &amp; 70 in SCA7 patient LCLs</td>
<td></td>
<td>(Tsai et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Derived from individuals with 10, 51, 59 or 66Q.</td>
<td>Analysis of ATXN7 expression</td>
<td></td>
<td>(Sopher et al., 2011)</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>Swiss mouse embryo fibroblast cell line</td>
<td>Transfected with truncated ATXN7 cDNA constructs with 10 or 60 CAG repeats.</td>
<td>Are TFFC/STAGA subunits recruited into nuclear inclusions by mutant ATXN7?</td>
<td>(Helmlinger et al., 2004b)</td>
</tr>
<tr>
<td>PC12</td>
<td>Derived from pheochromocytoma of the rat adrenal medulla. Can be induced to a neural phenotype by the addition of nerve growth factor.</td>
<td>Tetracycline-regulated expression of HA-tagged full-length ATXN7 Q10 or Q100.</td>
<td>ATXN7 Q100 forms aggregates in PC12 cells, co-immunoprecipitates with APLP2.</td>
<td>(Takahashi-Fujigasaki et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline-regulated expression of N-terminal FLAG &amp; C-terminal GFP ATXN7 with 10Q or 65Q.</td>
<td>Expanded ATXN7 causes toxicity by inducing ROS production from NADPH oxidase complexes.</td>
<td>(Ajayi et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline-regulated expression of N-terminal FLAG &amp; C-terminal GFP ATXN7 with 10Q or 65Q.</td>
<td>Inhibition of Autophagy via p53-Mediated Disruption of ULK1.</td>
<td>(Yu et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline-regulated expression of N-terminal FLAG &amp; C-terminal GFP ATXN7 with 10Q or 65Q.</td>
<td>Differential degradation of full-length &amp; cleaved ATXN7 fragments.</td>
<td>(Yu et al., 2012)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Human Neuroblastoma cell line subcloned from SK-N-SH.</td>
<td>Expressing normal (10Q) and mutant (100Q) full-length &amp; truncated forms of ATXN7 with tags or GFP.</td>
<td>In SCA7 cells proteins are recruited in inclusions &amp; caspase-3 is activated.</td>
<td>(Zander et al., 2001)</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>Human Neuroblastoma cell line</td>
<td>Transfected with constructs expressing tagged truncated or full-length ATXN7 (10Q or 65Q).</td>
<td>The role of wild-type and expanded ATXN7 in transcriptional regulation.</td>
<td>(Ström et al., 2005)</td>
</tr>
</tbody>
</table>
1.5.2 Model organisms

Studies showing that the ATXN7 yeast orthologue, SaGa associated Factor 73 kDa (Sgf73) was a component of the SAGA (Spt/Ada/Gcn5 acetylase) complex lead to the central discovery that human ATXN7 formed part of the mammalian equivalent, STAGA (Helmlinger et al., 2004b; Sanders et al., 2002). This functional homology between human and yeast ATXN7 has proven to be particularly useful, since the yeast genome and biology is well documented, and in vitro studies can be conducted with relative ease. Investigations of Sgf73 have provided valuable insights into the functioning of wild-type and mutant ATXN7 (Köhler et al., 2008; McMahon et al., 2005). While yeast can aid in modelling pathways and mechanisms in neurodegenerative diseases such as HD and Parkinson's, this simple organism fails to recapitulate the more advanced processes involved in the pathophysiology of these disorders (Miller-Fleming et al., 2008).

The complex nervous system of *Drosophila melanogaster* has been beneficial to the study of neurodegenerative diseases, including SCA7. A conditional *Drosophila* model of SCA7 expressing truncated mutant ATXN7 in neurons showed nuclear inclusions along with reduced locomotion and shortened lifespan (Latouche et al., 2007). More recently, Mohan et al., (2014) demonstrated that loss of endogenous ATXN7 expression in Drosophila resulted in neural and retinal degeneration, suggesting that loss of wild-type function may contribute of disease pathogenesis (Mohan et al., 2014b). The zebrafish homologue of ATXN7 was characterised by Yanicostas and colleagues (Yanicostas et al., 2012). Oligonucleotide-mediated knock-down of ATXN7 resulted in gross developmental abnormalities, indicating the requirement for the wild-type protein in differentiation of Purkinje cells, granule cells and photoreceptors.

The use of transgenic mice has become a popular strategy for the study of SCA7 pathogenesis. Multiple SCA7 mouse models exist (Table 6), with the majority expressing wild-type or expanded ATXN7 under the control of a promoter specific to the brain and/or retina. Yvert and colleagues developed a mouse model designed to overexpress mutant ATXN7 containing 90 glutamines, under the control of the RHO promoter (Yvert et al., 2000). These mice developed progressive retinal
degeneration from 4 weeks of age, and exhibited ubiquitinated nuclear inclusions containing a cleaved N-terminal fragment of ATXN7, along with proteasome subunits. It was also shown that rod photoreceptors in these mice developed abnormal morphologies, with a subset of cells migrating into other retinal layers (Yefimova et al., 2010). Rods also surrendered to apoptotic and non-apoptotic cell death. The SCA7 knock-in mouse developed by Yoo and colleagues contained human ATXN7 exon 3 with 266 CAG repeats, and recapitulated many of the symptoms associated with the disease, such as ataxia, retinopathy and premature death (Yoo et al., 2003). Since SCA7 is progressive disease that advances over a course of decades in humans, successful modelling of the degenerative process may prove difficult in mice (and other models) that have a relatively short lifespan (Ingram et al., 2012). Therefore it may be necessary to utilise mice over-expressing ATXN7 with a large expansion, such as 92 or 266 glutamines (La Spada et al., 2001; Yoo et al., 2003), in order to produce detectable phenotypes.

There are a number of distinct differences between the mouse and human eye, which are of particular relevance to the study of the pathophysiology in SCA7 (reviewed in Swaroop et al., 2010). The retinal degeneration associated with SCA7 is classified as a cone-rod dystrophy, since dysfunction progresses from the central region of the eye (fovea) towards the periphery (Aleman et al., 2002). In mice, rod photoreceptors outnumber cone cells by 30:1, which is significantly different from the ratio of 18-20:1 as seen in humans. Additionally, most mammals (including mice) have two types of cone opsin (S opsin and M opsin), whilst humans have a third cone opsin (L opsin) which confers trichromatic colour vision. Mouse cone cells can express both opsins, but human cone cells are capable of expressing only one type of opsin. These differences in murine and human eye anatomy and physiology may result in differences in developmental and disease-related studies. In most cases, transgenic mice retain full expression of the normal murine homolog of the gene of interest, creating further complications in disease-modelling. Recently, the first "fully humanised" transgenic mouse model of HD has been developed, which contains two human huntingtin (HTT) genes (heterozygous for the pathogenic expansion), with no mouse Hdh genes (the murine homolog of HTT)
(Southwell et al., 2012). The mice displayed many of the expected disease-associate features, including motor and cognitive defects. It is likely that similar models for other neurological conditions will be valuable for in vivo investigations and testing of gene-based therapies.
Table 6: Mouse models of SCA7 (adapted from Figiel et al., 2012)

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
<th>General phenotype</th>
<th>Retinal phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7E</td>
<td>90Q under Pcp2 promoter, expressed in PCs</td>
<td>Defects in motor performance &amp; learning. ATXN7 translocates to nucleus and forms large aggregates. Degeneration of PCs and deep cerebellar nuclei.</td>
<td></td>
<td>(Helmlinger et al., 2004a; Yvert et al., 2000; Yvert et al., 2001)</td>
</tr>
<tr>
<td>R7E</td>
<td>90Q under Rho promoter, expressed in retina</td>
<td>Nuclear inclusion formation, photoreceptor dysfunction, neurodegeneration. Normal lifespan.</td>
<td>Inclusions in ONL, changes in gene expression, neuronal layer disorganisation, decrease in neuronal layer thickness, photoreceptor dysfunction.</td>
<td>(Helmlinger et al., 2006a; Yefimova et al., 2010; Yvert et al., 2000)</td>
</tr>
<tr>
<td>Gfa2-SCA7-90Q</td>
<td>90Q under Gfa2 promoter, limited expression in astrocytes including Bergmann glia.</td>
<td>Ataxia phenotype, Purkinje cell degeneration, abnormal gait, poor co-ordination. No premature death. Nuclear inclusions in Bergmann glia. Decreased glutamate uptake. Normal lifespan.</td>
<td></td>
<td>(Custer et al., 2006)</td>
</tr>
<tr>
<td>Prp-SCA7-c92Q</td>
<td>Expresses human ATXN7 cDNA with 92Q</td>
<td>Small body, premature death. Ataxia, retinopathy, motility dysfunction, degeneration of PCs and Bergmann glia.</td>
<td>Cell loss, nuclear inclusions, decrease in neuronal layer thickness, photoreceptor dysfunction, visual impairment.</td>
<td>(Clarke et al., 2007; Garden et al., 2002; Grote and La Spada, 2003; La Spada et al., 2001)</td>
</tr>
<tr>
<td>Knock-in SCA7 266Q</td>
<td>Insertion of human exon 3 of ATXN7 with 266Q</td>
<td>Rapid progression, ataxia, myoclonic seizures, retinopathy, infertility, premature death. Decrease in size of PCs. Nuclear inclusions in glial cells, PCs, cones, rods, retinal ganglion cells.</td>
<td>Cell loss, abnormal morphology, nuclear inclusions, gene expression changes, decrease in neuronal layer thickness, photoreceptor dysfunction.</td>
<td>(Noma et al., 2012; Yoo et al., 2003)</td>
</tr>
<tr>
<td>B7E2; ataxin 7- Q52</td>
<td>Expresses human ATXN7 cDNA under PDGF promoter (neuronal expression).</td>
<td>Balance and gait alterations, alteration of neuronal morphology, aggregates</td>
<td></td>
<td>(Yvert et al., 2001)</td>
</tr>
<tr>
<td>PrP-floxed, SCA7- 92Q BAC</td>
<td>ATXN7 cDNA under PrP promoter (expression in central nervous system).</td>
<td>Balance and coordination alterations, loss of glial processes, decrease in neuronal layer thickness.</td>
<td></td>
<td>(Furrer et al., 2011; Ramachandran et al., 2014a; Ramachandran et al., 2014b)</td>
</tr>
</tbody>
</table>

*Q = glutamines
1.6 Stem cells as in vitro disease models

1.6.1 Induced pluripotent stem cells

In 2006 Takahashi and colleagues reported the first case of induction of pluripotency in mouse fibroblast cultures (Takahashi and Yamanaka, 2006). Through the introduction of four key embryonic transcription factors, namely *POU domain class 5 transcription factor 1* (OCT3/4), *SRY-box containing gene 2* (SOX2), *Kruppel-like factor 4* (KLF4) and *myelocytomatosis oncogene* (c-MYC), somatic cells could be reprogrammed to an embryonic stem cell-like state. These induced pluripotent stem cells (iPSCs) possessed all the key molecular and phenotypic hallmarks of embryonic stem cells (ESCs), such as morphology, expression of pluripotency markers and *in vitro* and *in vivo* differentiation capacity. These findings were soon replicated using human dermal fibroblasts (Takahashi et al., 2007). Dermal fibroblasts obtained from a skin biopsy are the most common somatic donor cell type for reprogramming experiments, although additional cell types such as B cells, melanocytes and keratinocytes have been successfully reprogrammed (Robinton and Daley, 2012).

The discovery of iPSC technology has been hailed as one of the most important breakthroughs in molecular and cellular biology, since it can allow for patient-specific disease modelling *in vitro*, and derived cells may be used for future therapeutic transplantations (Figure 6). The iPSCs can be maintained indefinitely in culture under the appropriate conditions, or various differentiation protocols may be used (usually through the introduction of growth factors) to induce differentiation into the cell type of choice. Through the differentiation of iPSCs into cell types such as neurons, hepatocytes or cardiomyocytes, multiple diseases have been effectively modeled *in vitro*, including HD, Duchenne muscular dystrophy and Retinitis pigmentosa (Robinton and Daley, 2012).
Figure 6: Medical applications of iPSCs. Reprogramming technology and iPSCs have the potential to be used to model and treat human disease. In this example, the patient has a neurodegenerative disorder. Patient-specific iPSC cells — in this case derived by ectopic co-expression of transcription factors in cells isolated from a skin biopsy — can be used in one of two pathways. In cases in which the disease-causing mutation is known (for example, familial Parkinson’s disease), gene targeting could be used to repair the DNA sequence (right). The gene-corrected patient-specific iPSCs would then undergo directed differentiation into the affected neuronal subtype (for example, midbrain dopaminergic neurons) and be transplanted into the patient’s brain (to engraft the nigrostriatal axis). Alternatively, directed differentiation of the patient-specific iPSCs into the affected neuronal subtype (left) will allow the patient’s disease to be modelled in vitro, and potential drugs can be screened, aiding in the discovery of novel therapeutic compounds. From Robinton and Daley (2012), reproduced with permission by the Nature Publishing Group.

1.6.2 iPSC-based models of polyglutamine disease
Several studies have attempted to use iPSCs and their differentiated derivatives to study polyglutamine diseases. In a study comparing iPSCs and differentiated cells from a single SCA2 patient and unaffected control, it was shown that patient-derived iPSCs underwent abnormal neural stem cell rosette formation, and the resulting NSCs expressed less ATXN2 than the control-derived cells (Xia et al., 2012).
These results indicated that decreased expression of ATXN2 may play a role in pathogenesis, particularly during neuronal maturation.

In a neuronal cell model derived from SCA3 patient iPSCs, it was shown that SDS-insoluble aggregates of ATXN3 were formed in patient neurons, but not neurons from healthy controls (Koch et al., 2011). The iPSC-derived model system enabled the investigators to confirm that the protease calpain was responsible for the cleavage and aggregation of the ATXN3 protein, since calpain inhibition abolished the phenotype. Researchers at the National Institute of Neurological Disorders and Stroke (part of the National Institute of Health in the USA) have recognised the value of iPSC-based research, and have formed three iPSC consortia to facilitate studies on Parkinson's Disease, Amyotrophic lateral sclerosis and HD. In a large-scale study studying 14 iPSC lines from HD patients and controls, numerous differences were noted in gene and protein expression patterns, cellular physiology and neuronal phenotypes (HD Consortium, 2012). These studies have demonstrated the potential utility of iPSC-derived cells for the in vitro modelling of polyglutamine disease, as well as their value in testing potential therapies.

A single study has thus far used iPSCs as an in vitro model for SCA7 (Luo et al., 2012). Fibroblasts from a 56-year old SCA7 patient (with 45 CAG repeats) were reprogrammed retrovirally to create iPSCs. Following characterisation, the iPSCs were subjected to a general neuronal differentiation protocol, and were shown to be capable of differentiating into cells positive for nestin and β-III tubulin. Although a human ESC line was used for comparison, no downstream experiments were used to determine whether the SCA7 iPSC line or its derivates exhibited any abnormal cellular or molecular phenotypes.

### 1.6.3 iPSC-based models of retinal disease

A multitude of studies have investigated the use of iPSCs for the investigation of retinal diseases (Tucker et al., 2014). The vast majority of these are exploring the use of iPSC-derived retinal cells (usually RPE cells) for therapeutic purposes, rather than for in vitro modelling. The eye lends itself as a model system for the advancement of stem cell-based replacement therapies due to its surgical
accessibility, low immune response and small size (therefore fewer cells are required for a meaningful effect) (Tucker et al., 2014). Furthermore, it can be imaged non-invasively (Cramer and MacLaren, 2013). The US National Institutes of Health clinical trials registry lists numerous trials to study the safety and tolerability of human embryonic stem cell derived RPE implantations in Stargardt's macular dystrophy and age related macular degeneration patients (ClinicalTrials.gov). Numerous other types of stem cells are being considered for therapeutic purposes, including adult RPE stem cells and limbal stem cells (Blenkinsop et al., 2012; Stern and Temple, 2014). To date, only a single study by Masayo Takahashi and colleagues from the RIKEN Center for Developmental Biology (Japan) is exploring the use of iPSC-derived RPE for transplantation (Cyranoski, 2013; Kamao et al., 2014).

Photoreceptors derived from iPSCs are still being tested for transplantation efficacy in mice and other mammals. Zhou and colleagues demonstrated that swine iPSC-derived photoreceptors could integrate into the swine neural retina, following treatment with iodoacetic acid to remove rod photoreceptors (Zhou et al., 2011). Within three weeks after the subretinal injection, approximately 1% of the rhodopsin-expressing donor cells had integrated into the outer nuclear layer at the injection site, and showed outer segment-like projections. Others have shown that mouse iPSC-derived photoreceptor precursors can engraft into the retina and partially restore retinal function (Tucker et al., 2011). Although these studies have demonstrated the proof-of-principle of transplantation of iPSC-derived cells into the neural retina, significant concerns regarding the reprogramming method, differentiation process and purification procedure have been raised (Cramer and MacLaren, 2013). If patient-specific iPSCs were to be used in cases of inherited diseases, the underlying genetic mutations would require correction prior to transplantation, which would introduce an additional experimental procedure to be accounted for (Tucker et al., 2014).

A limited number of studies have utilised iPSC-derived photoreceptors for in vitro disease modelling (Table 7). Jin et al. (2011) generated iPSC lines from five RP patients with mutations in RP1, RP9, PRPH2 or RHO (retinitis pigmentosa 1, retinitis
pigmentosa 9, peripherin 2 or rhodopsin) (Jin et al., 2011). It was shown that rod photoreceptors differentiated from the iPSC lines were capable of normal electrophysiological function, however they underwent degeneration in vitro when compared to cells derived from control samples, as well as other retinal cells types such as cone photoreceptors and bipolar cells. Furthermore, in cells derived from patients with a RP9 mutation, it was found that a subset of cells co-expressed cytoplasmic 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. Since the antioxidant α-Tocopherol has been suggested as a dietary therapy for RP, the authors investigated the effects of α-Tocopherol treatment on the survival of differentiated rod photoreceptors. The treatment significantly increased the number of surviving rod photoreceptors in the cultures derived from RP9 patients, but had no effect on the cells derived from RP patients with other mutations, demonstrating the potential utility of this model system in the testing of prospective therapies. The authors went on to refine their approach in a separate study, by using the integration-free Sendai virus to deliver the reprogramming factors (Jin et al., 2012). The rod photoreceptors differentiated from a patient with RHO mutations exhibited markers of endoplasmic reticulum stress, consistent with previous studies.

Tucker and colleagues neatly demonstrated the utility of the iPSC modelling approach in a patient with autosomal recessive RP (Tucker et al., 2013). Following next-generation sequencing to identify the disease-causing mutations in USH2A, the authors used iPSC-derived photoreceptors to show that one of the mutations causes exonification of an intron, a translation frameshift and a premature stop codon. Furthermore, western blots revealed that the other mutation caused endoplasmic reticulum stress. The differentiated cells were capable of integrating into the retina of retinal degenerative mice, indicating that degeneration in these cells was occurring via a post-developmental process.

Although they are limited in number, these studies have adequately demonstrated the potential utility of iPSC-derived retinal cells in the study of retinal degenerative diseases. However, some refinement in experimental strategy is required in order
to address the issues of differentiation efficiency, cell population purity and methods of characterisation.

Table 7: Studies utilising iPSC-derived photoreceptors for *in vitro* disease modelling.

<table>
<thead>
<tr>
<th>Patient details</th>
<th>Reprogramming method</th>
<th>Differentiation method</th>
<th>Experiments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 RP patients from 4 families</td>
<td>Retroviral transduction of OCT3/4, SOX2, KLF4, c-MYC in dermal fibroblasts.</td>
<td>Floating culture, treated with LEFTY2 and DKK1. Followed by adherent culture, treated with retinoic acid &amp; taurine. Treated with α-tocopherol, ascorbic acid, and β-carotene from day 120-127.</td>
<td>Cells infected with virus containing GFP under NRL or RHO promoter (for sorting). Immunocytochemistry. Patch clamp experiments.</td>
<td>(Jin et al., 2011)</td>
</tr>
<tr>
<td>(mutations in RP1, PRPH2, RHO, RP9)</td>
<td></td>
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<tr>
<td>1 RP patient with a RHO mutation.</td>
<td>Sendai virus infection of OCT3/4, SOX2, KLF4, c-MYC in dermal fibroblasts.</td>
<td>Floating culture, treated with LEFTY2 (or SB-431542) and DKK1 (or CKI-7). Followed by adherent culture, treated with retinoic acid &amp; taurine.</td>
<td>Immunocytochemistry.</td>
<td>(Jin et al., 2012)</td>
</tr>
<tr>
<td>1 RP patient with a USH2A mutation.</td>
<td>Sendai virus infection of OCT3/4, SOX2, KLF4, c-MYC in keratinocytes.</td>
<td>Floating aggregates treated with NOG, DKK1, IGF-1, bFGF, DAPT.</td>
<td>Immunocytochemistry qPCR Western blot analysis Next-generation DNA sequencing Transplantation into mice.</td>
<td>(Tucker et al., 2013)</td>
</tr>
</tbody>
</table>
1.7 **Aim and objectives:**

Although SCA7 is relatively rare worldwide, SA has a high prevalence of the disease, and the affected population is genetically homogenous. Previous studies have highlighted four questions/areas which can be addressed through the investigation of this unique cohort.

**Broad aim:**

Through the accomplishment of the following objectives, it was sought to improve the molecular diagnosis and clinical management of South African SCA7 patients, and to develop an appropriate *in vitro* model for future studies.

**Objectives:**

1. To provide an update of the current estimates of the prevalence and frequencies of the polyglutamine Spinocerebellar ataxias in southern Africa.

2. To determine whether the South African SCA7-associated haplotype is present in additional SCA7 families from other African countries.

3. To develop an improved approach for the molecular diagnostic testing for SCA7, for implementation into the routine testing protocol at the National Health Laboratory Services.

4. To investigate and evaluate the utility of an iPSC-derived model of retinal degeneration associated with SCA7.
Chapter 2: The spectrum & frequency of the polyglutamine spinocerebellar ataxias in southern Africa
Note: An early form of the results included in this chapter were previously published in the South African Medical Journal (Smith et al., 2012). Sections of the article have been reproduced with permission from the co-authors and publisher.

2.1 Introduction

2.1.1 Testing of the polyglutamine SCAs in South Africa

Patients with symptoms of Spinocerebellar ataxia have been assessed at Groote Schuur Hospital in Cape Town, South Africa, for many years. From 1972, the details of these families were recorded within the Division of Human Genetics at the University of Cape Town (UCT). In 1987 a paper-based filing system was implemented (later changed to an electronic database). The first family recorded in this database contained individuals with clinical symptoms associated with SCA1; however, a molecular diagnosis could only be offered once the pathogenic mutation was identified in 1993 (Orr et al., 1993). The first individuals with SCA2, SCA3 and SCA6 were recruited to the Division in 1999, 2002 and 1995 respectively. The first family to receive a molecular diagnosis of SCA7 at Groote Schuur Hospital was tested in 1999, two years after the ATXN7 gene was identified (David et al., 1997).

In 1994 Dr Alan Bryer completed a PhD thesis based on the investigation of the polyglutamine SCAs in South Africa, with a specific focus on the Western Cape region. Later, this study was extended to other parts of SA, and the findings of this broader investigation were published in 2003 (Bryer et al., 2003). This was the first report on an estimation of the occurrence of the SCAs in SA. A total of 54 families from around SA were subjected to a clinical and molecular investigation (234 individuals, 132 of which were affected). Ten of these families did not carry any of the five mutations that were tested (SCA1, SCA2, SCA3, SCA6, SCA7). Half of the SCA-positive families had the SCA1 genotype (Figure 7), whereas 12 families were diagnosed with SCA7. Seven families carried the SCA2 mutation, however only 3 families were found to have SCA3 or SCA6 (2 and 1 respectively).
These results were somewhat surprising, since the frequencies of SCA1 and SCA7 were higher than had previously been reported elsewhere, and the percentages of SCA2 and SCA3 families were lower than expected. The reasons for these differences in distribution and frequency were unclear, although it was thought that it could be attributed to the unique and heterogeneous populations present in SA, which contrasted with the largely European- and Asian-based populations from other studies. A high proportion of the SA SCA1 patients were within the Mixed Ancestry population group (also referred to as "coloured"), which describes a population unique to SA, containing an admixture of European, Khoesan, Asian and black African ancestries (de Wit et al., 2010). In 1997 a molecular investigation revealed that two distinct haplotypes were present in the SA SCA1 Mixed Ancestry population (Ramesar et al., 1997), suggestive of two independent origins of the mutation in that group.

2.1.2 The South African SCA7-associated haplotype

An additional noteworthy finding from the report by Bryer revealed that all the SA SCA7 patients identified were of black African ancestry. Although SCA7 patients of African American ancestry had been previously described (Benton et al., 1998; Gouw et al., 1995), the only other population showing a similarly high proportion of
SCA7 patients was in Scandinavia, where a SCA7 founder effect had been reported (Jonasson et al., 2000). Following from these studies, the SA SCA7 patient population was investigated for the presence of a similar founder effect. In 2006 Greenberg and colleagues studied five extended SA SCA7 families and constructed a disease-associated haplotype consisting of two microsatellite markers and a transcribed SNP flanking the *ATXN7* expansion (Greenberg et al., 2006). The authors proposed that a founder effect existed within this patient population, and suggested that any black African SCA patients be tested for SCA7 first in any molecular investigations. The SA SCA7-associated haplotype has yet to be investigated in any additional countries. SCA7 patients from other African countries are occasionally referred to the NHLS for molecular confirmatory testing, and it was postulated that the SA SCA7-associated haplotype may be shared amongst these families.

Of all the SCA7 patients tested during the SA haplotype study, 43% were found to be heterozygous for the included SNP (rs3774729). This finding formed the foundation for a later study, which aimed to develop an allele-specific RNAi-based therapy for South African SCA7 patients (Scholefield et al., 2009). RNAi-based therapies utilise the endogenous pathway that exists within cells that functions to fine-tune gene expression (Figure 8). Short hairpin RNAs (shRNAs), short interfering RNAs (siRNAs) or microRNA (miRNA) mimics are exogenously introduced into the cell. Each molecule is designed to complement an endogenous mRNA target within the cell, and through the exploitation of the cellular RNAi machinery the exogenous RNAi effector can bring about cleavage or translational repression of the target mRNA (Figure 8).
Figure 8: Overview of the endogenous and exogenous RNAi pathways. During the endogenous RNAi process, RNA is transcribed from a microRNA (miRNA) or mirtron locus, and folds to form primary miRNA (pri-miRNA) molecules, or a mirtron lariat. These are processed by the endonuclease Drosha to yield pre-miRNAs. After exportin 5-mediated transport into the cytoplasm, the hairpin structure is removed by the Dicer, to yield a short double-stranded RNA molecule. The RNA-induced silencing complex (RISC) is directed by the 'guide' strand of the RNA complex towards the targeted complementary RNA. A complete base-pairing to this RNA results in cleavage and degradation of the target mRNA, whereas partial complementarity results in translational repression. Exogenous therapeutic effectors can enter the pathway at various levels, as miRNA mimics, short hairpin RNAs (shRNAs) or short interfering RNAs (siRNAs). Adapted from Smith et al., (2013).

With dominant disorders such as SCA7, a common RNAi-based strategy involves the design of RNAi effectors capable of selectively targeting the mutant allele, thus permitting the wild-type allele to continue to perform its normal cellular function. Whilst it may be possible to target the mutant allele alone based on the pathogenic CAG expansion, this method is beset with technical challenges, since adequate discrimination between alleles is required, and the prevention of off-target effects may be problematic. Therefore a common approach to achieve allele-specific silencing utilises a SNP linked to the pathogenic mutation, since SNPs are more amenable to targeting by RNAi effectors. Since the SNP rs3774729 had been found to be linked to the pathogenic SCA7 mutation in SA patients, Scholefield and colleagues were able to design RNAi hairpins to selectively target the A allele of the SNP in an artificial cell model (Figure 9). These findings were later confirmed in SCA7 patient-derived fibroblast cells (Scholefield et al., 2014). Although an allele-
specific silencing approach is only possible in patients who are heterozygous for the targeted SNP, future research is likely to reveal whether both alleles of the gene may be silenced without deleterious effects. A murine knock-out model of SCA7 is yet to be produced in order to demonstrate the necessity of ATXN7 during development, however it has been shown that non-allele specific knock-down is well tolerated in a mouse model of SCA7 (Ramachandran et al., 2014a; Ramachandran et al., 2014b), as well as models of SCA3 and HD (Alves et al., 2010; Boudreau et al., 2009). Since the potential utility of the designed RNAi-based therapy relies on the geographical extent of the South African SCA7 haplotype, it would be beneficial to study SCA7 families from other African countries.

Figure 9: RNAi-based silencing therapy for SCA7. The A allele of rs3774729, linked to the pathogenic SCA7 CAG repeat expansion in the mutant ATXN7 mRNA, can be effectively targeted by an RNAi effector, thus preventing the production of the mutant protein. In heterozygous patients the G allele is not silenced, and the wild-type protein continues to be expressed.
2.1.3 Aims & Objectives

The aim of this study was to provide a broad update and overview of the state of the polyglutamine SCAs in southern Africa, to be achieved by addressing the following objectives:

1. To provide the updated spectrum & frequency of the polyglutamine Spinocerebellar ataxias in South Africa.

2. To investigate the presence or absence of the SA SCA7-associated haplotype in SCA7 families from other African countries.
2.2 Methods

2.2.1 The spectrum & frequency of the polyglutamine SCAs in South Africa.

Ethical approval for the study was obtained from the University of Cape Town Human Research Ethics committee (HREC REF 460/2010) and is renewed annually.

The electronic patient database within the Division of Human Genetics at UCT was interrogated in order to determine the number of individuals and families who had received a confirmed molecular diagnosis of SCA1, 2, 3, 6, 7, 12, or 17 from 1987 to the end of 2013. The molecular diagnostic tests for these individuals had been undertaken within the NHLS laboratory at Groote Schuur Hospital in Cape Town. The NHLS receives referrals from clinicians from all over South Africa, and is the only centre in Africa offering molecular diagnostic testing for the polyglutamine SCAs.

All DNA isolations, molecular testing and analysis was performed by a registered medical scientist within the NHLS. DNA was isolated from whole blood using the QIAmp DNA Blood mini kit (Qiagen). Thereafter 100 nanograms (ng) of DNA was utilised in a multiplex PCR reaction to test for the presence of pathological expansions at the loci responsible for SCA1, 2, 3, 6 and 7, as described by Dorschner et al. (2002). Primers flanking each CAG repeat region (Appendix 2 – Primer sequences, pg 171) were used in a PCR reaction as described by the authors, and PCR products were analysed by capillary electrophoresis on the ABI 3100 Genetic Analyzer (Applied Biosystems). The GeneScan 500 Rox size standard (Applied Biosystems) was used for size estimation of PCR products, and results were analysed using GeneMapper software (version 3, Applied Biosystems). Positive control samples were included in each test run. A size correction formula was used to calculate the repeat length of each allele of the five genes included in the assay (Dorschner et al., 2002). From 2007, a duplex PCR designed by the NHLS laboratory was used to test for SCA12 and SCA17, based on the assays published by Holmes and Fujigasaki (Fujigasaki et al., 2001; Holmes et al., 1999). Repeat sizes were calculated based on fragment sizes, and molecular diagnoses were reported based on the EMQN guidelines (Sequeiros et al., 2010a; Sequeiros et al., 2010b) (Table 8).
Table 8: Reference ranges for CAG repeat sizes at the main SCA loci (adapted from Sequeiros et al. (2010). Values indicate the number of CAG repeats.

<table>
<thead>
<tr>
<th>SCA type</th>
<th>Normal range</th>
<th>Uncertain</th>
<th>Reduced penetrance</th>
<th>Full penetrance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCA1</td>
<td>6-38;</td>
<td>-</td>
<td>-</td>
<td>39-44 uninterrupted; 45-91</td>
</tr>
<tr>
<td></td>
<td>39-44 CAT</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>interrupted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCA2</td>
<td>14-31</td>
<td>32-34</td>
<td>-</td>
<td>35-500</td>
</tr>
<tr>
<td>SCA3</td>
<td>11-44</td>
<td>45-59</td>
<td>-</td>
<td>61-87</td>
</tr>
<tr>
<td>SCA6</td>
<td>4-18</td>
<td>-</td>
<td>19</td>
<td>20-33</td>
</tr>
<tr>
<td>SCA7</td>
<td>4-19</td>
<td>28-33</td>
<td>34-35</td>
<td>36-460</td>
</tr>
<tr>
<td>SCA12</td>
<td>4-32</td>
<td>40-45</td>
<td>-</td>
<td>51-78</td>
</tr>
<tr>
<td>SCA17</td>
<td>25-42</td>
<td>-</td>
<td>43-48</td>
<td>49-66</td>
</tr>
</tbody>
</table>

For the database search, the CAG repeat genotype (size of both alleles) was noted for each SCA patient tested, along with their ethnicity (self- or clinician-reported, if available) and date of referral. Due to changes in referral, recording and reporting protocols spanning the 27 year period, this information was not always available for each patient. Paper-based records were used wherever possible to provide additional information.

2.2.2 Investigation of the South African SCA7-associated haplotype in African families

In order to determine whether the SA SCA7-associated haplotype existed within non-South African SCA7 families, each available SCA7 patient and any unaffected family members for whom DNA was available was genotyped at 3 loci: an intronic AC repeat between exons 1 and 2 (called "AC1"), a SNP (rs3774729), and a microsatellite marker (D3S1287) as previously reported by Greenberg et al. (2006) (Figure 10). Primers flanking each marker were utilised in a standard 25 µl PCR reaction containing 1x GoTaq buffer (Promega), 200 µM dNTPs, 0.4 µmol/L (µM) of each primer, 0.5U GoTaq polymerase (Promega) and 100ng DNA. For the AC1 and D3S1287 reactions the cycling conditions consisted of 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 40 seconds; and a single
cycle of 72°C for 7 minutes on the MultiGene thermocycler (Labnet International). The resulting PCR product was subjected to agarose gel electrophoresis to determine the success of the reaction (details in Appendix 4 – Molecular reagents and protocols, pg 175). PCR products were further analysed by capillary electrophoresis as described previously. Microsatellite alleles were called based on size, and organised into "bins" as specified by Greenberg et al., (2006) (given in Appendix 4 – Molecular reagents and protocols, pg 176). At least two samples from the Greenberg study cohort were included with each experiment to ensure consistency of allele calls.

The SNP rs3774729 was genotyped by direct sequencing. The PCR was carried out in a 25µl reaction with 1x GoTaq Buffer (Promega), 200µM dNTPs (Bioline), 0.4µM of each primer, 0.5U GoTaq Polymerase (Promega), and 100ng DNA. Cycling conditions were 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 40 seconds; single cycle of 72°C for 7 minutes. PCR products were sequenced using a standard cycle sequencing reaction and conditions. In a 10µl reaction: 1x BigDye Terminator Sequencing Buffer (Applied Biosystems), 1µl BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems), 0.4uM forward primer, 2µl PCR product. Cycling: 98°C for 5 minutes, followed by 25 cycles of 96°C for 10 seconds, 50°C for 15 seconds, 60°C for 4 minutes. Sequencing products were precipitated with ethanol and analysed on the ABI 3100 Genetic Analyzer. Sequence alignments were performed using the BioEdit Sequence Alignment Editor (version 7.0.9.0). For haplotyping analysis, the phase of the alleles was inferred manually in each pedigree, and confirmed using the program PHASE (version 2.1, (Stephens et al., 2001). Where an unaffected control (mutation negative) cohort was available, the Fisher's Exact test was used to determine association.
Figure 10: Diagram showing the positions of the three SCA7 haplotype markers SCA7-AC1, D3S1287 and the SNP rs3774729 (SCA7-SNP1) on chromosome 3. Diagram courtesy of the authors of Greenberg et al., (2006) (unpublished).
2.3 Results

2.3.1 The spectrum & frequency of the polyglutamine Spinocerebellar ataxias in southern Africa

From 1987 until 2013, approximately 1470 individuals from 1120 families had been referred to the Division of Human Genetics/NHLS for testing for one or more of the SCAs. Initially, the majority of referrals were for SCA1 testing since a few large SCA1 families had been identified during the 1980’s, but from 1995 there was an increase in the number of tests being performed. During 2012 and 2013 more than 120 multiplex assays were completed per year (Figure 11).

Figure 11: Number of diagnostic tests performed per year, per disorder (SCA1, SCA2, SCA3, SCA6 and SCA7).

During this 27-year period, 313 individuals from 215 families received a molecular diagnosis of SCA1, SCA2, SCA3, SCA6 or SCA7. By the end of 2013, the highest percentage of affected families had been diagnosed with SCA1 (36%), followed by SCA2 and SCA7 (both 28%) (Figure 12). Since the initiation of SCA12 and SCA17 testing in 2007, no individuals were found to have pathogenic alleles at either locus. A single individual had a SCA17 allele within the zone of reduced penetrance (42 repeats), therefore remaining results will focus on the more common types of polyglutamine SCA in SA, namely SCA1, 2, 3, 6 and 7.
Figure 12: Distribution of the five polyglutamine SCAs in affected southern African families (n=215 families).

In some cases the ethnicity of the tested individual was stated on the referral form. The ethnicities of many of the confirmed individuals was unknown, but the available data could give an indication of specific trends and distribution (Figure 13). The majority of the SCA1 cases of known ethnicity were of Mixed Ancestry, with a smaller proportion of Caucasian individuals. Within the SCA2 and SCA7 cohorts the largest percentage of individuals were of black African ancestry. Most of the individuals diagnosed with SCA3 and SCA6 were of unknown ethnicity. All four population groups were represented in the cohort of undiagnosed individuals of known ethnicity, with patients of Mixed Ancestry representing the highest proportion (Figure 13).
2.3.2 Allele distribution in South African SCA patients

In recent years the allele sizes at each of the five loci has been recorded for the majority of tested individuals. The CAG repeat alleles for the SA population fell within the boundaries specified by the EMQN (Table 8, Table 9). The full allele distribution graphs are presented in Appendix 9 – Allele distribution graphs (pg 193). No recorded alleles were within the "uncertain" or "zone of reduced penetrance" ranges, apart from two individuals carrying alleles of 34 and 35 repeats at the SCA7 CAG repeat locus.

Figure 13: Distribution of ethnicities within each patient group (SCA1, SCA2, SCA3, SCA6, SCA7). The "undiagnosed" group refers to individuals of known ethnicity who did not receive a molecular diagnosis of SCA1, 2, 3, 6, or 7. The undiagnosed individuals of unknown ethnicity are not represented. The ethnic distribution of the remaining undiagnosed individuals is unknown.
Table 9: Summary of allele ranges at the CAG repeat loci for SCA1, 2, 3, 6 and 7 in South African patients (full graphs are given in Appendix 9 – Allele distribution graphs).

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Normal allele (number of CAG repeats)</th>
<th>Expanded allele (number of CAG repeats)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of alleles</td>
<td>Smallest allele</td>
</tr>
<tr>
<td>SCA1</td>
<td>1677</td>
<td>14</td>
</tr>
<tr>
<td>SCA2</td>
<td>1329</td>
<td>12</td>
</tr>
<tr>
<td>SCA3</td>
<td>1620</td>
<td>11</td>
</tr>
<tr>
<td>SCA6</td>
<td>1742</td>
<td>5</td>
</tr>
<tr>
<td>SCA7</td>
<td>1277</td>
<td>4</td>
</tr>
<tr>
<td>SCA12</td>
<td>953</td>
<td>5</td>
</tr>
<tr>
<td>SCA17</td>
<td>1011</td>
<td>27</td>
</tr>
</tbody>
</table>

2.3.3 Investigation of the South African SCA7 haplotype in non-South African SCA7 families

2.3.3.1 Family 637

In 2009, DNA samples from a family in rural northern Namibia were referred to the NHLS laboratory for SCA testing. The family consisted of 8 children, all of whom were clinically affected with cerebellar dysarthria and blindness. The deceased mother was reported to be affected, along with her father (also deceased). The 7th child was deceased, and the 6th child had a 13 year old daughter, who at that time did not show clinical symptoms. DNA was obtained from 5 of the affected children, along with the 13 year old grand-daughter. The family was coded as Family 637, and each of the individuals were given a unique code of 637.1, 637.2, etc (Table 10). The genotyping and haplotype analysis for Family 637 was completed by Dr Lauren Watson.

The CAG repeat size of each individual was determined via standard methods by the NHLS laboratory. All 5 affected children were found to have pathogenic repeats at the SCA7 locus, with sizes ranging from 56 to 61 repeats (Table 10). The unaffected grand-daughter had two wild-type alleles (11 and 12 repeats). Within the research
laboratory, all 6 samples were genotyped at the 3 loci associated with the SA SCA7 haplotype, as previously described. The previously published haplotype-associated alleles for SA SCA7 patients were allele 6 for AC1, allele A for rs3774729 and allele 1 for D3S1287 (Greenberg et al., 2006). All 5 affected children from Family 637 had each of the SA haplotype-associated alleles, whilst the unaffected grand-daughter (637.5) did not have any of the associated alleles (Table 10).

Table 10:  Family 637 genotypes at the SA SCA7-associated haplotype loci.  The SA haplotype-associated alleles are in bold.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Clinical Status</th>
<th>AC1 genotype</th>
<th>CAG repeat genotype</th>
<th>rs3774729 genotype</th>
<th>D3S1287 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>637.1</td>
<td>Affected</td>
<td>6/7</td>
<td>8/56</td>
<td>A/A</td>
<td>1/3</td>
</tr>
<tr>
<td>637.2</td>
<td>Affected</td>
<td>6/7</td>
<td>8/60</td>
<td>A/A</td>
<td>1/3</td>
</tr>
<tr>
<td>637.3</td>
<td>Affected</td>
<td>6/7</td>
<td>8/58</td>
<td>A/A</td>
<td>1/3</td>
</tr>
<tr>
<td>637.4</td>
<td>Affected</td>
<td>5/6</td>
<td>11/61</td>
<td>A/G</td>
<td>1/3</td>
</tr>
<tr>
<td>637.5</td>
<td>Unaffected</td>
<td>4/5</td>
<td>11/12</td>
<td>G/G</td>
<td>3/5</td>
</tr>
<tr>
<td>637.6</td>
<td>Affected</td>
<td>5/6</td>
<td>11/60</td>
<td>A/G</td>
<td>1/3</td>
</tr>
</tbody>
</table>

Since DNA from a limited number of family members was available for genotyping, no complete trios (both parents and a child) were represented. For this reason the phase of each allele could not be established unequivocally for each individual; however, due to the presence of the mother-child pair (individuals 637.4 and 637.55), the haplotypes for each individual could be inferred manually. For the AC1 and D3S1287 markers, the alleles that were shared between all affected individuals were allele 6 and allele 1 respectively. At the SNP locus all affected patients carried at least one A allele, whilst the unaffected grand-daughter had a GG genotype, suggesting that her mother’s G allele was associated with the non-pathogenic CAG repeat allele. Together, these results suggested that the SA SCA7-associated haplotype was present within this Namibian family (Figure 14). No DNA from additional unaffected individuals from the same region was available for analysis.
Figure 14: Pedigree of Family 637 with inferred haplotypes and CAG repeat sizes. The genotypes at the microsatellite marker AC1, the \ATXN7\ CAG repeat, the SNP rs3774729, and the microsatellite marker D3S1287 are shown in order from the telomere to the centromere, along with the South African SCA7-associated haplotype genotypes (see key). Shaded symbols indicate subjects affected with SCA7. Circles denote females, squares males. A diagonal line through a symbol indicates that the individual is deceased. Haplotypes are presented as black and white bars. A black bar indicates the haplotype co-segregating with the disease.

2.3.3.2 Zambian Family A and B

In 2011, an affected male from the city of Kitwe in central Zambia was referred to the NHLS for molecular testing for the polyglutamine SCAs. His results revealed an expanded allele of 56 CAG repeats at the \ATXN7\ locus. A formal collaboration was entered into with the Zambian clinician, subsequent to ethical approval and a Material Transfer Agreement. After consultation with the Zambian clinician (Dr Masharip Atadzhanov, University of Zambia School of Medicine), DNA samples were received from 13 family members (both affected and unaffected, called Family A), as well as 4 individuals from an additional unrelated family in the town of Mongu (western Zambia) (Family B) (Table 11). The collaborators in Zambia had coded each of these samples as P1, P2, etc. DNA was also received from 40 unaffected control (mutation negative) individuals from the same region (coded C1, C2, etc).
The DNA samples had been isolated in Zambia from blood plasma, which typically yields very small quantities of DNA. Approximately 300 ng of DNA was received for each individual, therefore each of the PCR assays was adapted to use a smaller quantity of DNA (50 ng, rather than 100 ng). However, due to the low yield and poor quality of the DNA, the genotypes of some individuals could not be obtained for all the haplotype markers or CAG repeats. Firstly, the CAG repeat genotype of each individual was determined by singleplex PCR. In cases of apparent homozygosity, TP PCR was used to determine whether the individual carried an expanded allele (discussed in more detail in Chapter 3). In these cases the exact number of CAG repeats could not be determined. Once the CAG repeat genotyping, each sample was genotyped at each of the three remaining haplotype marker loci (AC1, rs3774729 and D3S1287) (Table 11).

Table 11: Zambian individual genotypes at the SCA7-associated haplotype loci. Where the CAG repeat size was determined by TP PCR, "N" designates an allele within the wild-type range, and "E" designates an allele within the expanded/pathogenic range. The SA haplotype-associated alleles are in bold.

<table>
<thead>
<tr>
<th>Family</th>
<th>Individual</th>
<th>Reported clinical status</th>
<th>AC1 genotype</th>
<th>CAG repeat genotype</th>
<th>rs3774729 genotype</th>
<th>D3S1287 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>P2</td>
<td>Unaffected</td>
<td>2/5</td>
<td>N/N</td>
<td>A/A</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>Unaffected</td>
<td>4/5</td>
<td>N/N</td>
<td>A/G</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>Affected</td>
<td>6/7</td>
<td>9/50</td>
<td>A/A</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>Affected</td>
<td>6/7</td>
<td>9/55</td>
<td>A/G</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>Affected</td>
<td>6/6</td>
<td>N/E</td>
<td>A/A</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>Unaffected</td>
<td>6/4</td>
<td>N/E</td>
<td>A/G</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>Unaffected</td>
<td>6/7</td>
<td>N/N</td>
<td>A/A</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>P9</td>
<td>Affected</td>
<td>5/6</td>
<td>9/50</td>
<td>A/A</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>P10</td>
<td>Unaffected</td>
<td>4/5</td>
<td>N/N</td>
<td>G/G</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>P11</td>
<td>Unaffected</td>
<td>6/7</td>
<td>N/E</td>
<td>A/A</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>P12</td>
<td>Unaffected</td>
<td>4/-</td>
<td>N/N</td>
<td>A/G</td>
<td>3/-</td>
</tr>
<tr>
<td></td>
<td>P13</td>
<td>Unaffected</td>
<td>6/6</td>
<td>N/E</td>
<td>-</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>P14</td>
<td>Unaffected</td>
<td>5/7</td>
<td>N/N</td>
<td>A/A</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>P15</td>
<td>Affected</td>
<td>6/7</td>
<td>N/N</td>
<td>A/A</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>P16</td>
<td>Unaffected</td>
<td>6/7</td>
<td>N/N</td>
<td>A/A</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>P17</td>
<td>Unaffected</td>
<td>2/7</td>
<td>10/10</td>
<td>A/A</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>P18</td>
<td>Unaffected</td>
<td>4/5</td>
<td>N/N</td>
<td>A/G</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>P19</td>
<td>Affected</td>
<td>5/6</td>
<td>9/52</td>
<td>-</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>P20</td>
<td>Affected</td>
<td>5/6</td>
<td>N/E</td>
<td>-</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>P21</td>
<td>Unaffected</td>
<td>1/5</td>
<td>10/12</td>
<td>A/A</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>P22</td>
<td>Unaffected</td>
<td>5/6</td>
<td>10/48</td>
<td>A/A</td>
<td>1/3</td>
</tr>
</tbody>
</table>
Four clinically non-symptomatic individuals (as reported by the Zambian clinician) were found to have CAG repeat expansions within the disease-causing range (P7, P11, P13 and P22). All of the patients with a pathogenic repeat had the SA SCA7 haplotype-associated alleles at each of the three markers (allele 6 at AC1, allele A at rs3774729, and allele 1 at D3S1287). Within the unaffected control group, allele 6 of AC1 was found in 11 individuals, whilst allele 1 of D3S1287 was not found in any of the control cohort (Table 12). The SA SCA7 haplotype alleles for these two markers were significantly associated with the patient group (p<0.0001), suggesting that the haplotype is associated with the disease, and is not simply at a high frequency in the tested control population. The A allele of rs3774729 was found at a high frequency in both groups (100% of patients and 91% of control samples).

Table 12: Frequencies of the SA SCA7-associated haplotype alleles in Zambian patients and unaffected control (mutation negative) individuals.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Controls with allele (%)</th>
<th>Patients with allele (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1 allele 6</td>
<td>14/47 (30%)</td>
<td>11/11 (100%)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>rs3774729 allele A</td>
<td>41/45 (91%)</td>
<td>8/8 (100%)</td>
<td>1</td>
</tr>
<tr>
<td>D3S1287 allele 1</td>
<td>0/50 (0%)</td>
<td>11/11 (100%)</td>
<td>p&lt;0.0001</td>
</tr>
</tbody>
</table>

Together, these results provided evidence to suggest that the Zambian SCA7 patients from the two families shared the SA SCA7-associated haplotype. To substantiate these findings, the pedigree of each family was used to manually infer the phase of each allele. The PHASE haplotype analysis program was also used to confirm these results (Stephens et al., 2001). Although no samples from complete parent-child trios were available to unequivocally determine the phase of each haplotype marker, pedigree analysis provided further evidence to support the supposition that the SA SCA7-associated haplotype was shared within both families (Figure 15 and Figure 16).
Figure 15: Pedigree of Zambian Family A with inferred haplotypes and CAG repeat sizes. The genotypes at the microsatellite marker AC1, the ATXN7 CAG repeat, the SNP rs3774729, and the microsatellite marker D3S1287 are shown in order from the telomere to the centromere, along with the South African SCA7-associated haplotype genotypes (see key). The CAG repeat genotype is given as a number (= number of repeats), or "N" to indicate a wild-type allele, or "E" to indicate an expanded allele. Shaded symbols indicate subjects diagnosed with SCA7. Circles denote females, squares males. A diagonal line through a symbol indicates that the individual is deceased. Haplotypes are presented as black or coloured bars (green, blue, orange, turquoise or purple). A solid black bar indicates the haplotype co-segregating with the disease. To maintain anonymity, the sex and order of birth have been changed in the pedigree.
Figure 16: Pedigree of Zambian Family B with inferred haplotypes and CAG repeat sizes. The genotypes at the microsatellite marker AC1, the ATXN7 CAG repeat, the SNP rs3774729, and the microsatellite marker D3S1287 are shown in order from the telomere to the centromere, along with the South African SCA7-associated haplotype genotypes (see key). The CAG repeat genotype is given as a number (= number of repeats), "N" to indicate a wild-type allele, or "E" to indicate an expanded allele. Shaded symbols indicate subjects diagnosed with SCA7. Circles denote females, squares males. A diagonal line through a symbol indicates that the individual is deceased. Haplotypes are presented as black or white bars. A black bar indicates the haplotype co-segregating with the disease.

2.3.3.3 Family 986

In 2013, a Nigerian family (Family 986) was referred to the NHLS for molecular diagnostic testing. Samples were received from the proband, his affected mother and 2 affected brothers. A family history of blindness was reported, therefore a molecular diagnosis of SCA7 was anticipated. All 4 individuals were found to carry CAG repeats that were marginally within the pathogenic size range and all 4 individuals were genotyped at the three haplotype marker loci (Table 13).
Table 13: Family 986 genotypes at the SCA7-associated haplotype loci. The SA haplotype-associated alleles are in bold.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Clinical Status</th>
<th>AC1 genotype</th>
<th>CAG repeat genotype</th>
<th>rs3774729 genotype</th>
<th>D3S1287 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>986.1</td>
<td>Affected</td>
<td>4/5</td>
<td>10/39</td>
<td>A/A</td>
<td>2/3</td>
</tr>
<tr>
<td>986.2</td>
<td>Affected</td>
<td>4/5</td>
<td>10/39</td>
<td>A/A</td>
<td>2/3</td>
</tr>
<tr>
<td>986.3</td>
<td>Affected</td>
<td>4/6</td>
<td>9/39</td>
<td>A/A</td>
<td>3/7</td>
</tr>
<tr>
<td>986.4</td>
<td>Affected</td>
<td>4/5</td>
<td>10/39</td>
<td>A/A</td>
<td>2/3</td>
</tr>
</tbody>
</table>

The phase of the haplotype-associated loci was inferred manually by pedigree analysis. At the AC1, SNP and D3S1287 loci, alleles 4, A and 3 (respectively) were shared between the affected individuals (Figure 17). At the D3S1287 locus the affected mother carried allele 3, along with an allele that could not be grouped into an allele bin as specified by Greenberg et al., (2006). These results suggested that the disease-associated haplotype alleles within this family were 4, A, and 3, which differed from the SA-associated haplotype alleles of 6, A and 1. Of interest, all four individuals displayed the same CAG repeat size, indicating that little or no instability of the repeat region during transmission from the mother to each child. Detailed clinical information was not provided for this family, therefore it is unknown whether the affected individuals experienced a similar age of onset of symptoms.
Figure 17: Pedigree of Family 986 with inferred haplotypes and CAG repeat sizes. The genotypes at the microsatellite marker AC1, the ATXN7 CAG repeat, the SNP rs3774729, and the microsatellite marker D3S1287 are shown in order from the telomere to the centromere, along with the South African SCA7-associated haplotype genotypes (see key). Shaded symbols indicate subjects diagnosed with SCA7. Circles denote females, squares males. A diagonal line through a symbol indicates that the individual is deceased. Haplotypes are presented as black and white bars. A black bar indicates the haplotype co-segregating with the disease.
2.4 Discussion
Updated figures on the number of individuals and families who have been molecularly diagnosed with SCA1, 2, 3, 6 or 7 in SA have been presented. An early version of these results were published in 2012 (Smith et al., 2012), and represented the first update on the prevalence of the polyglutamine SCAs in SA since Bryer et al., (2003). The previously reported high frequencies of SCA1 and SCA7 in SA remain, and may be attributed to the founder effects within the SCA1 Mixed Ancestry population (Ramesar et al., 1997) and the SCA7 black African population (Greenberg et al., 2006). This is supported by the fact that the Mixed Ancestry and black African ethnic groups represented the highest proportion of patients affected with SCA1 and SCA7, respectively (Figure 13). A single study has reported on three black African SCA7 families from the Gauteng region of SA (Modi et al., 2000), but it is not known whether any of these individuals have undergone molecular testing through the NHLS laboratory.

A high proportion of SCA2 families were also of black African ancestry, however preliminary laboratory-based investigations have not yielded any clear evidence for an associated haplotype (Wood, 2013). Of particular interest is the low frequency of SCA3 found in SA. Over 26 years of testing, only 11 individuals from 9 families have been molecularly diagnosed with SCA3. SCA3 is commonly reported as the most frequent of the polyglutamine SCAs, with relative frequencies ranging upwards of 30% in regions of Europe and the Americas (Figure 18). The reason for the low prevalence of SCA3 in SA is unclear, although it may be due to the relatively low proportion of individuals of European ancestry in SA, compared to the indigenous black African and Mixed Ancestry population groups.

Within each patient group, the ethnicity of a high percentage of individuals was listed as "unknown", since many referring clinicians did not list the ethnicity of patients on the referral form. For example, the ethnicity of almost all of the SCA6 patients was unconfirmed, but surname analysis suggested that the majority of these individuals were of Caucasian ancestry. Whilst the knowledge of a patient's ethnic background is not essential in the molecular diagnosis process, it may be useful for identifying trends and founder effects, as has been demonstrated for
SCA1 and SCA7. The knowledge of patient ethnicities may also be useful from an epidemiological perspective, since it can allow for comparisons with other countries and regions.

The distribution of these five polyglutamine SCAs in SA remains markedly different to trends seen elsewhere in the world (Figure 18). SCA7 is usually found at a significantly lower frequency when compared to SCA1, SCA2, SCA2 and SCA6, apart from a region in Scandinavia, where 63% of the individuals tested had SCA7 expansions when compared to the other four SCAs (Juvonen et al., 2005). Although disease-associated haplotypes have been established in both the SA and Scandinavian SCA7 patient population, only one marker is shared between the two haplotypes (D3S1287). Due to the lack of reporting of allele calling methods for D3S1287 in the Scandinavian publication it is unclear whether the South African and Scandinavian patients share the same allele at this locus. Similarly, a haplotype-based study was undertaken in Mexico in 2013, and two of the investigated markers included D3S1287 and rs3774729, which form part of the SA SCA7-associated haplotype (García-Velázquez et al., 2013). Ninety individuals from 19 families from the Veracruz region of Mexico were genotyped, and found to share a common haplotype. Once again, due to differences in allele reporting methods between the Greenberg and García-Velázquez studies, it can not be determined whether the Mexican and SA SCA7 patients share the same alleles at the two marker loci, but this may warrant further investigation.
Whilst almost 1500 individuals had been referred for molecular testing over the 27-year period, only 313 (21%) received a confirmed molecular diagnosis. Firstly, this is likely to be an underrepresentation of the number of South Africans affected with the SCAs, since financial constraints and deficient healthcare infrastructure limits the number of clinical referrals from low-income and rural areas. Secondly, it is of concern that almost 80% of the referred individuals did not receive a molecular diagnosis. Whilst this may be due to poor or unwarranted clinical referrals, it is possible that a proportion of these patients are affected with an additional type of SCA that does not form part of the current testing panel. Clinical or familial information rarely accompanies the biological samples, therefore it is unknown whether these undiagnosed individuals come from families with multiple affected members, or if their condition was isolated. It is also not known whether any particular population group is overrepresented in this "undiagnosed" cohort, since
the ethnicities of only a small proportion of this group is known (Figure 13). Studies undertaken in other countries may be useful in determining whether other types of SCA should be included in the testing panel, but the South African population is highly heterogeneous and unique, and no other broad SCA-based epidemiological investigations have been completed in additional African countries. International diagnostic companies commonly include DRPLA, as well as SCA types 5, 8, 10, 13, 14 and 28 as part of a testing panel for the autosomal dominant cerebellar ataxias (Table 14). The majority of these disorders have been reported in multiple countries, but none have been reported in Africa. The NHLS does provide molecular testing for DRPLA upon request, but the prevalence of this condition in SA has not yet been investigated.
Table 14: Disorders commonly included as part of autosomal dominant cerebellar ataxia testing panels (in addition to SCA1, 2, 3, 6 and 7).

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Mutation</th>
<th>Reported countries</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRPLA</td>
<td>CAG expansion in ATN1</td>
<td>Japan, Britain</td>
<td>(Tomoda et al., 1991), (Warner et al., 1994)</td>
</tr>
<tr>
<td>SCA5</td>
<td>Mutations in SPTBN2</td>
<td>USA, France, Germany</td>
<td>(Ranum et al., 1994), (Stevanin et al., 1999), (Burk et al., 2004)</td>
</tr>
<tr>
<td>SCA8</td>
<td>CAG expansion in ATXN8, CTG expansion in ATXN8OS</td>
<td>Japan, Italy, USA, Canada, Mexico, Japan</td>
<td>(Ikeda et al., 2000), (Cellini et al., 2001), (Ikeda et al., 2004)</td>
</tr>
<tr>
<td>SCA10</td>
<td>ATTCTT expansion in ATXN10</td>
<td>Mexico, Brazil, Argentina</td>
<td>(Grewal et al., 2002), (Gatto et al., 2007), (Teive et al., 2004)</td>
</tr>
<tr>
<td>SCA13</td>
<td>Mutations in KCNC3</td>
<td>France, Philippines</td>
<td>(Herman-Bert et al., 2000), (Waters et al., 2005)</td>
</tr>
<tr>
<td>SCA14</td>
<td>Mutations in PRKCG</td>
<td>Japan, Netherlands, France, Japan, Britain</td>
<td>(Yamashita et al., 2000), (van de Warrenburg et al., 2003), (Stevanin et al., 2004), (Morita et al., 2006), (Sailer et al., 2012)</td>
</tr>
<tr>
<td>SCA27</td>
<td>Mutations in FGF14</td>
<td>Netherlands</td>
<td>(van Swieten et al., 2003)</td>
</tr>
<tr>
<td>SCA28</td>
<td>Mutations in AFG3L2</td>
<td>Italy</td>
<td>(Cagnoli et al., 2006b)</td>
</tr>
</tbody>
</table>

In cases of families with a strong family history, but with an unknown genetic cause, it may be beneficial to employ next generation technologies such as whole genome or exome sequencing. Nemeth and colleagues demonstrated the utility of this approach, by using next generation sequencing in an attempt to determine the underlying mutations in 50 patients with familial or early-onset ataxia, who had previously been tested for SCA1, 2, 3, 6, 7 and Friedreich's ataxia (Nemeth et al.,
Targeted capture performed on 117 candidate genes revealed 13 pathogenic mutations (nine of which were novel) in the patient cohort, indicating that the inclusion of this strategy may be advantageous in diagnostic laboratories. However, it should be noted that this method may be accompanied by new challenges, such as increased cost and difficulty in interpreting the pathogenicity of new variants, therefore it may be some time before it is adopted into routine practice in low-resource countries such as SA.

In this study, evidence for the existence of the SA SCA7-associated haplotype in SCA7 families from Namibia and Zambia has also been presented. A confirmed molecular diagnosis has an immediate benefit to these families, since testing can be offered to other family members, the family can be counselled with regard to the management of affected members, and risks for current and future offspring can be discussed. These haplotyping results also suggested that the South African, Namibian and Zambian SCA7 families tested share a common founder. Although some background control individuals were genotyped from the Zambian population, future studies should focus on including additional unaffected individuals from the studied regions. In light of these haplotyping results, it is likely that additional SCA7 families exist in neighbouring countries such as Botswana and Zimbabwe, and countries further north such as Angola, Tanzania and the Democratic Republic of Congo. The Nigerian family studied did not have the SA SCA7-associated haplotype, which was not unexpected given the geographical distance and differing population structures. Further investigations will be required to determine whether additional Nigerian SCA7 families share the same haplotype as Family 986. A shared haplotype between large groups of affected individuals may be beneficial for the design of future therapeutic interventions. The RNAi-based therapy designed by Scholefield and colleagues targets the A allele of the SNP linked to the pathogenic CAG repeat in patients with the SA SCA7-associated haplotype (Scholefield and Wood, 2009; Scholefield et al., 2014). Similarly, a panel of five siRNAs have been designed to target three-quarters of US and European HD patients (Pfister et al., 2009), demonstrating the value of population-based haplotype studies.
There is a significant lack of knowledge with regard to the extent, distribution and frequency of the dominant inherited ataxias in Africa. Future work should concentrate on establishing and broadening relationships with neurologists on the continent, with the aim of increasing the awareness of this group of disorders, and to boost the number of clinical referrals for molecular testing of affected individuals. Pilot studies should also be implemented with the aim of determining other types of SCAs in African populations. Overall, this body of work would have a significant impact on the lives of many African patients with ataxia, since they would receive a molecular diagnosis for their condition, allowing for better healthcare management, planning and future treatment. It would also contribute to the greater understanding and study of the polyglutamine SCAs in southern Africa, and allow for more appropriate therapy design and implementation.
Chapter 3: An improved strategy for the molecular diagnosis of SCA7 in South Africa
Note: The results included in this chapter were previously published in the Journal of Molecular Diagnostics (Smith et al., 2013a). Sections of the article have been reproduced with permission from the co-authors and publisher.

3.1 Introduction

3.1.1 Molecular diagnosis of SCA7

Eighteen human neurological conditions are classified as triplet (or trinucleotide) repeat disorders, including SCA7. The pathogenic mutations are dynamic, and triplet repeats within the disease-causing ranges are unstable and prone to further expansion in both somatic and germline tissues (Stevanin et al., 1998). Instability in the germline leads to the phenomenon of anticipation, whereby the pathogenic repeat expands within successive generations in a family, resulting in progressively earlier ages of disease onset. It has also been hypothesised that somatic instability within key tissues may exacerbate disease symptoms (Lin et al., 2009). The causal mechanisms behind this instability have yet to be elucidated, but they are likely to be linked to the formation of secondary structures during DNA replication, repair, recombination and transcription (Lin et al., 2009; McMurray, 2010).

Since the discovery of the disease-causing mutation in \textit{ATXN7} in 1997, the most common method of molecular diagnosis of SCA7 has used fluorescent PCR-based genotyping. This assay utilises two gene-specific PCR primers designed to flank the CAG repeat region (Figure 19). One of the primers is fluorescently tagged, which allows for an estimation of the size of the PCR fragments after capillary electrophoresis.

![Figure 19: Assay design for conventional end-labelled PCR testing for triplet repeat disorders.](image)

Three potential electropherogram results may be generated by the end-labelled PCR method. In the case of an individual who is unaffected, two peaks within the unexpanded size range may be detected, representing two wild-type alleles (Figure 20a). The profile of an affected individual should show a single wild-type allele
peak, along with a series of peaks within the pathogenic size range, indicating the disease-associated unstable expanded allele (Figure 20b). Multiple peaks are frequently evident in the pathogenic size range, due to somatic instability. Finally, an electropherogram result may show a single peak within the wild-type allele range (Figure 20c). This result may indicate that the individual has two wild-type alleles of the same size (homoallelism), or they may carry a single wild-type allele along with an expanded allele that has not been detected by the assay.

![Electropherogram Results](image)

**Figure 20:** Examples of electropherogram results from the end-labelled PCR method for SCA7 CAG repeat genotyping. Red peaks indicate the Rox500 size standard. Green peaks show PCR fragments. Relative fluorescent units are represented on the y-axis, the x-axis shows fragment size in base pairs. (a) Representative profile of an unaffected individual, showing two alleles within the wild-type allele size range. (b) Representative profile of an individual affected with SCA7, showing a single wild-type allele and a number of alleles within the expanded size range. (c) Profile showing a single peak, indicative of an unaffected individual with two wild-type alleles of the same size, or an affected individual with an undetected expanded allele.

### 3.1.2 Triplet repeat primed PCR

In cases of apparent homozygosity (single peak), a patient cannot be given a confirmed molecular diagnosis. The EMQN recommends that the parents be tested (where possible) to confirm homozygosity, or that alternative methods such as
Southern blot analysis or triplet repeat primed PCR (TP PCR) be employed (Sequeiros et al., 2010a). The process of Southern blotting is labour- and time-consuming, and the NHLS laboratory at Groote Schuur Hospital does not currently have the facilities available to perform these tests. For these reasons the utility of implementing a TP PCR assay as a confirmatory test in cases of apparent homozygosity at the SCA7 locus was investigated.

The dilemma of drop-out of large alleles in molecular diagnostic testing is not restricted to SCA7, but is common to all triplet-repeat disorders. Full penetrance alleles can be as large as 500 repeats in SCA2 patients (Ramocki et al., 2008), or 4500 repeats in individuals with SCA10 (Matsuura et al., 2000). In an effort to create a simple confirmatory test, Warner and colleagues developed the TP PCR assay (Warner et al., 1996). Instead to two flanking primers, the TP PCR test consists of a single fluorescent flanking primer, along with a pair of primers with a common 5' sequence (Figure 21). The first paired primer contains a sequence complementary to the CAG repeat, whilst the second paired primer only binds to the 5' tail region of the first primer. The tail region is a random DNA sequence that is not complementary to any part of the human genome.

![Figure 21: Assay design for triplet repeat primed PCR (TP PCR) testing for triplet repeat disorders.](image)

The repeat-binding primer is supplied at one tenth of the molar ratio of the remaining two primers, and primes at multiple sites within the CAG repeat locus during the initial amplification cycles. Once the repeat-binding primer is exhausted, the locus-specific forward primer and the tail primer amplify the PCR products produced by the forward primer and repeat-binding primer in the early cycles. The molar ratio difference of the primers was designed to prevent the gradual shortening of the average PCR product size. Upon capillary electrophoresis, the results show a distinctive ladder-like electropherogram, with the peaks with the
greatest height indicating the wild-type CAG repeat allele, and the diminishing peaks indicating the presence of an expansion (Figure 22). This method was used to correctly identify 85 individuals affected with myotonic dystrophy, who had been previously diagnosed by Southern blot analysis (Warner et al., 1996). TP PCR protocols have also been developed for HD, Friedreich ataxia, SCA2 and SCA7 (Cagnoli et al., 2006a; Ciotti et al., 2004; Falk et al., 2006). Although the accurate size of the CAG repeat alleles can not be determined using TP PCR, controls of a known genotype can be analysed in parallel with unknown samples to give an indication of the repeat size range.

![Figure 22: Example of an electropherogram result from the TP PCR method for CAG repeat genotyping. Peaks show PCR fragments. Relative fluorescent units are represented on the y-axis, the x-axis shows fragment size in base pairs.](image)

At the SCA7 locus, the most common wild-type alleles in the South African population are 9 and 10 CAG repeats (see Appendix 9 – Allele distribution graphs, pg 193), therefore a high proportion of tested individuals show apparent homozygosity (close to 50%). In 2012, analysis of the patient registry at the Division of Human Genetics identified a total of 294 individuals (111 black Africans and 183 individuals of the remaining ethnic groups) who had received inconclusive SCA7 test results based on apparent homozygosity since the molecular test had been introduced (approximately 30% of referrals), and for whom DNA was available for additional testing. With the implementation of a confirmatory test, the majority of referred patients could receive a confirmed molecular result in the future.
3.1.3 Aims & Objectives

1. To optimise a SCA7 TP PCR assay for implementation into the NHLS diagnostic laboratory.

2. To re-test all the individuals who had previously been shown to be homozygous at the ATXN7 CAG repeat locus, using either singleplex PCR or TP PCR, in order to detect any additional pathogenic alleles.
3.2 Methods

3.2.1 Patient cohort

Ethical approval for the study was obtained from the University of Cape Town Human Research Ethics committee (HREC REF 229/2010) and is renewed annually. All samples have been de-identified to protect anonymity.

The majority of the samples referred for a molecular SCA diagnosis had been tested using the multiplex PCR method as described previously (Dorschner et al., 2002). In most cases the genotype of each sample at each of the five tested loci (SCA1, 2, 3, 6 and 7) was recorded on an electronic database. This database was mined for individuals who had received a homozygous result at the \textit{ATXN7} CAG repeat locus, and divided into two groups. The first group consisted of high risk individuals of black African ancestry, whilst the second low risk group consisted of individuals representing the remaining ethnic groups, and individuals of unknown ethnicity. The number of individuals who had been found to be homozygous at the remaining tested loci (SCA1, 2, 3 and 6) was also determined, in order to establish the utility of future confirmatory tests for these diseases.

3.2.2 SCA7 singleplex PCR

Within the research laboratory, an alternative set of primers were designed (Ms Fiona Baine, personal communication) to amplify the SCA7 locus in a singleplex PCR reaction. Within a 10 microlitre (µl) reaction, 100ng of DNA was included with 1x Failsafe Premix buffer J (Epicentre Biotechnologies), 0.4 µM of each primer and 0.6 units of GoTaq DNA polymerase (Promega). Cycling conditions were as follows: 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 53°C for 6 seconds, and 72°C for 40 seconds; and a single cycle of 72°C for 7 minutes on the MultiGene thermocycler (Labnet International). The resulting PCR product was subjected to agarose gel electrophoresis to determine the success of the reaction (Appendix 4 – Molecular reagents and protocols, pg 175). PCR products were further analysed by capillary electrophoresis as described previously. The number of CAG repeats per allele was calculated using the following formula (adapted from Dorschner et al., 2002):
3.2.3 Triplet repeat primed PCR (TP PCR)

The TP PCR protocol for SCA7 was optimised and modified from Majouie et al. (2007). The 25µl reaction contained 1x GoTaq buffer (Promega), 200 µM deoxyribonucleotide triphosphates (dNTPs), 0.04 µM repeat-binding primer, 0.4µM each of FAM-labelled flanking primer and tail primer, 6% dimethyl sulfoxide, 0.5U GoTaq polymerase (Promega) and 100ng DNA. Primer sequences are given on page 171. Cycling conditions were as follows: 95°C for 15 minutes; 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes; and a single cycle of 72°C for 10 minutes. Capillary electrophoresis was performed as described previously.

3.2.4 Sequencing of multiplex primer binding sites

Primers flanking the multiplex SCA7 primer-binding sites were designed (Figure 23) and used in a 10-µL reaction: 1x Failsafe Premix buffer J (Epicentre Biotechnologies), 0.4 mmol/L primers (forward and reverse), 0.6 U GoTaq DNA polymerase (Promega), and 100 ng DNA. Primer sequences are given on page 171. Cycling conditions were as follows: 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 60°C for 15 seconds, and 72°C for 40 seconds; and a single cycle of 72°C for 7 minutes. PCRs were subjected to standard cycle sequencing using the BigDye Terminator, version 3.1, Cycle Sequencing Kit (Applied Biosystems) and purified by ethanol precipitation. Sequencing was performed on the ABI3100 Genetic Analyzer (Applied Biosystems), and results were analysed using BioEdit sequence alignment software version 7.0.9.0 (Ibis Biosciences, Carlsbad, CA). Samples were sequenced in both directions.

\[
\text{Number of repeats} = \frac{(\text{PCR product size} - 266)}{3} \times 1.1211 + 5.0958
\]

Figure 23: Positions of primer binding around the ATXN7 CAG repeat. The sequence surrounding the ATXN7 CAG repeat is given (5' to 3'), with the positions of the sequencing primers highlighted in green, the multiplex primers underlined, and the repeat region in blue.
The high risk cohort of black African individuals was tested using the singleplex PCR to determine whether any additional expansions could be detected. Thereafter the whole cohort (both groups) was tested using the TP PCR assay. A positive control sample was included with each assay (SCA7-positive control with 54 repeats).
3.3 Results

3.3.1 Index case
The motivation for re-testing all the individuals with apparent homoallelicism at the SCA7 locus stemmed from an initial index case. A 17-year-old boy of black African ancestry was referred to the NHLS laboratory for molecular testing for the SCAs due to his clinical symptoms of body and limb ataxia, poor vision and ophthalmoplegia. The results from the multiplex PCR assay showed a single allele peak corresponding to 10 CAG repeats within \textit{ATXN7}. Since the patient fitted the clinical criteria for SCA7, and was of black African ancestry, it was suspected that the assay had not detected a pathogenic allele. The DNA was re-tested using the SCA7 singleplex PCR, and an expanded allele with 56 CAG repeats was identified.

3.3.2 Re-testing of “homozygous” individuals
A cohort of 111 black African individuals and 183 individuals of the remaining ethnicities (for which there was DNA available for molecular testing) was selected from the electronic database. All of these samples had previously shown a single unexpanded allele at the \textit{ATXN7} CAG repeat locus. The cohort of black African individuals was re-tested using the singleplex PCR assay, and SCA7 CAG repeats within the pathogenic size range were detected in six samples (5.4% of tested cases). The allele sizes ranged from 52 to 74 repeats. Both cohorts (total of 288 individuals) were then tested using the TP PCR assay, and no additional expansions were detected. Samples with and without pathogenic expansions could be easily distinguished based on the electropherogram results (Figure 24).
Figure 24: Electropherogram results from the TP PCR method for CAG repeat genotyping. a. Electropherogram from a sample without an expanded CAG repeat. b. Electropherogram from a sample containing a CAG repeat expansion (affected individual). Large peaks show PCR fragments. Small peaks (<500 relative fluorescent units) indicate the ROX500 size standard. Relative fluorescent units are represented on the y-axis, the x-axis shows fragment size in base pairs.

3.3.3 Sequencing of multiplex primer binding sites

The six newly identified positive samples were sequenced to determine whether polymorphisms at the multiplex primer binding sites had contributed to the lack of detection in the assay. No polymorphisms were identified in any of the samples tested (Figure 25). The referring clinicians of each of the six patients were contacted by a clinical geneticist and offered an updated molecular results report.
Figure 25: Sequencing of the multiplex primer binding regions in ATXN7. Sequencing chromatograms of three representative samples (a, b and c), showing no evident polymorphisms within the binding regions of the multiplex primers (forward and reverse).

3.3.4 Determination of homozygosity at tested SCA loci

The percentage of previously tested individuals who had been shown to be homozygous at the SCA1, 2, 3, 6 and 7 loci was calculated, in order to estimate the utility of confirmatory tests. Over the 26-year period of testing, 49% of the tested individuals showed apparent homozygosity at the SCA7 locus. The highest level of apparent homozygosity was at the SCA2 locus, where 66% of individuals showed a single peak. The remaining loci showed lower levels, with 16%, 23% and 29% of individuals showing apparent homozygosity at the SCA1, SCA3 and SCA6 loci respectively.
3.4 Discussion

The dynamic nature of the polyglutamine SCA7 mutation presents unique challenges to molecular diagnosis. Diagnostic laboratories are required to meet stringent quality standards and should adhere to published guidelines regarding assay design, control samples, and methods of reporting results (Sequeiros et al., 2010a). Here, the importance of using additional testing methods when presented with inconclusive results has been illustrated, as is standard good laboratory practice.

An individual with clinical symptoms of SCA7 was tested using a standard published multiplex PCR method. When these results revealed a single allele within the normal size range, the sample was retested using alternative PCR primers in singleplex and a pathogenic allele was detected. Retesting of a group of high-risk individuals revealed a lack of detection in 5.4% of previously tested samples. Preferential amplification of smaller repeat alleles is a common phenomenon in PCR assays; however, the range of the undetected alleles in this study was 52 to 74 repeats, which fell within the standard range of pathogenic alleles found in this patient population group. Larger alleles of up to 82 repeats had been previously identified using the multiplex method. Therefore, it was unlikely that the detection failure could be attributed to an abnormally high number of repeats. The multiplex SCA7-specific primers were able to detect the expansions when used in isolation, and there was significant overlap in the binding positions of the multiplex and singleplex primers (Figure 23). It was hypothesized that the lack of detection was linked to the dynamics of the multiplex assay, rather than the SCA7- specific primers. Polymorphisms in the regions surrounding the CAG repeat in the HD gene have previously been shown to interfere with diagnostic assays (Gellera et al., 1996; Margolis et al., 1999), but sequencing and bioinformatic analyses revealed no known polymorphic regions at the sites of binding of the ATXN7 multiplex primers (Figure 25), nor any predicted homodimer or heterodimer formations between the primers that could reduce target binding. Since the SCA7- associated expansion in South Africa is found almost exclusively within the indigenous black African population, it was not possible to determine whether there was a population- or
It remains to be investigated whether a similar detection inefficiency is apparent for the other SCA loci included in the multiplex assay. Preliminary retesting of 60 samples previously shown to be homozygous at the ATXN2 locus has revealed no pathogenic expansions.

It is unlikely that the potential limitations of this multiplex assay are restricted to the South African diagnostic laboratory. Furthermore, the application of the multiplex assay has been extended beyond the diagnostic arena, since several publications have used this method for epidemiological studies of the SCAs, without using confirmatory testing (Magaña et al., 2013; Sharma et al., 2012). Therefore, these findings are relevant to scientists within research and diagnostic laboratories who want to avoid a potential underestimation of SCA7 patient numbers. In this study, a singleplex PCR assay was initially used to rescreen samples for pathogenic alleles, and TP PCR was later employed as an additional confirmatory method. Singleplex PCR assays may still result in uncertainty if a single peak is detected, whilst a TP PCR assay is more likely to supply unequivocal results. TP PCR is not generally intended for accurate allele sizing; therefore, further analysis using Southern blotting should be performed if sizing of large alleles is required.

Molecular diagnostic laboratories are commissioned to provide results that are accurate, cost-effective, and delivered in a timely manner. To meet these needs, laboratories may incorporate assays that can jointly test multiple disorders, such as the multiplex SCA assays (Bauer et al., 2005; Dorschner et al., 2002). Although these methods are effective and can be relied on in many cases, the importance of verifying all test results and having alternative methods to address inconclusive results has been demonstrated. The Best Practice Guidelines for genetic testing for the SCAs, published by the EMQN, recommend implementation of complementary techniques to confirm homoallelicism (Sequeiros et al., 2010a). In the absence of additional confirmatory testing, the assay limitations should be clearly stated in the test reports. The findings presented here emphasize the importance of the investigation of inconclusive results. The SCA7 TP PCR assay has been successfully introduced into routine use in the NHLS diagnostic laboratory, and is used to confirm cases of apparent homoallelicism. In approximately 12 months since the
introduction of the TP PCR test, three individuals who would have otherwise received an inconclusive result have been given a confirmed diagnosis of SCA7. Almost 50% of the individuals tested to date have shown results of apparent homozygosity at the SCA7 locus. Similar confirmatory tests for the remaining tested SCAs, such as SCA2, will be explored in future studies. In this investigation, the necessity of adhering to the suggested guidelines and recommendations for molecular genetic testing has been emphasised, in order to contribute to the continual improvement and quality of test results.
Chapter 4: The use of induced pluripotent stem cells to investigate retinal degeneration associated with Spinocerebellar ataxia type 7
4.1 Introduction

The retinal degeneration seen in SCA7 patients is a feature that distinguishes this disease from all the other SCAs. Individuals with SCA7 suffer from disabilities associated with the degeneration of cerebellar Purkinje neurons, such as difficulty with walking, talking and swallowing, but the development of visual impairment adds to the significant burden of the disease. Visual symptoms may precede, accompany or follow the onset of neurological degeneration (Miller et al., 2009). With the initial onset of visual disability, patients most commonly complain of an intolerance or sensitivity to bright light, colour blindness or blurred vision (Miller et al., 2009).

Cell- and animal-based models have been able to provide some indications of the pathogenic mechanisms involved in the onset and development of retinal degeneration in SCA7 patients. However, there have been no in vitro models to explore the pathology of the condition using patient-derived photoreceptors. With the development of iPSC-based technology and specific differentiation protocols, this study sought to develop an in vitro model of SCA7 retinal degeneration using iPSC-derived retinal cells. This approach has been useful in modelling other retinal degenerative conditions such as retinitis pigmentosa (Jin et al., 2011; Jin et al., 2012; Tucker et al., 2013). It was therefore hypothesised that these patient-derived cells may provide valuable insights into SCA7 pathogenesis, and could be utilised to test future therapies.

4.1.1 iPSCs as a tool for disease modelling

Since the publication of the seminal technique for reprogramming of mouse and human adult dermal fibroblasts into cells representing ESCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), a multitude of publications have explored the utility of these cells for in vitro disease modelling and potential transplantation. Takahashi and colleagues generated the first human iPSCs from adult dermal fibroblasts and other somatic cells by retroviral transduction of four transcription factors (OCT3/4, SOX2, KLF4 and c-MYC, originally chosen from a pool of 24 genes). These transcription factors are known to be essential for the maintenance of pluripotency. OCT3/4 and SOX2 up-regulate pluripotency genes and down-regulate
genes associated with differentiation, whilst KLF4 and c-MYC remodel the chromatin structure and promote cell proliferation (Schmidt and Plath, 2012; Takahashi et al., 2007). The resultant cells exhibited a morphology similar to that of ESCs (tightly packed colonies of cells with a high nuclear-to-cytoplasm ratio) and fulfilled various criteria to confirm pluripotency, including the following:

1. Expression of ESC markers (including OCT3/4, SOX2, NANOG, GDF3, REX1, FGF4, ESG1, DPPA2, DPPA4, SALL4, E-CADHERIN, hTERT, SSEA1, SSEA-3, SSEA4, TRA-1-60, TRA-1-81, TRA-2-49/6E).
2. Demethylation of promoter regions of pluripotency genes (including OCT3/4, REX1, NANOG).
3. High telomerase activity and exponential growth.
4. An ability to differentiate in vitro into cell types derived from all three embryonic germ layers (endoderm, mesoderm and ectoderm).
5. A normal karyotype.

Although each iPSC line should ideally be fully characterised in line with the original criteria demonstrated by Takahashi in 2006, this process is expensive in terms of time and resources. Therefore alternative characterisation criteria have been proposed to be used in cases of in vitro, rather than in vivo applications (Ellis et al., 2009; Maherali and Hochedlinger, 2008). This set of "minimal" criteria states that the iPSCs should show the correct morphological characteristics (including unlimited self-renewal), express key pluripotency genes, show transgene independence, and demonstrate functional differentiation through the highest-stringency test acceptable (in vitro differentiation or teratoma formation).

Subsequent studies have modified the reprogramming strategy in various ways in order to address issues relating to reprogramming efficiency and safety. It has now been recognised that a number of variables must be addressed for each reprogramming experiment, and these factors may change depending on the desired aim and outcome (reviewed in Maherali and Hochedlinger, 2008). With regard to the reprogramming factors, some have been found to be dispensable, or they may be substituted with another factor. For example, it may be advisable to
replace c-MYC with LIN28, due to its oncogenic properties (González et al., 2011). Reprogramming may also be enhanced by the inclusion of small molecules such as the histone deacetylase inhibitor valproic acid (Huangfu et al., 2008).

One of the greatest considerations for any reprogramming experiment is the method of factor delivery. Takahashi and Yamanaka originally used a Moloney-based retrovirus, which is effectively silenced in the pluripotent cells, but carries a risk for genomic integration and associated insertional mutagenesis (Maherali and Hochedlinger, 2008; Takahashi et al., 2007). Lentivirus vector delivery systems carry a similar risk, but the vectors are able to transduce both dividing and non-dividing cells. Other non-integrative strategies can be effective, however they often require multiple applications and can have lower levels of expression (Maherali and Hochedlinger, 2008). More recently, replication-defective and persistent Sendai virus vectors (SeVdp) have been investigated, since they can carry all 4 transgenes within a single vector, and show a high level of reprogramming efficiency (Nishimura et al., 2011).

One of the appeals of iPSCs is that they can be used to derive cell types that would have otherwise not been available for study. This is particularly pertinent to the investigation of neurological conditions. The cells differentiated from the iPSCs share the same genetic background as the original patient cells from which they were derived, and therefore don't require any additional genetic manipulation. Furthermore, these cells allow for the study of development- and disease-associated mechanisms and pathways in a controlled environment, and can be used to test potential therapeutic interventions.

4.1.2 Differentiation of iPSCs into retinal cells

4.1.2.1 Control of human eye development

The mammalian eye is formed from cells derived from the mesodermal and ectodermal germ layers. The Wnt and Nodal pathways control key signalling networks involved in the developing embryo, where they control axis patterning, cell fate and migration. The inhibition of the Wnt and Nodal pathways results in the specification of the eye field in the anterior neural plate (Fuhrmann, 2008). The
The first morphological sign of eye development is the evagination of the optic vesicles from the ventral forebrain. Contact between the optic vesicle and the overlying surface ectoderm leads to the invagination of the lens placode, resulting in a double-layered optic cup (Gregory-Evans et al., 2013). The formation of the optic cup is dependent on the expression of *paired box 6* (*PAX6*), which causes congenital aniridia in humans when mutated (Quiring et al., 1994; Shaham et al., 2012). The inner layer of the optic cup forms the neural retina, containing the light-sensing photoreceptor cells (rod photoreceptors expressing rhodopsin and cone photoreceptors expressing S-, M- or L-opsin). The outer layer of the optic cup forms the RPE in response to the localised expression of *MITF* (Ramón Martínez-Morales et al., 2004). The iris and cilary body are formed at the boundary between the inner and outer layers of the optic cup.

The transcriptional control of mammalian neural retina development is a highly complex process involving the co-ordinated interaction of numerous genes. At the cellular level, the development of photoreceptors takes place in distinct stages (reviewed in Swaroop et al., 2010) (Figure 26). During early retinogenesis, multipotent retinal progenitor cells divide to produce either additional multipotent progenitors, or lineage-restricted progenitors which can give rise to photoreceptors. Once they have exited the cell cycle, the photoreceptor precursors are directed to become immature rods or cones. These cells start to express photoreceptor-specific genes (such as those required for phototransduction), grow axons to form functional synapses, and form outer segments. These cells become terminally differentiated, functional photoreceptors.
Figure 26: Stages of photoreceptor development. Early in retinogenesis, multipotent retinal progenitor cells (RPCs) divide and produce additional multipotent progenitors (thick circular arrow) or progenitor cells that become restricted in their competence to generate various cell types (thin circular arrow). Some of these proliferating cells become restricted to a lineage that will give rise to at least one photoreceptor cell and possibly to non-photoreceptor cells. After cell cycle exit, postmitotic precursors can remain plastic. During cell type specification of photoreceptors, precursors are directed to become cones or rods that eventually express photopigments (M opsin and S opsin in cones, and rhodopsin in rods), and form outer segments and synapses. Adapted from Swaroop et al., (2010) with permission by Nature Publishing Group.

Mouse-based studies have led to a "transcriptional dominance" model of this process of photoreceptor cell fate determination, involving the interplay between OTX2 (orthodenticle homeobox 2), CRX, NRL (neural retina leucine zipper), and other key regulators (Swaroop et al., 2010) (Figure 27). OTX2 is initially responsible for the steering of a retinal progenitor to a photoreceptor precursor fate (Nishida et al., 2003), whilst CRX and RORβ (RAR-related orphan receptor beta) induce the expression of S-opsin. The model proposes that the common postmitotic photoreceptor precursor follows a default pathway to become an S cone. RORβ also induces NRL expression, which results in CRX and NRL acting synergistically to activate NR2E3 (Nuclear Receptor Subfamily 2, Group E, Member 3). NRL and
NR2E3 form a complex that suppresses the expression of cone-related genes, and the NRL-CRX complexes promote the expression of rod-related genes (Oh et al., 2008; Peng et al., 2005) (Figure 27). Early photoreceptor development results in a high proportion of S cones within the neural retina, whereafter the actions of NRL and NR2E3 start to produce rod cells. An additional pathway involving interactions between RORα (RAR-related orphan receptor alpha), T3 (triiodothyronine) and TRβ3 (thyroid hormone receptor β2) controls the development and maturation of L, M and S cones (reviewed in Swaroop et al., 2010). In this way, changes in gene expression control the lineage choices of retinal progenitor cells, and cells become committed to a particular rod or cone fate. In humans, the first cones and rods are born around foetal weeks 8 and 10 respectively, and photoreceptors remain immature at birth. Rearrangement, packing and maturation of outer segments occurs postnatally.
Figure 27: Transcriptional dominance model of photoreceptor cell fate determination. A generic photoreceptor is formed under the control of homeobox protein OTX2 and other undetermined signals. This precursor is programmed to possess a ‘default’ S cone state under the control of OTX2 (and/or cone–rod homeobox protein (CRX)) and nuclear receptor RORβ unless diverted into a rod or M cone state by additional signals. Induction of neural retina leucine zipper protein (NRL) and its target, photoreceptor-specific nuclear receptor (NR2E3), induces a rod state and suppresses cone genes, which consolidates the rod fate. Adapted from Swaroop et al., (2010) with permission by Nature Publishing Group.

4.1.2.2 Approaches to modelling retinal development and differentiation in vitro

The development of the cells of the neural retina is a process that is tightly regulated, both temporally and spatially, and takes place over many months as described above. These cells are highly specialised, and their spatial organisation within the retinal tissue is a key feature that dictates their effective function. For these reasons it has been difficult to develop an effective protocol to differentiate photoreceptors from pluripotent stem cells, since one can currently not fully recapitulate the 3D developmental environment in vitro, and many of the complexities of the developmental process are still unknown. In spite of these challenges, many investigators have been successful in deriving retinal cells and optic cup-like structures from human and mouse ESCs and iPSCs (Table 15).
Table 15: Selected published protocols for differentiation of retinal cells from pluripotent stem cells. (SFEB - Serum-free embryoid body; PLF – poly-lysine, laminin, fibronectin; PR – photoreceptor)

<table>
<thead>
<tr>
<th>Original cell type</th>
<th>Differentiation system</th>
<th>Differentiation factors (apart from N2 and B27)</th>
<th>Differentiation period</th>
<th>Final cell type</th>
<th>Selected measures of cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse ES cells</td>
<td>SFEB culture. Plated on PLF plates after 5 days. Co-cultured with embryonic retinal cells to promote PR differentiation.</td>
<td>DKK1, LEFTY2, Activin A</td>
<td>17 days</td>
<td>Retinal progenitor-like cells</td>
<td>RX, PAX6</td>
<td>(Ikeda et al., 2005)</td>
</tr>
<tr>
<td>Human ES cells</td>
<td>SFEB. Plated on Matrigel or laminin after 3 days. Co-cultured with embryonic retinal cells to promote PR differentiation.</td>
<td>IGF, DKK1, NOG.</td>
<td>3 weeks</td>
<td>Retinal neurons</td>
<td>PAX6, CHX10, CRX, S-OPsin, RHO, NRL, PKCA (bipolar cells), PROX-1 (horizontal cells)</td>
<td>(Lamba et al., 2006)</td>
</tr>
<tr>
<td>Human ES cells</td>
<td>SFEB. Plated on PLF plates after 20 days.</td>
<td>DKK1, LEFTY2 (120 days). Retinoic acid, taurine (last 30 days)</td>
<td>150 days</td>
<td>Putative photoreceptors</td>
<td>RX, CRX, RHO, OPSINS, GNAT1, PDC, PEE6b, PDE6c, CNGA1, CRK1, SAG, ARR3, RDH12</td>
<td>(Osakada et al., 2008)</td>
</tr>
<tr>
<td>Human ES cells</td>
<td>SFEB. Plated on PLF plates after 21 days.</td>
<td>DKK1, LEFTY2, Y-27632. Retinoic acid, taurine (last 60 days)</td>
<td>150 days</td>
<td>Photoreceptors</td>
<td>RX, PAX6, MITF, CHX10, CRX, OPSINS, RHO, RCVRN</td>
<td>(Osakada et al., 2009a)</td>
</tr>
<tr>
<td>Human ES and iPS cells</td>
<td>SFEB. Plated on PLF plates after 21 days.</td>
<td>Y27632, CKI-7, SB431542. Retinoic acid, taurine (last 50 days)</td>
<td>150 days</td>
<td>Photoreceptors</td>
<td>CRX, PAX6, RX, CHX10, MITF, RCVRN, NRL, PDC, PDE6b, PDE6c</td>
<td>(Osakada et al., 2009b)</td>
</tr>
<tr>
<td>Human ES and iPS cells</td>
<td>SFEB. Plated on laminin plates after 6 days.</td>
<td>EGF, FGF2, Heparin</td>
<td>40 days</td>
<td>Photoreceptors</td>
<td>CHX10, CRX, MITF, NOG, OPSINS, OTX2, PAX6, RCVRN, RX</td>
<td>(Meyer et al., 2009)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>Cell clumps plated on Matrigel dishes. Neural clusters lifted on day 16 and grown as aggregates. Vesicle-like aggregates separated from day 20.</td>
<td>NOG, DKK1, IGF-1.</td>
<td>5 weeks</td>
<td>Photoreceptors</td>
<td>RCVRN, RHO, PAX6, S-OPsin, NRL, OTX2, CRX</td>
<td>(Lamba et al., 2010)</td>
</tr>
<tr>
<td>Mouse iPS cells</td>
<td>SFEB. Plated on lysine, collagen, laminin, fibronectin dishes after 5 days</td>
<td>NOG, DKK1, IGF-1, bFGF, DAPT, aFGF</td>
<td>33 days</td>
<td>Photoreceptors</td>
<td>PAX6, RX, NRL, OPSIN, RCVRN, RHO, CRX</td>
<td>(Tucker et al., 2011)</td>
</tr>
<tr>
<td>Human ES and iPS cells</td>
<td>SFEB. Plated on laminin after 6 days. Neural clusters lifted on day 16 and grown as aggregates. Vesicle-like aggregates separated from day 20.</td>
<td>NOG, DKK1, EGF, FGF2, Heparin</td>
<td>120 days</td>
<td>Optic vesicle-like structures</td>
<td>CHX10, CRX, LH2X, MITF, NRL, OTX2, PAX6, RCVRN and others.</td>
<td>(Meyer et al., 2011)</td>
</tr>
<tr>
<td>Cell Type</td>
<td>SFEB Conditions</td>
<td>Matrigel Additives</td>
<td>Days</td>
<td>Recruitment Stage</td>
<td>Marker Genes</td>
<td>References</td>
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<tr>
<td>Mouse ES cells</td>
<td>SFEB in 2% Matrigel solution</td>
<td>Laminin, entactin, Nodal</td>
<td>18</td>
<td>Optic cup-like structures</td>
<td>PAX6, CHX10, RX, PKCA, RCVRN, OTX2, BRN3, RHO</td>
<td>(Eiraku et al., 2011)</td>
</tr>
<tr>
<td>Human ES cells</td>
<td>SFEB in 1% Matrigel solution</td>
<td>Wnt inhibitor IWR1e, Hedgehog agonist SAG, CHIR99021</td>
<td>26</td>
<td>Optic cup-like structures</td>
<td>RX, CHX10, PAX6, MITF, RCVRN, NRL, RHO, S-OPsin</td>
<td>(Nakano et al., 2012)</td>
</tr>
<tr>
<td>Human ES and iPS cells</td>
<td>SFEB. Plated on poly-L-ornithine and laminin from day 30.</td>
<td>NOG, DKK1, IGF-1, SHH, thyromine, bFGF, retinoic acid, taurine, Activin A</td>
<td>60</td>
<td>Photoreceptors</td>
<td>CHX10, CRX, S-OPsin, L-OPsin, RHO, NEUROD, NESTIN, RCVRN, AR3</td>
<td>(Mellough et al., 2012)</td>
</tr>
<tr>
<td>Mouse ES cells</td>
<td>SFEB with 2% Matrigel.</td>
<td>Taurine, retinoic acid</td>
<td>36</td>
<td>Photoreceptor precursors</td>
<td>CRX, GNAT1, LHX2, NRL, NR2E3, NTE5, OTX2, PAX6, PRPH2, RAX, RCVRN, RH0, SIX3, SIX6, VSX2</td>
<td>(Gonzalez-Cordero et al., 2013)</td>
</tr>
<tr>
<td>Human ES and iPS cells</td>
<td>Clumps embedded in 2% Matrigel solution.</td>
<td>Retinoic acid, taurine, bFGF, aFGF, SHH</td>
<td>30</td>
<td>Photoreceptors</td>
<td>PAX6, RX, CRX, MITF, NRL, RHO</td>
<td>(Boucherie et al., 2013)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>SFEB. Plated on Synthemax dishes after 5 days.</td>
<td>DKK1, NOG, IGF-1, bFGF, aFGF, DAPT</td>
<td>90</td>
<td>Photoreceptors</td>
<td>OTX2, RCVRN, NF200, BRN3B, PAX6, CHX10, CRX, NRL, RHO</td>
<td>(Tucker et al., 2013)</td>
</tr>
<tr>
<td>Human iPS cells</td>
<td>SFEB. Plated on Matrigel after 7 days. Neural retina domains dissected and cultured in suspension.</td>
<td>Heparin, taurine, retinoic acid</td>
<td>98</td>
<td>Optic cup-like structures</td>
<td>LHX2, RX, VSX2, OTX2, RCVRN, TUJ1, MITF, OPSINS, RHO, PAX6</td>
<td>(Zhong et al., 2014)</td>
</tr>
</tbody>
</table>
The published protocols are all somewhat similar in their general strategy, and differ slightly in their culture system and/or factors (Table 15). Most studies have used a serum-free embryoid body-like (SFEB) culture system, where the pluripotent stem cells are initially grown as floating aggregates of cells for a period of time (ranging from a few days to weeks) before being re-plated on adherent dishes. This system is designed to mimic the 3D cellular environment of the early developing embryo. A multitude of growth factors and small molecules have been investigated for their capacity to mimic the molecular environment surrounding the cells of the developing neural retina. The inhibition of the Wnt and Nodal pathways can be mimicked \textit{in vitro} by using the recombinant proteins dickkopf-1 (DKK1) and left-right determination factor 2 (LEFTY2, or Lefty-A), or the small molecules CKI-7 and SB431542 (Ikeda et al., 2005; Osakada et al., 2009b). The differentiation of retinal cells can also be enhanced by the addition of noggin (NOG), retinoic acid, taurine and sonic hedgehog protein (SHH) (Table 15). The inhibition of bone morphogenic protein by NOG results in the neural induction of pluripotent cells (Dottori et al., 2010) and SHH promotes the proliferation and differentiation of photoreceptor cells (Levine et al., 1997). Retinoic acid also supports the differentiation and maturation of photoreceptor cells by activating NRL expression (Khanna et al., 2006) and taurine is necessary to prevent the degeneration of retinal ganglion cells and photoreceptors (Gaucher et al., 2012). The timing of differentiation varies between the protocols, with mouse studies ranging between 17 and 36 days, and human experiments ranging from 21 to 150 days, indicating that the chemical and physical culture conditions have an effect on the temporal differentiation process. The outcomes of the various methods also differ, with some methods producing early retinal progenitors or putative photoreceptors, and others generating whole optic cup-like structures (Table 15).

\textbf{4.1.3 In vitro modelling of SCA7 using iPSC-derived retinal cells}

Cone-rod dystrophy is considered to be a feature of SCA7, but the precise cause for the selected degeneration of photoreceptors remains elusive. This is particularly relevant in light of the fact that ATXN7 is expressed in many other tissues in the body. As previously discussed, the current hypothesis behind the development of
the SCA7 phenotype stems from the role that ATXN7 plays within the TFTC and STAGA transcriptional activator complexes (Helmlinger et al., 2004b; Helmlinger et al., 2006a), that leads to transcriptional changes in photoreceptor genes. Transcriptional dysregulation has been proposed to be a key pathogenic event associated with SCA7 and many other polyglutamine diseases (Carlson et al., 2009; Helmlinger et al., 2006b). It is also considered to be a primary event in disease progression (Helmlinger et al., 2006b). Transcriptional changes have been investigated in SCA7 mice models, revealing significant alterations in expression of photoreceptor-related genes, including the opsins, NRL and CRX (Abou-Sleymane et al., 2006; Chou et al., 2010; La Spada et al., 2001; Yoo et al., 2003). However, due to the lack of disease-relevant cell models for in vitro studies, these changes have not been investigated in human SCA7 patient-derived cells. Therefore, the aim of this study was to investigate whether SCA7 patient fibroblasts could be reprogrammed into iPSCs, which in turn could be differentiated into retinal cells, and whether these cells could be utilised for the investigation of SCA7-associated pathogenesis (with a focus on transcriptional dysregulation). The retinal differentiation protocol described by Boucherie and colleagues (2013) was selected for these experiments, since it was considered to be efficient and less expensive, and it spanned a shorter time frame than previously described methods. Using a system of embedding clumps of iPSCs in an extracellular matrix and culturing the cells with SHH, retinoic acid and taurine, Boucherie et al. were able to derive a high percentage of cells with a photoreceptor identity after 4 weeks. It was therefore anticipated that this method may be suitable for the investigation of transcriptional changes in SCA7 patient-derived cells.
4.1.4 Aims and Objectives

Broad Aim: To develop and assess an iPSC-derived model for the study of retinal degeneration associated with SCA7.

Objectives:

1. To reprogram dermal fibroblasts obtained from SCA7 patients and an unaffected family member into induced pluripotent stem cells (iPSCs).

2. To characterise the iPSCs, with respect to expression of pluripotency markers, silencing of the Sendai virus, in vitro differentiation capacity and karyotypic normality.

3. To differentiate the iPSCs into retinal cells, using a published protocol.

4. To characterise the retinal cells by monitoring expression of various markers by quantitative PCR and immunocytochemistry.

5. To determine whether there are transcriptional differences between SCA7 patient-derived fibroblasts, iPSCs and retinal cells when compared to unaffected control-derived cells.
4.2 Methods

4.2.1 Creation of SCA7 patient- and control-derived iPSCs

Two SCA7 families were contacted by a genetic nursing sister from the Division of Human Genetics at UCT. The families were selected from the patient registry and database based on their physical location (residents of the greater Cape Town area) and their family composition (each family consisted of both affected and unaffected individuals). The families attended the neurogenetics clinic at Groote Schuur Hospital, where they were counselled by a genetic counsellor and a clinical geneticist. Following the informed consent process, a 4mm skin biopsy was taken by a nurse or doctor, transported to the tissue culture laboratory in cell culture medium, and cultured as described below. A total of five individuals agreed to participate in the study (four affected and one unaffected). The participants consented to their cells being utilised for SCA7-based research to be undertaken within the Division of Human Genetics at the University of Cape Town, subject to approval by the institutional Human Research Ethics committee (details can be found in Appendix 1- Ethics (pg 168). Cells from three of these individuals (two patients and one mutation negative individual) were utilised for the experiments described here. All samples have been de-identified to protect anonymity.

4.2.1.1 Skin biopsies and dermal fibroblast isolation

Skin biopsies were taken from the inner forearm. The area was wiped with a sterile swab before administration of a local anesthetic. A sterile circular biopsy punch (4 millimeter diameter, Miltex,) was used to biopsy a section of skin (deep enough to include the dermis), which was placed in a sterile container containing standard culture medium (Appendix 5 – Cell culture medium and preparation of additives, pg 177). Skin biopsies were transported at room temperature and processed within 48 hours. Dermal fibroblasts were isolated and cultured as described in Appendix 6 – Cell culture protocols (pg 180).

4.2.1.2 Preparation of mouse embryonic fibroblast feeder cells (MEFs)

Mouse embryonic fibroblasts (MEFs) were used as feeder cells during the derivation, maintenance and culture of iPSCs. The MEFs were isolated from day
14.5 embryos (BALB/c strain), and inactivated at passage 5 to create mitotically inactive MEFs or iMEFS (details in Appendix 6 – Cell culture protocols, pg 181). In some cases commercially available MEFs were used (ATCC SCRC-1040). iMEF feeders were plated one day before iPSCs. Wells were coated with a 0.1% gelatin solution (Appendix 6 – Cell culture protocols, pg 182). For culture in a 6-well culture plate (35cm wells), approximately $0.3 \times 10^6$ iMEFs were thawed and added to each well in MEF culture medium. The cells were incubated overnight at 37°C. The following day, each well was washed three times with 1x phosphate buffered saline (PBS) solution to remove floating cells and traces of MEF medium, and replaced with fresh iPSC culture medium. Whereafter iPSCs were added to the well following thawing or passaging.

### 4.2.1.3 Reprogramming of dermal fibroblasts

The reprogramming of the patient- and control-derived fibroblasts was carried out by Dr Robea Ballo during a research visit to the James Martin Stem Cell Facility (within the Sir William Dunn School of Pathology) at the University of Oxford, where she was assisted by Dr Sally Cowley. Reprogramming of dermal fibroblasts into iPSCs was achieved through the introduction of Sendai virus vectors (SeVdp) containing *OCT4, SOX2, KLF4* and *c-MYC* (Nishimura et al., 2011), and detailed methods can be found in Appendix 6 – Cell culture protocols (pg 184). Initial iPSC colonies from each reprogramming experiment were manually picked using a sterile needle, and clonally expanded. Each iPSC clonal line was assigned a unique name. Putative iPSC lines were frozen and transported to the Division of Human Genetics at the University of Cape Town for characterisation and further experiments. All characterisation and differentiation experiments were carried out by Danielle Smith, unless otherwise stated.

### 4.2.1.4 Culture of iPSCs

Depending on the downstream application or experiment, iPSC colonies were either cultured as patches on iMEFs (as described previously) in iPSC culture medium, or were adapted to feeder-free conditions (Appendix 6 – Cell culture protocols, pg 185). When grown as patches on iMEFs, 50% of the iPSC culture medium was replaced with fresh medium every day. Every 5-7 days the patches were manually
dissected with a sterile needle, and placed onto fresh iMEFs with 10µM Y-27632 (Sigma Aldrich). Under feeder-free conditions, the iPSCs were grown on huES-qualified Matrigel matrix (Corning) in mTESR medium (a defined, feeder- and serum-free maintenance medium, StemCell Technologies), and passaged enzymatically every 5-7 days (Appendix 6 – Cell culture protocols, pg 186).

4.2.2 Characterisation of iPSC lines

iPSCs were cultured and maintained in the Tissue Culture Laboratory within the Division of Human Genetics. Since the cells and their derivatives were to be used for in vitro studies, each line was characterised according to a list of minimal criteria (Maherali and Hochedlinger, 2008). In order to determine whether the reprogrammed dermal fibroblasts were pluripotent and resembled human embryonic stem cells, a battery of tests was performed on each iPSC line.

4.2.2.1 Expression of pluripotency markers

iPSCs were cultured and prepared for immunocytochemistry (Appendix 8 – Immunocytochemistry, pg 192) to confirm the expression of two selected pluripotency markers (OCT4 and TRA-1-60). iPSCs were analysed as colonies grown on iMEFs, since the feeder cells could act as an internal negative control (showing no expression of pluripotency markers). Details of image processing and antibodies are supplied in Appendix 8 – Immunocytochemistry and Appendix 3 – Antibodies used for immunocytochemistry (pg 174 and pg 192). These experiments were completed by Dr Lauren Watson.

4.2.2.2 Silencing of reprogramming virus

In addition to confirming the expression of pluripotency markers, immunocytochemistry using an antibody raised against the Sendai virus vector was used to verify that the virus was silenced in the reprogrammed cells. The Sendai virus contained a microRNA (miRNA) target binding site designed to be targeted by mir-302a, a miRNA selectively expressed in embryonic stem cells (Nakanishi et al., 2012). Therefore fully reprogrammed iPSCs would silence the Sendai virus through the endogenous miRNA pathway. iPSCs cultured as colonies on iMEFs were fixed and processed for immunocytochemistry, and fluorescence levels were compared
to newly-infected fibroblast cells. These experiments were completed by Dr Lauren Watson.

4.2.2.3 Expression of pluripotency genes

The expression of selected pluripotency genes (OCT4, SOX2, NANOG) was confirmed by quantitative PCR (qPCR). Three 35mm wells of each iPSC line were cultured in feeder-free conditions until confluent. The cells were lifted enzymatically and centrifuged at 1500 revolutions per minute (rpm) for 5 minutes to form a cell pellet, which was either stored at -80°C until needed, or immediately subjected to RNA isolation. The RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions. Following extraction, the RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer and NanoDrop 1000 software (NanoDrop Technologies). Complementary DNA (cDNA) was synthesised from 1µg of RNA using the High Capacity Reverse Transcription Kit (Applied Biosystems). Excess RNA was stored at -80°C. The cDNA for each isolated sample was diluted in nuclease-free water to a final concentration of 10ng/µl and stored at -20°C.

The qPCR assays were completed using the Power SYBR Green PCR Master Mix (Life Technologies, according to the manufacturer's instructions) with 10ng of cDNA, on the CFX96 Real-Time PCR Detection System (Bio-Rad). All samples were completed in technical triplicate. Wherever possible, methods and analyses adhered to guidelines stipulated by Bustin et al., (2009). For the purposes of this study, the relative quantification method of analysis was used, since the aim of the study was to compare the differences in expression levels between SCA7 patient- and unaffected control-derived cells. The β-actin was selected as the reference gene. In order to use the relative standard curve method of analysis, a dilution series (containing cDNA of known concentrations, prepared from pooled representative samples) was prepared (25, 12.5, 6.25, 3.125, 1.625 ng/µl), and used in qPCR reactions with each primer pair. These samples were used to prepare standard curves for each test gene (each primer pair), by plotting the log of the concentration of each sample (x-axis) versus the corresponding Ct value (y-axis). The equations of the resulting straight line graphs were obtained, to be used for analysis of the test samples. Ct values for each test sample were attained using the CFX Manager.
software (version 3.0, Bio-Rad). In order to calculate the concentration of cDNA in each unknown sample (test samples), the experimentally obtained Ct value was used in the equation acquired from the standard curve previously prepared for the gene of interest. This value could then be normalised to the β-actin value obtained for the same sample (as the endogenous control), to account for different amounts of starting material. These values could then be used for downstream analyses. The experiments for iPSC lines P1a, P2a and C1a were completed by Dr Lauren Watson.

4.2.2.4 Karyotyping

In addition to standard pluripotency tests, it was also necessary to confirm the absence of genetic aberrations acquired during the reprogramming or culture process (Maherali and Hochedlinger, 2008). This was achieved through karyotype analysis (G-banding). iPSCs were cultured in feeder-free conditions, and a confluent 25 cm² flask was transferred to the NHLS Cytogenetics laboratory at Groote Schuur Hospital for processing and analysis (Appendix 6 – Cell culture protocols, pg 186).

4.2.2.5 Confirmation of iPSC in vitro differentiation capacity

Each iPSC line underwent directed in vitro differentiation in order to confirm the iPSC capacity to form cell types derived from each of the three embryonic germ layers (endoderm, mesoderm and ectoderm). iPSCs were cultured as colonies on iMEFs (as described previously) for up to 7 days. Thereafter each colony was mechanically dissected using a sterile syringe needle into numerous patches. The number of patches cut from each colony depended on the total size (usually 16 to 25 patches). The patches were cultured in poly(2-hydroxyethyl methacrylate)-coated wells (poly-HEMA coated, Appendix 6 – Cell culture protocols, pg 187) for 24 hours in iPSC culture medium, in order to form embryoid bodies (EBs, spheres of iPSCs grown in suspension).

In order to induce differentiation into cells of an endodermal lineage, the culture medium was changed to embryoid body culture medium (EB medium, Appendix 5 – Cell culture medium and preparation of additives, pg 178) after 24 hours and cultured for 2 to 3 days. Thereafter the EBs were seeded into culture dishes
previously prepared containing coverslips with a coating of 0.1% gelatin (Appendix 6 – Cell culture protocols, pg 182). The medium was changed every 2-3 days for an additional 10-15 days, whereafter the dishes were processed for immunocytochemistry. For mesodermal differentiation, iPSC medium was changed to EB medium supplemented with 0.5mM ascorbic acid and cells were grown and plated as described for endodermal differentiation. For ectodermal differentiation, iPSC medium was changed to culture medium containing N2 and B27 supplements. The EBs were seeded into wells containing a monolayer of confluent inactivated PA6 cells (newborn mouse bone marrow-derived stromal cells). The culture medium was changed every 2 to 3 days for an additional 10 to 15 days. Details outlining the preparation of culture media and supplements are given on pg 178. At the end of the culture period the cells were fixed and processed for immunocytochemistry (Appendix 8 – Immunocytochemistry, pg 192). Different antibodies were used to detect markers characteristically expressed in each specific germ layer (Table 16). The processing and imaging for iPSC lines P1a, P2b C1a was completed by Dr Janine Scholefield.

Table 16: Antibodies for in vitro differentiation immunocytochemistry

<table>
<thead>
<tr>
<th>Germ Layer</th>
<th>Antibody</th>
<th>Full name</th>
<th>Cell/tissue types (from the Human Protein Atlas &amp; Abcam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoderm</td>
<td>Anti-FOXA2</td>
<td>Forkhead box A2</td>
<td>Airway epithelium, breast, urinary bladder, prostate, intestinal cells</td>
</tr>
<tr>
<td></td>
<td>Anti-AFP</td>
<td>Alpha-fetoprotein</td>
<td>Liver cells</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>Anti-ASA</td>
<td>Sarcomeric alpha actinin</td>
<td>Skeletal and cardiac muscle</td>
</tr>
<tr>
<td></td>
<td>Anti-SMA</td>
<td>Smooth muscle actin</td>
<td>Smooth muscle cells of blood vessels and parenchymal tissue of intestine, testis and ovary</td>
</tr>
<tr>
<td>Ectoderm</td>
<td>Anti-GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>Astrocytes</td>
</tr>
<tr>
<td></td>
<td>Anti-TUBB3</td>
<td>Tubulin, beta 3 class III</td>
<td>Central nervous system &amp; peripheral nerves</td>
</tr>
</tbody>
</table>

4.2.3 Differentiation of iPSCs into retinal cells

Initial iPSC-based studies within the Division of Human Genetics utilised retrovirally-reprogrammed cells, and the first attempt at generating retinal cells used the small molecule protocol described by Osakada and colleagues (Osakada et al., 2009b). Although this protocol resulted in a small percentage of cells expressing CRX and
recoverin (RCVRN), it was considered to be expensive, since the differentiation period spanned over 150 days. Shortly thereafter the newly reprogrammed Sendai virus iPSCs became available, along with the publication of shorter retinal differentiation protocols, therefore the initial study was not included in these results, but is available on request.

The 1-month differentiation protocol followed that previously described by Boucherie et al. (2013). Prior to differentiation, the iPSCs were cultured in feeder-free conditions on Matrigel in mTESR medium. The cells were dissociated enzymatically and plated onto Matrigel-covered dishes in neural differentiation medium (Appendix 7 - Differentiation of iPSCs into retinal cells, pg 190) containing N2 and B27 supplements (Life Technologies). After settling for an hour, adhered cells were covered in a 2% Matrigel solution in order to create a "sandwich" system. The following day the medium was replaced with neural differentiation medium without Matrigel, and cells were fed with fresh medium every second day. From day 10 the medium was supplemented with 3nM recombinant sonic hedgehog (SHH), 50ng/µl acidic fibroblast growth factor (aFGF), 10ng/µl basic fibroblast growth factor (bFGF), 1mM taurine and 500nM retinoic acid, factors previously shown to play significant roles in the development and maturation of photoreceptors (Levine et al., 1997). Cells were pelleted and frozen at -80°C at various time points over the differentiation period, or fixed and processed for immunocytochemistry (at the end of the culture period). An overview of the differentiation protocol is given in Figure 28, and full details are given in Appendix 7 - Differentiation of iPSCs into retinal cells (pg 190).
Figure 28: Overview of 1-month photoreceptor differentiation protocol (based on Boucherie et al., 2013). The iPSCs were cultured embedded in Matrigel for a period of 30 days in medium supplemented with N2 and B27. From day 10 the medium was supplemented with recombinant sonic hedgehog (SHH), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), taurine and retinoic acid.

4.2.4 Characterisation of retinal cells

During the differentiation period the cells were monitored in order to determine whether they were exhibiting the expected morphology at specific time points. At these stages (approximately every 10 days), cells from selected wells were dissociated with TrypLE, pelleted, and frozen at -80°C to be used for qPCR experiments (as described on page 98). A set of 14 retinal genes was selected from the literature, and the expression of a subset of these was monitored over the differentiation period. These included ATXN7, CRX, PAX6, OTX2 and NRL. The expression of the remaining genes was determined at the end of the culture period (Day 30). These included RHO, opsin 1 (cone pigments, long-wave-sensitive (OPN1LW), opsin 1 (cone pigments) medium-wave-sensitive (OPN1MW), opsin 1 (cone pigments) short-wave-sensitive (OPN1SW), RCVRN, retinal pigment epithelium-specific protein 65kDa (RPE65), Microphthalmia-associated transcription factor (MITF), arrestin 3 (ARR3) and guanine nucleotide binding protein (G protein), \textit{alpha transducing activity polypeptide 1} (GNAT1). A number of these genes have also been shown to be dysregulated in SCA7 mouse models (Table 17). At the end of the differentiation period the remaining wells of cells were fixed and processed for immunocytochemistry using antibodies against ATXN7, CRX and RCVRN.
4.2.5 Determination of transcriptional differences between SCA7- and control-derived fibroblasts, iPSCs and retinal cells

With the aim of determining whether any transcriptional differences could be detected between SCA7 patient- and unaffected control-derived cell types, a panel of candidate genes was selected. These genes were chosen based on previous transcriptional studies using mouse model retinal and cerebellar tissue (Abou-Sleymane et al., 2006; Chou et al., 2010; Yoo et al., 2003). The panel included the following genes: ATXN7, brain expressed, X-linked 1 (BEX1), DnaJ (Hsp40) homolog, subfamily A, member 1 (DNAJA1), glutamate receptor, ionotropic, AMPA 2 (GRIA2), heat shock protein 27 (HSP27), heat shock protein 70 (HSP70), heat shock protein 105 (HSP105), oligodendrocyte transcription factor 1 (OLIG1), and ubiquitin carboxyl-terminal esterase L1 (UCHL1). (Table 17). The expression of these genes was determined in fibroblasts, iPSCs and retinal cells (at various time points) derived from SCA7 patients and unaffected control individuals. The details of these individuals are given in Section 4.3.1 (pg 109).
Table 17: Transcriptionally dysregulated genes identified in SCA7 models.

<table>
<thead>
<tr>
<th>Gene name and function</th>
<th>Gene symbol (human)</th>
<th>Function</th>
<th>Model</th>
<th>Tissue/cell type</th>
<th>Up or down-regulated</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cone arrestin</td>
<td>ARR3</td>
<td>Retinal signal transduction</td>
<td>R7E, R7N, R6/2</td>
<td>Retina</td>
<td>Down</td>
<td>Affymetrix, qPCR</td>
<td>(Abou-Sleymane et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCA7-92Q</td>
<td>Retina</td>
<td>Down</td>
<td>qPCR</td>
<td>(La Spada et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7E</td>
<td>Retina</td>
<td>Down</td>
<td>qPCR</td>
<td>(Helmlinger et al., 2006a)</td>
</tr>
<tr>
<td>Brain-expressed X-linked 1</td>
<td>BEX1</td>
<td>Neuronal differentiation</td>
<td>Ataxin 7-Q52</td>
<td>Cerebellum</td>
<td>Up</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
</tr>
<tr>
<td>Cone-rod homeobox containing gene</td>
<td>CRX</td>
<td>Photoreceptor differentiation</td>
<td>R7E, R7N, R6/2</td>
<td>Retina</td>
<td>Down</td>
<td>Affymetrix, qPCR</td>
<td>(Abou-Sleymane et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7E</td>
<td>Retina</td>
<td>Down</td>
<td>qPCR</td>
<td>(Helmlinger et al., 2006a)</td>
</tr>
<tr>
<td>DnaJ (HSP40) homolog, subfamily A, member 1</td>
<td>DNAJA1</td>
<td>Chaperone protein</td>
<td>Ataxin 7-Q52</td>
<td>Cerebellum</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCA7-266Q</td>
<td>Eye &amp; Brain</td>
<td>Down</td>
<td>Northern blot</td>
<td>(Yoo et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7E, R7N, R6/2</td>
<td>Retina</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Abou-Sleymane et al., 2006)</td>
</tr>
<tr>
<td>Rod transducin alpha subunit</td>
<td>GNAT1</td>
<td>Modulator of visual impulses</td>
<td>R7E, R7N, R6/2</td>
<td>Retina</td>
<td>Down</td>
<td>Affymetrix, qPCR, Northern blot</td>
<td>(Abou-Sleymane et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SCA7-92Q</td>
<td>Retina</td>
<td>Down</td>
<td>qPCR</td>
<td>(La Spada et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7E</td>
<td>Retina</td>
<td>Down</td>
<td>qPCR</td>
<td>(Helmlinger et al., 2006a)</td>
</tr>
<tr>
<td>Subunit of glutamate AMP A receptor</td>
<td>GRIA2</td>
<td>Neurotransmitter receptor</td>
<td>Ataxin 7-Q52</td>
<td>Cerebellum</td>
<td>Up</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
</tr>
<tr>
<td>Heat shock protein, 105 kDa</td>
<td>HSP105</td>
<td>Heat shock protein</td>
<td>Ataxin 7-Q52</td>
<td>Cerebellum</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
</tr>
<tr>
<td>Heat shock protein, 27 kDa</td>
<td>HSP27</td>
<td>Heat shock protein</td>
<td>SCA7-92Q</td>
<td>Transformed lymphoblasts</td>
<td>Down</td>
<td>Semi-quantitative RT-PCR &amp; Western blot</td>
<td>(Tsai et al., 2005)</td>
</tr>
<tr>
<td>Protein</td>
<td>Gene Symbol</td>
<td>Function</td>
<td>Location</td>
<td>Expression</td>
<td>Assay</td>
<td>References</td>
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<td>---------------------------------</td>
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<tr>
<td>Heat shock protein, 70 kDa</td>
<td>HSP70</td>
<td>Heat shock protein</td>
<td></td>
<td>Down</td>
<td>Semi-quantitative RT-PCR &amp; Western blot</td>
<td>(Tsai et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Neural retina leucine zipper</td>
<td>NRL</td>
<td>Regulator of rod-specific genes</td>
<td>R7E, R7N, R6/2</td>
<td>Down</td>
<td>Affymetrix, qPCR</td>
<td>(Abou-Sleymane et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>Olig1 bHLH protein</td>
<td>OLIG1</td>
<td>Formation of oligodendrocytes</td>
<td>Ataxin 7-Q52</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Green cone opsin</td>
<td>OPN1MW</td>
<td>Photopigment</td>
<td>R7E, R7N, R6/2</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Abou-Sleymane et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>Blue cone opsin</td>
<td>OPN1SW</td>
<td>Photopigment</td>
<td>SCA7-266Q</td>
<td>Down</td>
<td>Northern blot</td>
<td>(Yoo et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Recoverin</td>
<td>RCVRN</td>
<td>Cycling of rhodopsin</td>
<td>R7E, R7N, R6/2</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Abou-Sleymane et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>RHO</td>
<td>Photopigment</td>
<td>SCA7-266Q</td>
<td>Down</td>
<td>Northern blot</td>
<td>(Yoo et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Ubiquitin carboxy-terminal</td>
<td>UCHL1</td>
<td>Processing of ubiquitinated proteins</td>
<td>Ataxin 7-Q52</td>
<td>Down</td>
<td>Affymetrix microarray</td>
<td>(Chou et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>hydrolase LI</td>
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</tr>
</tbody>
</table>
For the fibroblast experiments, RNA was extracted and cDNA synthesised from a confluent 25cm² flask of cells. iPSC-based experiments were completed on cDNA derived from RNA isolated from a confluent 35mm dish of iPSCs grown on Matrigel (feeder-free system). For experiments based on retinal cells, RNA was isolated from differentiated dishes of cells on Day 30 (35mm dishes). Unless otherwise stated, all extractions were completed in biological replicate or triplicate (RNA was isolated from 2-3 dishes or flasks of cells grown in parallel). RNA extractions, cDNA synthesis and qPCR experiments were performed as previously described.

Statistical analyses to compare patient- and control-derived samples were completed using the Mann-Whitney test for unpaired samples, utilising a two-tailed distribution and assuming unequal variances. Analysis was performed in Microsoft Office Excel 2007, and significance was defined as p≤0.05. An overview of the full experimental procedure is presented in Figure 29.
Figure 29: Overview of experimental procedure. Dermal fibroblasts were isolated from two SCA7 patients (P1 and P2), and an unaffected control individual (C1, sister of P2). The fibroblasts were infected with a reprogramming Sendai virus to derive five different iPSC lines (P1a, P2a, P2b, C1a, C1b). The iPSC lines were characterised to confirm pluripotency. Thereafter they were differentiated into retinal cells in three separate experiments (1, 2 and 3). The retinal cells were characterised to confirm expression of retinal cell markers. Transcriptional differences between the patients and control were determined by qPCR using RNA from the fibroblasts, iPSCs and retinal cells.

It has also been hypothesised that somatic instability within key tissues may exacerbate disease symptoms in triplet repeat disorders (Lin et al., 2009). In order to evaluate the length of the CAG repeat in patient- and control-derived cultured
cells (fibroblasts, iPSCs and differentiated cells), a PCR reaction was designed and optimised to enable genotyping of the CAG repeat using cDNA as a template. Within a 10 µl reaction, 50ng of cDNA was included with 1x Failsafe Premix buffer J (Epicentre Biotechnologies), 0.4 µM/L of each primer and 0.6 units of GoTaq DNA polymerase (Promega). Cycling conditions were as follows: 95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 53°C for 6 seconds, and 72°C for 40 seconds; and a single cycle of 72°C for 7 minutes on the MultiGene thermocycler (Labnet International). The resulting PCR product was subjected to agarose gel electrophoresis to determine the success of the reaction (Appendix 4 – Molecular reagents and protocols, pg175). PCR products were further analysed by capillary electrophoresis as described previously. The expected size of the PCR fragment was 325 bases, excluding the CAG repeat, therefore the number of repeats could be calculated.
4.3 Results

4.3.1 Patient material

iPSC lines were generated from three individuals from two families. Patient 65.3 (hereafter referred to as P1) was a male from a SCA7 family who had initially presented with symptoms at age 34. Molecular diagnostic tests at the age of 37 revealed that he carried an expansion in \( ATXN7 \) with 47 repeats (Table 18). At the time of the skin biopsy he was 50 years old. He could no longer walk (fully dependent on a wheelchair) and was completely blind.

Patient 213.2 (hereafter referred to as P2) was a 44 year old female from a family with an affected mother. At the time of the biopsy the patient had not undergone molecular testing for SCA7, but had complained of visual problems. Blood was taken and the patient was given a molecular diagnosis of SCA7 (41 repeats). A biopsy was also taken from the patient’s 33 year old unaffected sister. PCR-based testing showed a single allele corresponding to 10 repeats, and TP PCR was used to confirm that the individual did not carry a pathogenic expansion. This individual (213.3, hereafter referred to as C1) served as an unaffected control (mutation negative) sample. For this study, a single iPSC clonal line was used from individual 65.3 (clone 65.3.1), since no additional characterised clonal lines were available at the initiation of experiments. Two clonal lines were used from individuals 213.2 and 213.3 (Table 18). In order to simplify results, the two patients were coded as "P1" or "P2", the control individual as "C1", and each clone was designated as "a" or "b", as outlined in Table 18.

<table>
<thead>
<tr>
<th>Individual code</th>
<th>CAG genotype</th>
<th>Age at diagnosis</th>
<th>Age at biopsy</th>
<th>iPSC lines</th>
<th>iPSC codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.3</td>
<td>10/47</td>
<td>37</td>
<td>50</td>
<td>65.3.1</td>
<td>P1a</td>
</tr>
<tr>
<td>213.2</td>
<td>10/41</td>
<td>44</td>
<td>44</td>
<td>213.2.2</td>
<td>P2a</td>
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<td>213.2.5</td>
<td>P2b</td>
</tr>
<tr>
<td>213.3</td>
<td>10/10</td>
<td>n/a</td>
<td>33</td>
<td>213.3.5</td>
<td>C1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>213.3.7a</td>
<td>C1b</td>
</tr>
</tbody>
</table>
4.3.2 Characterisation of iPSCs

4.3.2.1 Morphology

Reprogramming of fibroblasts from the three individuals (P1, P2 and C1) was completed in the James Martin Stem Cell Facility in Oxford as outlined in section 4.2.1.3 (pg 96). Following Sendai virus infection, iPSC colonies with the correct morphology appeared within 3 to 4 weeks. These colonies were flat and had distinct borders, and contained tightly packed cells with a high nucleus-to-cytoplasm ratio (Figure 30). The colonies were manually picked and clonally expanded in separate dishes on iMEFs.

![Figure 30: Morphology of iPSC colonies. Colonies could be seen as groups of tightly packed cells with a high nuclear-to-cytoplasm ratio. The colonies also had distinct borders that separated the iPSCs from the surrounding mouse embryonic feeder fibroblasts. a) iPSC colonies at 10x magnification. b) iPSC colony at 20x magnification. Images courtesy of Dr Robea Ballo.](image)

4.3.2.2 Expression of pluripotency markers

To determine whether each clonal iPSC line expressed markers associated with pluripotency, each line was subjected to various tests. Immunocytochemical analysis of all of the iPSC lines revealed iPSC colonies with distinct OCT4 nuclear staining, compared to the surrounding mouse embryonic feeder fibroblasts (Figure 31). The iPSC colonies also stained positive for TRA-1-60, a cell surface antigen expressed by undifferentiated human embryonic stem cells (Fong et al., 2009).
P1a

OCT4

a)

b)

P2a

c)

d)

P2b

e)

f)

C1a

g)

h)

C1b

i)

j)
The expression of pluripotency markers was also confirmed by real-time quantitative PCR (qPCR). The five iPSC lines expressed high levels of OCT4, SOX2 and NANOG, standard markers of pluripotency (Figure 32). Little or no expression was found in the donor fibroblasts or cells that had been subjected to a retinal differentiation protocol for 10 days. The expression levels of SOX2 and NANOG were similar across the five iPSC lines, but lines P2b and C1b showed lower levels of OCT4 expression compared to the remaining three lines (although still significantly higher than differentiated fibroblasts and retinal cells). Together, these results confirmed that all five iPSC lines expressed markers associated with pluripotency.
Figure 32: Expression of pluripotency markers in iPSC lines, determined by qPCR. All five iPSC lines (P1a, P2a, P2b, C1a, C1b) expressed OCT4 (a), SOX2 (b) and NANOG (c), compared to low expression levels in the original donor fibroblasts (pooled data from all three fibroblast lines), or cells subjected to the retinal differentiation protocol for 10 days (Day 10 PRs, pooled data from lines P2b and C1b). All levels shown relative to β-actin. Error bars show standard error from the mean. iPSC data for lines P1a, P2a and C1a courtesy of Dr Lauren Watson.

4.3.2.3 Silencing of Sendai virus

To determine whether the iPSC lines could show growth and maintenance independent of reprogramming gene expression, immunocytochemistry was used to confirm that the iPSC lines did not contain traces of the reprogramming Sendai virus. The Sendai virus vector used for reprogramming contained a miRNA target site to enable the silencing of reprogramming gene expression in pluripotent cells (Nishimura et al., 2011). The vector was also replication-defective, and would therefore be diluted with subsequent cellular divisions. The iPSC colonies were co-stained with primary antibodies against the pluripotency marker OCT4 and the nucleocapsid protein of the virus (anti-NP). All lines were assessed after passage 8. iPSC colonies from all five lines showed little or no evidence of NP staining (Figure
33b-d), compared to intense cytoplasmic staining in newly infected fibroblasts (Figure 33a), indicating that the Sendai virus had been effectively silenced.

![Image](attachment:image_url)

**Figure 33**: Silencing of the reprogramming Sendai virus. Newly infected fibroblasts (a) and iPSC colonies (b-d) co-stained with primary antibodies against OCT4 (red) and the viral nucleocapsid protein (green). Nuclei in blue. Images courtesy of Dr Lauren Watson.

### 4.3.2.4 In vitro differentiation

In order to test the differentiation capacity of the iPSC lines, they were cultured in various growth conditions to drive differentiation toward a particular cell lineage (endodermal, mesodermal or ectodermal cells). Dishes cultured with EB medium resulted in cells of an endodermal lineage, which stained positive for either forkhead box A2 (FOXA2) or alpha-fetoprotein (AFP) (Figure 34a, d, g, j, m). FOXA2 is expressed in airway epithelium, breast, urinary bladder, prostate, intestinal cells, whilst AFP is expressed in liver cells. Mesodermal cells were derived from culturing iPSCs in EB medium supplemented with ascorbic acid, and showed positive staining for sarcomeric alpha actinin (ASA, a marker of skeletal and cardiac muscle) or smooth muscle actin (SMA) (Figure 34b, e, h, k, n). Finally, iPSC cultured in EB medium containing N2 and B27 resulted in cells staining positive for glial fibrillary acidic protein (GFAP) and tubulin (beta 3 class III, TUBB3), indicative of astrocytes and nerves of the central and peripheral nervous systems (Figure 34c, f, i, l, o). All
five iPSC lines demonstrated the capacity to differentiate into cell types derived from the three embryonic germ layers.
4.3.2.5 Karyotyping

It was necessary to assess the karyotypic stability of iPSC lines, since it has been shown that human ESCs have a tendency to acquire large genetic aberrations over long culture periods (Draper et al., 2004; Maherali and Hochedlinger, 2008). The five iPSC lines were assessed by standard Giemsa banding analysis, which revealed no gross structural abnormalities in any of the lines (reports in Appendix 10 – iPSC karyotype reports, pg 198). All iPSC lines were assessed after passage 10.

4.3.3 Differentiation of iPSCs into retinal cells

In order to differentiate the iPSCs into retinal cells, the cells were subjected to a previously published differentiation protocol (Boucherie et al., 2013). Three separate differentiation experimental runs were performed, using identical procedures and timelines. The aim of the first experiment was to assess the reproducibility of the published protocol, and was carried out using iPSC lines P1a and C1b. This experimental run did not contain any biological replicates, and only one well of cells was analysed per line at each time point (Table 19). The second experiment used iPSCs derived from the other SCA7 patient (P2b), along with the same control line (C1b). The final differentiation experiment used iPSC lines from both SCA7 patients (P1a and P2a), with a different clonal line from the unaffected control individual (C1a) (Table 19). Experiments 2 and 3 were completed using biological replicates (two or three wells of cells were analysed per line at each time point). There were no further experimental differences between the three experiments.
### Table 19: Overview of experimental rounds of retinal differentiation

<table>
<thead>
<tr>
<th>Differentiation experiment</th>
<th>Patient iPSC line(s)</th>
<th>Unaffected control iPSC line</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P1a</td>
<td>C1b</td>
</tr>
<tr>
<td>2</td>
<td>P2b</td>
<td>C1b</td>
</tr>
</tbody>
</table>
| 3                         | P1a
P2a                     | C1a                         |

### 4.3.3.1 Changes in cell organisation and morphology

Before the initiation of the differentiation protocol, the iPSCs were changed to a feeder-free culture system and cultured on Matrigel for 2-3 passages in order to remove any contaminating iMEFs, which may have interfered with differentiation and subsequent expression analyses. On Matrigel the iPSCs grew as a monolayer and were passaged every 5-6 days (Figure 35a). The retinal differentiation protocol described by Boucherie et al., (2013) recommended that clumps of the iPSCs be embedded between two layers of Matrigel (1% and 2%), forming what is often termed a "Matrigel sandwich". This culture system created a 3-dimensional environment for the differentiating cells, which was designed to mimic the neural stem cell niche in the developing embryo, since the Matrigel provided a complex of laminin, collagen IV, nidogen and proteoglycans (Boucherie et al., 2013). Generally, the sandwich system facilitated a rapid self-organisation and differentiation of the pluripotent stem cells into structures containing cells morphologically indicative of columnar neuroepithelia (Figure 35b). These structures lost their integrity from day 4-5 (Figure 35c), and cells spread into an adherent monolayer. By day 10, cells with a neuronal morphology could be seen in areas of low confluence (Figure 35d). Later in the differentiation period (day 19), large adherent vesicle-like structures were visible in some dishes, similar in appearance to floating optic-vesicle-like structures previously described in other protocols (Gonzalez-Cordero et al., 2013; Nakano et al., 2012) (Figure 35e). The appearance of more cells with a neuronal morphology was observed towards the end of the 30 day culture period (Figure 35f-i).
Although the differentiating cells in each experiment generally exhibited a similar differentiation process as described, various inter- and intra-well differences were noted. For example, on day 4 of the first differentiation experiment, it was noted that some cells had organised into separate neuroepithelium-containing structures (Figure 36a, b), whilst in more confluent areas of the dish there were monolayers of cells containing neural rosettes (Figure 36c, d). Some wells contained cells with long neuronal processes (Figure 36e, f). On day 30, some areas of the same dish contained tightly-packed clumps of cells with indistinct morphologies (Figure 36g,
h), but in areas of low confluence the cells showed a more neuronal morphology (Figure 36i-l). These morphological observations suggested that the differentiation experiments produced a mixed population containing neuronal cells by Day 30.
4.3.3.2 Expression of retinal cell markers

To determine whether the differentiated cells expressed retinal cell markers, immunocytochemical analyses were performed on cells at the end of the differentiation period (day 30). The cells were stained for either the disease-causing protein ATXN7, or the retinal cell markers CRX and RCVRN (Figure 37). The transcription factor CRX is expressed in rod and cone precursors and mature photoreceptors, as well as rod bipolar cells, and is considered to be the earliest photoreceptor marker to be expressed in the developing retina (Hennig et al., 2008). RCVRN is a calcium-binding protein that is commonly used as a pan photoreceptor marker (Dizhoor et al., 1991).
The differentiated cells with a neuronal morphology revealed a diffuse expression of ATXN7 within both the nucleus and cytoplasm, consistent with patterns observed in SCA7 patient-derived tissues (Einum et al., 2001) (Figure 37). No consistent differences in ATXN7 expression levels or localisation were noted between patient- and control-derived cells. CRX and RCVRN showed similar patterns of expression (Figure 37). Analysis of iPSCs (non-retinal cells) confirmed the specificity of the CRX and RCVRN antibodies, since no positive staining was observed (Figure 37n-o).

As previously discussed, a high degree of morphological and cytological heterogeneity was observed, both within a single well of cells, and between each well. This made it difficult to note any differences between affected and unaffected cells, and it was also difficult to estimate the differentiation efficiency of the protocol. A high percentage of cells expressed the photoreceptor markers CRX and RCVRN (in accordance with the 60% efficiency as published by Boucherie et al.
and most of the dishes contained large, brightly-stained clusters of cells. No obvious intranuclear inclusions were observed in any of the iPSCs or the differentiated cells.

In addition to monitoring of cellular morphological and organisational changes over the differentiation period, cells were pelleted and frozen at regular intervals, to allow for analysis of gene expression over time. qPCR was performed using primers for various retinal genes, including ATXN7. No biological replicates were included in experiment 1. Experiment 3 contained results from two patient lines and a control line run concurrently with biological triplicates, and therefore represented the most robust results.

Four key regulatory genes involved in retinal development were monitored over the experimental differentiation period, namely PAX6, OTX2, CRX and NRL, as well as ATXN7. In all the differentiation experiments, PAX6 showed a steady increase in expression over the culture period (Figure 38). At the end of the culture period in experiments 1 and 3, the patient line P1a showed higher PAX6 expression than the control line, whilst in experiment 2 and the patient line P2b and P2a (derived from the same individual) showed a lower level of expression than the control. Experiments 1 and 2 showed an initial spike in OTX2 expression, followed by a decline, although this was not the case in the patient-derived cells in experiment 3 (Figure 39). CRX expression closely mimicked the patterns shown for PAX6, with a steady increase in expression over time (Figure 40). The cells derived from P1a showed higher expression levels than the control line in experiments 1 and 3, whilst the cells derived from individual P2 showed lower levels than the control. There was evidence for stable expression of NRL in the differentiating cells, although this was at a very low level in experiment 3 (Figure 41), indicating that that particular experiment may have contained a lower proportion of rod photoreceptors. There was a general trend indicating an increase in ATXN7 expression over the 30 day period across the three experiments (Figure 42), and ATXN7 showed higher expression levels in the patient-derived lines compared to the control lines at multiple time points.
Figure 38: qPCR results showing PAX6 expression in cells undergoing retinal differentiation in Experiment 1 (a), 2 (b) and 3 (c). In each case the unaffected control cell line is shown in green, with the patient lines in red or orange. Error bars show standard error. All results expressed relative to β-actin.
Figure 39: qPCR results showing *OTX2* expression in cells undergoing retinal differentiation in Experiment 1 (a), 2 (b) and 3 (c). In each case the unaffected control cell line is shown in green, with the patient lines in red or orange. Error bars show standard error. All results expressed relative to β-actin.
Figure 40: qPCR results showing CRX expression in cells undergoing retinal differentiation in Experiment 1 (a), 2 (b) and 3 (c). In each case the unaffected control cell line is shown in green, with the patient lines in red or orange. Error bars show standard error. All results expressed relative to β-actin.
Figure 41: qPCR results showing NRL expression in cells undergoing retinal differentiation in Experiment 1 (a), 2 (b) and 3 (c). In each case the unaffected control cell line is shown in green, with the patient lines in red or orange. Error bars show standard error. All results expressed relative to β-actin.
Figure 42: qPCR results showing ATXN7 expression in cells undergoing retinal differentiation in Experiment 1 (a), 2 (b) and 3 (c). In each case the unaffected control cell line is shown in green, with the patient lines in red or orange. Error bars show standard error. All results expressed relative to β-actin.
In order to better understand the composition of the final differentiated cell population, qPCR was performed on cells obtained from day 30 of experiments 2 and 3 (Figure 43). Primers were included for the pan-photoreceptor marker RCVRN, RHO, the cone opsins OPN1LW, OPN1MW and OPN1SW, as well as RPE65 and MITF (markers of RPE), and ARR3 and GNAT1 (downstream targets of CRX). The differentiated cells expressed varying levels of RCVRN, RHO, and the cone opsins, although RCVRN expression was generally higher than the opsins (Figure 43a-e). Compared to the opsin genes, RPE65 and MITF showed reasonably high levels of expression across all experiments (Figure 43f, g). Finally, the expression of ARR3 and GNAT1 was determined in the differentiated cells. Both genes are known targets of CRX, and have been shown to be downregulated in SCA7 mice (Abou-Sleymane et al., 2006; La Spada et al., 2001); however, no significant differences were noted (Figure 43h,i). The expression levels of the opsins, ARR3, and GNAT1 were extremely low compared to the reference gene β-actin, and therefore it is likely that the relative expression results were somewhat inaccurate, since they lay outside the confidence parameters of the assay.
4.3.4 Transcriptional dysregulation in patient-derived cells

4.3.4.1 Transcriptional changes in patient-derived differentiated retinal cells

RNA was isolated from the retinal cells at the end of each differentiation experiment (day 30), and used in qPCR experiments in order to identify potential transcriptional changes in SCA7-derived cells. Multiple transcriptional changes were noted in the SCA7-derived retinal differentiated cells (Table 20), therefore a selected number of genes that showed consistent results, or were of particular interest, will be discussed.

Many expression changes in retinal cell-associated genes have already been noted, but the selected panel contained an additional group of genes that were not restricted to retinal tissues. The expression of BEX1 was increased in both patient-
derived cell lines included in Experiment 3, when compared to the control line (Table 20). K(Lysine) Acetyltransferase 2A (KAT2A), also known as GCN5, the histone acetyltransferase that forms part of the STAGA transcriptional activator complex (Nagy and Tora, 2007), showed significant reduction in expression in both patient lines in experiment 3 of the retinal differentiation, and showed a trend towards down-regulation in patient cells in experiment 2 (Table 20). OLIG1 is a transcription factor expressed in the myelinating oligodendrocytes of the central nervous system, and is required for the repair of demyelinated lesions (Arnett et al., 2004). OLIG1 showed increased expression levels in patient cells in experiments 1 and 3. These results indicated that there were some transcriptional alterations in SCA7 patient-derived retinal cells, but none of the tested genes showed consistent dysregulation across all three experimental runs.
Table 20: Transcriptional changes in SCA7 patient-derived retinal cells, compared to unaffected control cells.

Gene expression graphs are given on page 203.

<table>
<thead>
<tr>
<th>Retinal-associated genes:</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>P1a vs C1b</td>
<td>P2b vs C1b</td>
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<td>NS</td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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<td>NS</td>
</tr>
<tr>
<td>RHO</td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
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<td>SIG UP</td>
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<tr>
<td>Other genes:</td>
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<td></td>
<td></td>
</tr>
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<td>BEX1</td>
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<td>NS</td>
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<td>NS</td>
</tr>
<tr>
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</tr>
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<td>NS</td>
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</tr>
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<td>UP</td>
<td>NS</td>
</tr>
<tr>
<td>UCHL1</td>
<td>DOWN</td>
<td>NS</td>
<td>DOWN</td>
</tr>
</tbody>
</table>

Key:
- **UP** = up-regulated (p≤0.2)
- **SIG UP** = significantly up-regulated (p≤0.05)
- **DOWN** = down-regulated (p≤0.2)
- **SIG DOWN** = significantly down-regulated (p≤0.2)
- **NS** = non-significant
- **ND** = not detected/determined
4.3.4.2 Transcriptional changes in fibroblasts and iPSCs

Although they are considered to be unaffected cell types, it was thought that it might be instructive to establish whether there are transcriptional changes in patient-derived fibroblasts and undifferentiated iPSCs. For the experiments in fibroblasts, cells from individuals P1, P2 and C1 were analysed, along with fibroblasts isolated from an additional SCA7 family. Family 926 consisted of five affected individuals from three generations. The affected father (926.3) had a genotype of 10 and 41 CAG repeats within \textit{ATXN7}, and the expanded allele had been passed on to three of his children (926.1, 926.2 and 926.5) (Table 21). The father had suffered from poor vision and unsteadiness from the age of 46. At the time of biopsy individuals 926.1 and 926.2 were suffering from severe spasticity and ataxia, accompanied by vision loss. Fibroblasts were also obtained from the unaffected mother (926.8) and one unaffected child (926.7).

<table>
<thead>
<tr>
<th>Individual code</th>
<th>CAG genotype</th>
<th>Age of onset (self-reported)</th>
<th>Age at biopsy</th>
</tr>
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<td>26</td>
</tr>
<tr>
<td>926.2</td>
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<td>17</td>
</tr>
<tr>
<td>926.3</td>
<td>9/41</td>
<td>46</td>
<td>52</td>
</tr>
<tr>
<td>926.7</td>
<td>9/10</td>
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<td>14</td>
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<tr>
<td>926.8</td>
<td>9/10</td>
<td>n/a</td>
<td>46</td>
</tr>
</tbody>
</table>

Transcriptional differences between the two patient fibroblasts lines (P1 and P2) were compared to the control line (C1) in two independent experiments (Table 22, Experiment 1 and Experiment 2), and fibroblasts from the additional SCA7 family were included in a third experiment (three affected, two unaffected individuals). Significant changes were only noted in experiment 2 when comparing fibroblasts from individuals P2 and C1 (sisters). \textit{ATXN7} was found to be significantly down-regulated in the patient-derived cells. Modest down-regulation of the heat shock proteins \textit{DNAJA1}, \textit{HSP27} and \textit{HSP70} was also noted, with an up-regulation of the ubiquitin-protein hydrolase \textit{UCHL1}. In the three experiments using the iPSC lines,
no transcripts showed consistent dysregulation across multiple experiments, apart from HSP27 which showed a trend towards down-regulation in experiments 2-4 (Table 23). ATXN7 showed no significant changes in expression, apart from experiment 4, where there was an increase in expression in P2 iPSCs, compared to the control. No transcripts showed significant changes when the results from all the fibroblast or iPSC samples were pooled.

Table 22: Transcriptional changes in SCA7 patient fibroblasts, compared to unaffected control fibroblasts. Family 926 comprised three patients and two unaffected controls. Gene expression graphs are given on page 205.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Experiment 1 P1 vs C1</th>
<th>Experiment 2 P2 vs C1</th>
<th>Family 926</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATXN7</td>
<td>NS</td>
<td>SIG DOWN</td>
<td>NS</td>
</tr>
<tr>
<td>BEX1</td>
<td>NS</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DNAJA1</td>
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<td>DOWN</td>
<td>NS</td>
</tr>
<tr>
<td>GRIA2</td>
<td>ND</td>
<td>NS</td>
<td>ND</td>
</tr>
<tr>
<td>HSP105</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HSP27</td>
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</tr>
<tr>
<td>OLIG1</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>UCHL1</td>
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<td>SIG UP</td>
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Key:
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- **DOWN** = down-regulated (p≤0.2)
- **SIG DOWN** = significantly down-regulated (p≤0.2)
- **NS** = non-significant
- **ND** = not detected/determined
Table 23: Transcriptional changes in SCA7 patient iPSCs, compared to unaffected control cells. Gene expression graphs are given on page 206.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Experiment 1 P1a vs C1b</th>
<th>Experiment 2 P2b vs C1b</th>
<th>Experiment 3 P1 vs C1a, C1b</th>
<th>Experiment 4 P2a, P2b vs C1a, C1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATXN7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>SIG UP</td>
</tr>
<tr>
<td>BEX1</td>
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</tr>
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<td>DNAJA1</td>
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<td>NS</td>
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- **UP** = up-regulated (p≤0.2)
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- **SIG DOWN** = significantly down-regulated (p≤0.2)
- **NS** = non-significant
- **ND** = not detected/determined

**4.3.4.3 Determination of the CAG repeat length in mRNA from SCA7 patient fibroblasts, iPSCs and retinal cells.**

A PCR-based assay was used to confirm the size of the ATXN7 CAG repeat alleles in mRNA from patient- and control-derived fibroblasts, iPSCs and retinal cells. The results were visualised on an agarose gel (Figure 44), and confirmed by fluorescent genotyping (data not shown). This assay confirmed that both the mutant and wild-type ATXN7 alleles were expressed by all three cell types, and there were no obvious differences in allele expression in affected or unaffected cells (Figure 44).
Figure 44: Determination of the CAG repeat length in ATXN7 mRNA from SCA7 patient fibroblasts, iPSCs and retinal cells, by semi-quantitative PCR. PCR products (5μl) were electrophoresed on a 3% (w/v) agarose gel at 160V for 30 minutes. Lanes 1-3 show the results from the fibroblasts, iPSCs and retinal cell mRNA of individual P1 (respectively). Lanes 4-6 show the results from the fibroblasts, iPSCs and retinal cell mRNA of individual P2 (respectively). Lanes 7-9 show the results from the fibroblasts, iPSCs and retinal cell mRNA of individual C1 (respectively). Lane 10 shows the no template control, and "L" indicates the molecular weight marker (GeneRuler 100bp Plus DNA ladder, Fermentas). Samples from all three individuals showed a single band at approximately 355bp, corresponding with a wild-type allele, whilst patient cell lines P1 and P2 showed an additional larger band corresponding to a mutant allele.
4.4 Discussion

The broad aim of the study was to determine whether iPSC-derived cells could be effectively created and employed to model SCA7 retinal degeneration in vitro. iPSCs were successfully generated from two SCA7 patients (P1 and P2), along with a control sample from a patient’s unaffected sister (C1). The cells were characterised in terms of expression of pluripotency markers and karyotypic stability, and were capable of differentiating in cell types derived from all three embryonic germ layers. Thereafter, the characterised iPSCs were subjected to a differentiation protocol with the aim of producing retinal cells. This was completed in three separate differentiation experiments, with each experiment using different patient and control iPSC clonal lines. The differentiated cells were assessed at various time points to confirm the expression of retinal cell markers. Finally, expression analyses were performed on the differentiated cells, to identify transcriptional differences between SCA7 patient and unaffected control-derived cells, which may give some insight into pathogenic mechanisms or consequences.

4.4.1 Can iPSCs differentiate into retinal cells in vitro?

Firstly, it was necessary to assess whether the chosen differentiation protocol was effective in producing retinal cells. The morphologies and organisation of the differentiating cells were monitored by microscopy throughout the three 30 day protocols (Figure 35, pg 119). Early observations indicated that some neuroepithelial cells were produced within the culture dishes, resulting in cells with a distinct neuronal morphology towards the end of the culture period. Immunocytochemistry experiments showed that a heterogeneous cell population was produced by the end of the 30 days, containing a large proportion of cells expressing the photoreceptor markers CRX and RCVRN (Figure 37, pg 123). Quantitative PCR experiments were completed at the end of the differentiation experiments, revealing expression of the retinal cell genes PAX6, OTX2, CRX and NRL in the differentiating cells (Figure 38 to Figure 41, pg 125). The cells also expressed varying levels of the photoreceptor genes RCVRN, RHO, and the cone opsins (Figure 43, pg 132). OPN1SW showed higher expression levels than OPN1LW, OPN1MW and RHO, consistent with the knowledge that S-opsin is expressed earlier in the
fetal retina (Xiao and Hendrickson, 2000). RPE65 and MITF showed reasonably high levels of expression across all experiments (Figure 43f, g). Although pigmented cells were only observed in two dishes in experiment 1, the expression levels of these genes suggest that the dishes were "contaminated" with cells differentiating towards an RPE fate. Together, these results confirmed that the retinal differentiation protocol was capable of producing a proportion of cells expressing markers of "mature" photoreceptors. However, the low expression levels of these genes at day 30 points to the likelihood that these cells were either produced at a very low percentage (the protocol was inefficient), or that 30 days was too short a time period to produce mature photoreceptor cells.

4.4.2 Is retinal development recapitulated in in vitro differentiation experiments?

In order to better understand the differentiation process, the expression levels of a selected number of retinal genes were monitored at specific time points (approximately every 10 days). These genes had been previously shown to be some of the key regulators of embryonic retinal development. Three of these genes, namely PAX6, CRX and NRL showed a steady increase in expression over the culture period in all three experiments (Figure 38, Figure 40, Figure 41, pg 125). In the mature retina, PAX6 expression is lost in post-mitotic photoreceptor precursors and bipolar neurons, but is maintained in other retinal cell types, including amacrine, horizontal and ganglion cells (Macdonald and Wilson, 1997; Shaham et al., 2012). These results suggest that there was either an increase in PAX6-expressing retinal progenitors over the 30 days, or that the final population of cells contained a significant percentage of amacrine, horizontal and ganglion cells (or other PAX6-expressing cell types). If the protocol was efficient in producing a high percentage of mature photoreceptors, one would expect an initial increase in PAX6 expression, followed by a decline in expression as the cells became post-mitotic.

CRX is predominantly expressed in retinal photoreceptors and pinealocytes (Chen et al., 1997). CRX acts synergistically with NRL to transactivate RHO, and is also responsible for transactivating multiple photoreceptor genes, including IRBP (interphotoreceptor retinoid-binding protein) and the other colour opsins, through
binding the consensus binding site C/TTAATCC (Chen et al., 1997; Furukawa et al., 1997; Livesey et al., 2000). In addition to its key role in the development of retinal tissues, CRX expression is maintained into adulthood. Microarray analysis of CRX-/mice revealed expression changes in various components of the phototransduction pathway (including RHO, RCVRN and ARR3), revealing a wide transcriptional network that is controlled by CRX (Livesey et al., 2000). In the retinal differentiation experiments presented here, CRX expression closely mimicked the patterns shown for PAX6, with a steady increase in expression over time (Figure 40, pg 127), consistent with results obtained from developmental studies.

NRL is expressed exclusively in rod photoreceptors (Swain et al., 2001), and acts synergistically with CRX to control the expression of rod-associated genes (Chen et al., 1997). The loss of NRL in mice results in the transformation of rods into functional S-cones, and expression of NRL under control of the CRX promoter results in mice with only rod photoreceptors (Mears et al., 2001; Oh et al., 2007). These experiments highlighted the role of NRL in the specification of rod cell fate, as well as the apparent plasticity of photoreceptor precursors during early retinogenesis (Oh et al., 2007). In the retinal differentiation experiments there was evidence for stable expression of NRL in the differentiating cells, although this was at a very low level in experiment 3 (Figure 41, pg 128), indicating that that particular experiment may have contained a low proportion of rod photoreceptors.

The fourth gene analysed, OTX2, showed an initial spike in expression in Experiments 1 and 2, followed by a decline, although this was not the case in the patient-derived cells in experiment 3 (Figure 39, pg 126). OTX2 is a homeobox transcription factor expressed in the anterior neuroectoderm of the developing embryo (Simeone et al., 1993). Mouse studies illustrated the role of OTX2 in photoreceptor development, since conditional knockout mice expressing inactivated OTX2 under control of the CRX promoter showed a complete loss of photoreceptors, and the developing cells were instead converted to amacrine neurons (Nishida et al., 2003). The same study showed that OTX2 is an upstream regulator of CRX. OTX2 is expressed in the mature retina, in photoreceptors, bipolar cells, and the retinal pigment epithelium (Béby et al., 2010). The reasons for the
decreased expression of OTX2 in the differentiation experiments is unclear, although it could further indicate that the differentiation protocol produced a high percentage of amacrine cells, which do not express OTX2.

It is challenging to reconcile the results obtained in this study with published models of retinal cell fate determination. Significant differences in expression of retinal genes were noted between differentiated cells in all three differentiation experiments, which bring the reproducibility of the protocol and the purity of the cell population into question. The interpretation of results associated with this type of experiment is difficult, since an increase in gene expression could indicate that the cells were up-regulating a specific gene, or more cells within the population were expressing the gene. Likewise, a decrease in expression could indicate a down-regulation of expression, or that some of the gene-expressing cells were dying. It was clear that the directed differentiation of pluripotent cells into retinal cells resulted in a heterogeneous cell population. This heterogeneity may have been due to the fact that the protocol started with clusters of cells of indeterminate sizes. Pluripotent cells are sensitive to both their spatial organisation, as well as the molecular cues provided by the culture medium, so different sized clusters may show variations in differentiation. It is also feasible that the culture medium did not provide strong enough cues to "push" the iPSCs toward the same lineage, particularly if there were any contaminating non-pluripotent cells in the initial culture. In order to fully understand the in vitro differentiation procedure, it would be necessary to monitor the expression of a large panel of genes and proteins throughout the differentiation period, in order to highlight the key mechanisms and pathways involved in this highly complex process.

4.4.3 Can SCA7 retinal degeneration be modelled in vitro in iPSC-derived cells?

The SCA7 patient- and unaffected control-derived iPSCs were differentiated into retinal cells with the ultimate aim of gaining some insight into pathogenic mechanisms of the disease. The expression of the disease-causing gene ATXN7 was monitored at various stages of the differentiation experiments. There was a general trend indicating an increase in ATXN7 expression over the 30 day period across the three experiments (Figure 42, pg 129). This was consistent with the knowledge that
ATXN7 forms part of the STAGA/TFTC transcriptional activator complexes, which mediate the expression of multiple photoreceptor-associated genes through the interaction with transcription factors such as CRX (Helmlinger et al., 2004b). It is therefore likely that ATXN7 is expressed at very low levels in the pluripotent stem cells, but is upregulated in response to retinal differentiation cues. ATXN7 showed higher expression levels in the patient-derived lines compared to the control lines at multiple time points across all three experiments. By day 30 of the retinal differentiation period the patient-derived lines showed lower expression of ATXN7 than the control line (Figure 42b, c), indicating that an endogenous mechanism may be involved in correcting the changes in expression. In 2011, Sopher and colleagues completed a study illustrating that ATXN7 expression is regulated by a convergently transcribed antisense noncoding RNA, called spinocerebellar ataxia-7 antisense noncoding transcript 1 (SCAANT1), which binds to and represses an alternative promoter within ATXN7 (Sopher et al., 2011). The authors found increased levels of ATXN7 expression correlating with larger repeat sizes in SCA7 fibroblasts and lymphocyte samples, and went on to show that expansion of the ATXN7 CAG repeat tract interferes with SCAANT1 promoter activity. Reduction in SCAANT1 resulted in de-repression of the alternative promoter, and increased expression of ATXN7. This mechanism may explain the increased expression levels in the SCA7 iPSCs and retinal cells at earlier time points, and could provide an alternative means of future therapeutic interventions.

Immunocytochemical analysis of ATXN7 expression in the retinal cells at the end of the differentiation period showed diffuse expression within the nucleus and cytoplasm of examined cells. In this study, no obvious intranuclear inclusions were observed in any of the iPSCs or the differentiated cells. This may be due to the likelihood that the differentiated cells represent a population of cells at an early stage of development, rather than adolescent or adult cells which were examined in the literature. It is also possible that an external stimulus or stressor is necessary for the formation of aggregates. This hypothesis was neatly demonstrated by Koch et al., who showed that excitation of SCA3 patient iPSC-derived neurons resulted in the cleavage and aggregation of ATXN3 (Koch et al., 2011). The issue of inducing
ageing in iPSC-derived cells is not restricted to SCA7, since many neurological
diseases display a late age of onset that may not be recapitulated in in vitro cell
culture conditions. Some novel methods of overcoming these problems have been
pursued, and will be discussed in Chapter 5. The utility of monitoring nuclear
inclusions in polyglutamine disease modelling has been questioned, since the
inclusions are often present in non-affected cell types such as the kidney or
pancreas (Jonasson et al., 2002). An alternative hypothesis has proposed that the
larger inclusions visible by microscopy play a protective role, whilst smaller
microaggregates interfere with cellular physiology (Todd and Lim, 2013). Additional
experiments would be required to determine whether SCA7 iPSC-derived
photoreceptors form aggregates in conditions more representative of the diseased-
state.

Since transcriptional dysregulation has been proposed to be a key mechanism
underlying pathogenesis in the polyglutamine disorders (Helmlinger et al., 2006b),
including SCA7 (Chou et al., 2010), the neurogenetics research group within the
Division of Human Genetics at UCT has compiled a panel of genes that have been
previously shown to be dysregulated in SCA7 mouse and cell models (Table 17, pg
104). The aim was to monitor the expression of these genes in various SCA7
patient-derived cell types, with the hope that key players in pathogenesis may be
identified, or that a "transcriptional signature" may be established for affected cells.
An additional advantage of having a panel of genes which are consistently
dysregulated in affected cells, is that these genes can act as biomarkers when
testing therapeutic interventions in vitro (Scholefield et al., 2014).

RNA was isolated from the retinal cells at the end of each differentiation
experiment (day 30), and used in qPCR experiments in order to identify potential
transcriptional changes in SCA7-derived cells. The evidence supports the hypothesis
that the differentiation experiments resulted in a highly heterogeneous population
of cells which differed across wells and experiments. Multiple transcriptional
changes were noted in the SCA7-derived retinal differentiated cells (Table 20, pg
134). However, it is likely that the majority of these changes may be attributed to
experimental differences, rather than pathogenic mechanisms, and little confidence
can be placed on these results without further refinement of the experimental model and follow-up experiments. For these reasons, a selected number of genes that showed consistent results, or were of particular interest, will be discussed here.

The interaction between ATXN7 and CRX has been hypothesised to be a key factor behind the development of retinal degeneration in SCA7 patients. In the experiments presented here, the cells derived from P1a showed higher expression levels than the control line in experiments 1 and 3, whilst the cells derived from individual P2 showed lower levels than the control. One might place a greater degree of confidence in the relationship between the patient P2 and the control C1, since they share a similar genetic background, but the exact reasons for these expression differences still remain unclear, and the potential effect of experimental differences can not be ignored. Western blots in 14-week-old SCA7 transgenic mice showed reduced levels of the CRX protein, although these mice had already shown significant photoreceptor cell loss compared to 7-week-old mice, who did not show a reduction in CRX expression. These results suggested that the reduction in CRX expression does not precede cell loss in the SCA7 retina, indicating that phororeceptor dysfunction, rather than cell loss, may the leading cause of blindness (La Spada et al., 2001). The expression of multiple known CRX targets, which were previously shown to be down-regulated in SCA7 mice, were included in the gene expression experiments. None of these target genes (including OPN1SW, ARR3, GNAT1 or RHO) showed consistent changes in patient cells across the three experiments.

The expression of BEX1 was increased in both patient-derived cell lines included in Experiment 3, when compared to the control line (Table 20, pg 134). These results were consistent with those observed by Chou and colleagues, who detected increased BEX1 expression in the cerebellum of ataxin 7-Q52 mice (Chou et al., 2010). BEX1 was initially identified as a small protein that was involved in neurotrophin signalling and neuronal differentiation (Vilar et al., 2006). Subsequent studies demonstrated that BEX1 is expressed at high levels in retinal ganglion cells, and accumulates in the nucleus after optic nerve stroke in rats (Bernstein et al.,
2006). Additionally, BEX1 has been associated with axon regeneration, since BEX1 knock-out mice show a reduced capacity for regeneration of peripheral and sciatic nerves after injury than wild-type mice (Khazaei et al., 2010). Therefore, the up-regulation of BEX1 in SCA7-affected retinal cells could point to an endogenous regeneration pathway.

KAT2A is also known as GCN5, the histone acetyltransferase that forms part of the STAGA transcriptional activator complex (Nagy and Tora, 2007). Although there are no established links between ATXN7 and the expression of KAT2A, numerous studies have identified a functional interaction between the two proteins, which results in changes in STAGA activity in in vitro and in vivo models of SCA7 (Burke et al., 2013; Chen et al., 2012; Helmlinger et al., 2006a; Palhan et al., 2005). These reports differ in their proposed mechanisms and effects of mutant ATXN7 on STAGA, but loss of GCN5 expression has been shown to result in increased retinal degeneration in SCA7 mice, although this was not accompanied by changes in expression of ATXN7 transcriptional targets (Chen et al., 2012). KAT2A expression was significantly reduced in both patient lines in experiment 3 of the retinal differentiation, and showed a trend towards down-regulation in patient cells in experiment 2 (Table 20, pg 134), which may be indicative of a negative feedback loop affecting the expression of KAT2A, and possibly other components of STAGA.

A clear link between the down-regulation of OLIG1 and the presence of mutant ATXN7 has not been established, although it was also found to be reduced in the cerebellum of SCA7 mice (Chou et al., 2010). OLIG1 is a transcription factor expressed in the myelinating oligodendrocytes of the central nervous system, and is required for the repair of demyelinated lesions (Arnett et al., 2004). OLIG1 showed increased expression levels in patient cells in experiments 1 and 3, perhaps indicating that there was a higher number of oligodendrocytes in these cultures. Retinal ganglion cells are not myelinated within the retina, but become myelinated in the distal region of the optic nerve (Bartsch et al., 1994). The first 10 days of the retinal differentiation protocol consisted of a "general neuronal differentiation" stage, before any additional factors were introduced to induce differentiation into retinal cells. It is therefore feasible that the final culture was contaminated with
neuronal cells which had already differentiated to an extent by day 10, and which were not influenced by the addition of the retinal factors.

Transcriptional changes in patient-derived fibroblasts and iPSCs were also determined. These cells express ATXN7, therefore transcriptional alterations may provide insight into potential protective mechanisms (for example, up-regulation of heat shock proteins), or they may indicate pathogenic pathways that are overridden by additional protective systems. Modest down-regulation of the heat shock proteins DNAJA1, HSP27 and HSP70 was noted, with an up-regulation of the ubiquitin-protein hydrolase UCHL1. The reason for the down-regulation of the heat shock proteins is unclear, but UCHL1 may be up-regulated as part of a ubiquitin proteosome system response to degrade the mutant ATXN7 protein. No significant changes were observed in fibroblasts derived from Family 926, which may be due to the larger cohort size, or the relatively "young" age of the fibroblasts (RNA was isolated at passage 2, compared to passages 5-9 for experiments 1 and 2). The iPSCs presented as an additional "unaffected" cell type which could be studied to identify transcriptional changes in vitro (Table 23, pg 137). No transcripts showed consistent dysregulation across multiple experiments, apart from HSP27 which showed a trend towards down-regulation in experiments 2-4 (p≤0.2). HSP27 belongs to the family of small heat shock proteins, and plays a role in the cellular stress response by acting as a molecular chaperone and a regulator of apoptosis (reviewed by Concannon et al., 2003). The role of HSP27 in pathogenesis has been extensively investigated in SCA3, since it was shown that it was down-regulated in both neuronal and non-neuronal cells expressing mutant ATXN3 (SK-N-SH neuroblastoma cells and patient-derived lymphoblasts) (Chang et al., 2009; Chang et al., 2005; Wen et al., 2003). However, prolonged culture of the cells resulted in an eventual increase in HSP27 expression, indicating a response to toxicity in "older" cells (Chang et al., 2005). Follow-up studies confirmed that mutant ATXN3 interferes with HSP27 protein synthesis, and over-expression of HSP27 results in reduced levels of apoptosis in vitro (Chang et al., 2009). Levels of HSP27 protein were also found to be reduced in SCA7 patient-derived lymphoblasts (Tsai et al., 2005), therefore it would be of interest to determine whether the transcriptional
changes observed in the iPSCs in these experiments are translated to the protein level.

A PCR-based assay was used to confirm the size of the ATXN7 CAG repeat alleles in mRNA from patient- and control-derived fibroblasts, iPSCs and retinal cells. Firstly, this served as a "fingerprinting" assay, to confirm that the iPSCs and retinal cells were derived from the same patient, and that no samples had been swapped during the experimental procedures. Since the assay utilised cDNA from cell-derived mRNA, it was semi-quantitative. It therefore confirmed that both the mutant and wild-type ATXN7 alleles were expressed by all three cell types, and there were no obvious differences in allele expression in affected or unaffected cells (Figure 44, pg 138). These results are consistent with those obtained from other iPSC-derived models of polyglutamine disorders. Western blot analysis confirmed the expression of both ATXN3 alleles in iPSC-derived neuroepithelial-like stem cells from SCA3 patients, coinciding with no changes in CAG repeat length for up to 47 passages (Koch et al., 2011). Similarly, HD patient neural stem cell lines displayed limited or no instability with passaging and differentiation (HD Consortium, 2012). The iPSC-based experiments described here indicated that both alleles were constantly expressed in vitro in multiple cell types, and it is unlikely that pathogenesis can be attributed to differing expression levels of the mutant and wild-type alleles, or a somatic increase in CAG repeat size.

4.4.4 Factors to consider in future studies

Transcriptional dysregulation of multiple potential disease-associated transcripts was noted in SCA7 patient-derived fibroblasts, iPSCs and differentiated retinal cells, which may warrant further investigation. These experiments provide the first description of SCA7 iPSC-derived retinal cells and their transcriptional phenotype. However, the experimental strategy requires significant refinement to allow for more confidence to be placed in these results. The differentiation protocol described by Boucherie et al. (2013) was selected based on its relative ease, low cost, and reported high efficiency, but alternative protocols should be investigated and evaluated in future studies (see Table 15, pg 90).
Due to the morphological changes and differences noted within and between different wells of cells during the differentiation protocol, along with the variation in results from immunocytochemistry and qPCR experiments, it was thought that the protocol used was either not highly efficient in producing photoreceptor cells, or that the 30 day culture period was not sufficient to produce mature photoreceptors. Various interventions may be used in future experiments to overcome the issues of cell population purity. Although culture conditions are designed to select for and promote the growth of the pluripotent cells, a small proportion may undergo some form of differentiation during routine culture. If this differentiated sub-population grows at a faster rate than the desired cell type, it may contaminate the well significantly once the differentiation protocol is initiated. It therefore may be advantageous to employ a technique such as fluorescence activated cell sorting (FACS) to derive a "pure" starting population of pluripotent cells at the initiation of the differentiation protocol. Abujarour and colleagues demonstrated that FACS utilising a combination of antibodies against SSEA4 (stage-specific embryonic antigen-4), TRA-1-81 and CD30 (cluster of differentiation 30) is highly efficient in deriving a population of cells expressing OCT4 and NANOG, and can thus be used to sort pluripotent cells from partially reprogrammed or differentiated sub-populations (Abujarour et al., 2013).

The issue of purity is a key concern with regard to using iPSC-derived cells for either in vitro modelling or therapeutic transplantation. Whilst some differentiation protocols are more effective than others, none have reported a 100% efficiency in differentiation from pluripotent cells. The protocol developed by Boucherie and colleagues utilised a "Matrigel sandwich" system to provide the spatial cues necessary for retinal differentiation, whilst additives to the culture medium (N2 and B27 supplements, retinoic acid, taurine, aFGF and bFGF) provided the chemical cues. Other protocols have used alternative culture systems and additives to derive retinal cells. These additives include activin-A, NOG and heparin, and additional inhibitors of the Wnt and Nodal pathways (Table 15, pg 90). Culturing the cells in hypoxic conditions which mimic physiological environment more closely can also be beneficial (Bae et al., 2011). Some of the differentiation protocols prescribe an
enrichment step, where optic-vesicle like structures or domains are dissected manually and cultured separately (Meyer et al., 2011; Zhong et al., 2014). However, these processes of culturing the cells as 3D clusters can be accompanied by additional technical difficulties, since cells within the structures can easily become necrotic (Dr Budd Tucker, personal communication). The process of human retinal development spans a period of over 24 weeks in the embryo (Swaroop et al., 2010), therefore it is feasible that longer differentiation protocols will be more efficient in producing photoreceptors of a mature phenotype, and these will be pursued in future studies.

Regardless of the differentiation protocol used, it is likely that one would need to introduce a method of purification before the cells can be used for the desired application. Furthermore, for therapeutic applications it would be necessary to remove any remaining pluripotent cells from the population, in order to reduce the risk of teratoma formation. With the introduction of a sorting method such as FACS in *in vitro* studies, one could determine the efficiency of the protocol in producing the desired cell type, as well as the cellular composition of the heterogeneous cell population (if multiple antibodies are used). FACS would also provide a homogeneous cell population which could be used in downstream experiments such as qPCR or western blots. The disadvantage of this strategy is that it relies on the identification of cell surface markers that are unique to each cell type of interest (for example, cone photoreceptors, rod photoreceptors, retinal ganglion cells and amacrine cells). Yuan and colleagues employed this strategy to identify markers for the isolation of neural stem cells, glia and neurons (Yuan et al., 2011). Using an initial pool of 190 antibodies, unique cell-surface signatures were identified for each of the 3 cell types, which could then be used in "immunophenotyping" screens. A similar investigation found four candidate cell surface antigens which could be used in the identification of photoreceptor precursors. FACS using two of these candidates, Cd24a and Nt5e, resulted in a pool of cells which effectively integrated into the outer nuclear layer of mutant mouse eyes (Lakowski et al., 2011). An alternative strategy, as demonstrated by Lamba et al. (2010), is to infect the differentiated cells with a viral vector expressing a marker from a promoter of
interest. Lamba utilised a lentivirus driving GFP from the \textit{interphotoreceptor retinoid-binding protein (IRBP)} regulatory sequence (Lamba et al., 2010). Although this method relies on further manipulation of the cells by viral infection, which is not advisable for therapeutic transplantation, the sorted cells showed some evidence of integration and survival in mouse retinas. It is likely that future studies will identify refined methods of deriving and purifying differentiated cells, which will allow for safe and effective transplantations, as well as high calibre \textit{in vitro} experiments.

In the case of \textit{in vitro} modelling experiments, it may be beneficial to select alternative or additional markers of pathogenesis to be monitored in the differentiated cells. In the experiments described here, the aim was to establish whether any transcriptional changes could be detected in the SCA7 patient-derived cells. This disease-associated feature was selected due to the role of ATXN7 within the TFTC/STAGA transcriptional activator complex. However, numerous other pathogenic mechanisms and pathways have been implicated in the polyglutamine repeat diseases, including inclusion formation, proteosome impairment and mitochondrial dysfunction. These pathways provide multiple potential avenues which may be explored in iPSC-derived cells in the future.
Chapter 5: Concluding Remarks
5.1 The inherited ataxias in Africa

The hereditary ataxias are characterised by progressive degeneration, leading to permanent disability. The inability to co-ordinate movement has a significant impact on the quality of life of the affected patients, as well as their family members and caregivers. Furthermore, palliative care and management of these individuals comes at a significant cost. The global epidemiology of the hereditary ataxias is largely unknown, but a recent meta-analysis of prevalence studies has lead to the estimation that an average of 2.7 per 10^5 individuals are affected with autosomal dominant hereditary ataxia (Ruano et al., 2014). Given the current population estimate of 1.11 billion (www.populationdata.net, accessed 30 June 2014), approximately 30 000 individuals on the African continent may be affected with one of these disorders.

It has been acknowledged that the body of literature on neurodegenerative disorders in Sub-Saharan Africa is limited, and that there have been few population-based studies (Lekoubou et al., 2014). Reports on the prevalence of the inherited ataxias on the African continent have been extremely scarce (Table 24). Many of the existing reports in the literature are not representative of a large geographical area, but rather of isolated cases and families. Published prevalence rates in Sub-Saharan Africa are almost non-existent. The NHLS laboratory at Groote Schuur Hospital in Cape Town is the only known laboratory offering a molecular diagnostic service for the inherited ataxias on the African continent. Additionally, the Division of Human Genetics at the University of Cape Town is the only known research group in Africa investigating this group of disorders at a molecular level. Although the expected prevalence of the inherited ataxias in Africa is significantly lower than other widespread diseases such as HIV and Tuberculosis, the potential impact of future research into these disorders can not be ignored.
Table 24: Published reports of the inherited ataxias in African countries. The reported number of families with each type of SCA is given, with the number of affected individuals in brackets (if available).

<table>
<thead>
<tr>
<th>Country</th>
<th>SCA1</th>
<th>SCA2</th>
<th>SCA3</th>
<th>SCA7</th>
<th>Other</th>
<th>Reference</th>
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</thead>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>1</td>
<td>(Cancel et al., 1997)</td>
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<td></td>
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<td></td>
<td>1</td>
<td>(Durr et al., 1996)</td>
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<tr>
<td>Egypt</td>
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<td></td>
<td>7 individuals with hereditary ataxia</td>
<td>(El Tallawy et al., 2010)</td>
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<td></td>
<td>(Durr et al., 1996)</td>
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<td></td>
<td>(Traoré et al., 2001)</td>
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<td></td>
<td>(Benomar et al., 1996)</td>
<td>(Cancel et al., 1997)</td>
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<td></td>
<td></td>
<td></td>
<td>(Durr et al., 1996)</td>
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<td>6 individuals with hereditary ataxia</td>
<td>(Aiyesimoju et al., 1984)</td>
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<td>South Africa</td>
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<td>7 (8)</td>
<td>50 (65)</td>
<td>5 individuals with SCA6</td>
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</tbody>
</table>

There are a number of potential reasons for the lack of testing for the hereditary ataxias in Africa. Firstly, relatively rare conditions such as these are commonly overshadowed by more pressing healthcare issues, such as poor nutrition and communicable diseases. Secondly, there is a significant disparity in the number of neurologists, medical geneticists, genetic counsellors and medical scientists on the African continent who are able to identify, counsel, test and treat these patients effectively. In 2004 the World Health Organization estimated that the neurological service in Western countries varied from 1 to 10 neurologists per 100 000 inhabitants (World Health Organization, 2004). In South Africa, one of the most resourced countries on the continent, there are currently 167 neurologists registered with the Health Professions Council of South Africa (www.hpcsa.co.za, accessed 30 June 2014). This translates to roughly 0.3 neurologists per 100 000 citizens. In many regions in Africa, the number of neurologists per 100 000 ranges
between 0 and 0.1 (World Health Organization, 2004). Primary healthcare physicians, particularly in rural areas, may not be adequately aware of the symptoms associated with this group of disorders. Finally, there is a considerable cost to molecular testing, which is particularly significant to those in low income groups. The current cost for molecular diagnosis of SCA1, 2, 3, 6 and 7 (a multiplex PCR test with DNA extraction) is approximately 2 500 South African rands (ZAR) (roughly USD240), which is equivalent to 20 days work at minimum wage in South Africa. Given that there are no current therapeutic interventions for these disorders, there may be little motivation to undergo confirmatory testing.

A large number of individuals and families would benefit from increased awareness, molecular testing and research for the inherited ataxias in Africa. A confirmed molecular diagnosis can enable more appropriate clinical management of the affected individuals, and family members can be counselled with regard to their own risk, as well as the risk for current and future offspring. Additionally, individuals with a confirmed diagnosis may be eligible for future clinical trials and therapeutic interventions. There are currently over ten international clinical trials recruiting patients with Spinocerebellar ataxia, many of which would benefit from including individuals of African ancestry (www.clinicaltrials.gov, accessed 30 June 2014).

The work presented in this study serves as the most recent update on the spectrum and prevalence of the polyglutamine SCAs in South Africa, and represents the largest patient cohort described on the African continent. A total of 313 individuals from 215 families have received a molecular diagnosis of SCA 1, 2, 3, 6 or 7 over a 26-year period of testing (1987 to 2013). Of concern is the proportion of individuals who did not receive a diagnosis of one of the tested SCAs (over 1 100), indicating the need for pilot studies to identify the "missing" causes of neurological symptoms in this patient group.

This study was primarily focussed on SCA7, due to the uniquely high number of South Africans affected with this disorder. Three SCA7 families from Zambia and Namibia have also been described, representing the first report of affected families
from Sub-Saharan countries apart from SA. These results signify that SCA7 is prevalent in other southern African regions, and there may be individuals similarly affected with other types of SCA in these countries. Future work will focus on increasing the awareness of the inherited ataxias amongst the neurological community in southern Africa, in order to improve the level of patient referrals for molecular testing. Relationships with neurologists from neighbouring African countries will also be nurtured in order to determine the prevalence of affected individuals in Sub-Saharan Africa. Where possible, additional haplotyping studies will be pursued to identify shared haplotypes amongst affected families, which may facilitate the design of custom therapeutics (Scholefield et al., 2014; Scholefield et al., 2009).

5.2 The molecular diagnosis of the inherited ataxias
The NHLS laboratory at Groote Schuur Hospital in Cape Town (affiliated to the University of Cape Town) is currently the only centre to offer molecular testing for the inherited ataxias (SCA 1, 2, 3, 6, 7, 12, 17) in Africa. The NHLS laboratory subscribes to the EMQN external quality assessment scheme for the Spinocerebellar ataxias (SCA 1-7), in order to ensure a high international standard of testing and reporting. However, until recently, no confirmatory methods were used to substantiate cases of apparent homozygosity. The importance of confirmatory testing was demonstrated in this study, where re-testing of 111 black African individuals (who had previously been given inconclusive results) revealed six individuals with pathogenic expansions. These findings indicated that the conventional multiplex PCR did not detect expansions in 5.4% of the referred cases. The NHLS laboratory at Groote Schuur Hospital is affiliated with the University of Cape Town, and works closely with the Division of Human Genetics. Since the NHLS laboratory is dedicated to service delivery, it is often unable to devote time and resources to the research and development of additional molecular tests. Therefore these aspects are often undertaken in the Human Genetics research laboratory. Future work within the research laboratory will focus on the optimisation and implementation of confirmatory TP PCR assays for the remaining
SCA types (SCA1, 2, 3, and 6), so that every individual referred for molecular testing may receive a conclusive result.

5.3 **In vitro modelling of SCA7**

5.3.1 **Sample availability**

Experiments and studies utilising patient-derived cells ideally require samples from a cohort of well-defined and characterised individuals, both affected and unaffected with the disease of interest. The genetic backgrounds of individuals can differ significantly, and any of these underlying differences can complicate the interpretation of results. In order to overcome this, one can use a large cohort of individuals to identify highly robust disease-associated traits. In neurodegenerative disease-based research, this can be somewhat difficult, since access to patients and their biological material can be limited. This is particularly problematic in the case of rare diseases, and in under-resourced, third world countries such as South Africa. Additionally, the creation and characterisation of iPSC lines is logistically difficult, time-consuming and expensive. In an attempt to overcome these issues, various consortia have been formed to create repositories of fibroblasts derived from PD, ALS and HD patients. It is likely that similar consortia will be formed in the future, with the aim of sharing biological material and resources to aid the investigation of additional inherited and neurodegenerative diseases.

Where large patient cohorts are not available, it may be advantageous to use samples from large families. In the case of SCA7, South Africa presents a unique resource of potential patient material, since the majority of patients share the same ethnic background, and evidence exists for the presence of a founder effect within the population. Whilst some SCA7 families reside in the Cape Town region, the majority of South African SCA7 families are from the rural Eastern Cape (the traditional home of the Xhosa population). In this study, characterised iPSC lines from only two SCA7 unrelated patients and an single unaffected individual were available, which was a potential limitation, since additional patients and unaffected control individuals should ideally have been included to substantiate the results. The Division of Human Genetics currently has fibroblasts banked from 13 individuals
from four South African SCA7 families, and five of these lines have been reprogrammed into iPSCs. These samples may provide a valuable resource if they are included in future investigations.

5.3.2 Retinal differentiation from iPSCs

Since SA has a large and homogeneous cohort of SCA7 patients, iPSC technology was employed to create a valuable biological resource for the in vitro study of the disease. SCA7 iPSCs generated from a single patient have been previously described in 2012 (Luo et al., 2012), but the experiments depicted here are the first to explore the derivation of SCA7 iPSC-derived retinal cells. A heterogeneous population of retinal-like cells was obtained from two SCA7 patient iPSCs in three separate experiments, and compared to the cells derived from an unaffected individual. The differentiated cells were monitored for transcriptional changes in a panel of selected genes, since transcriptional dysregulation has been proposed to be a primary event in pathogenesis (Helmlinger et al., 2006b). By the end of the 30-day differentiation period, numerous transcriptional differences were noted between patient- and control-derived cells, but the majority of these changes were not consistent across all three experiments. Some of these changes may warrant further investigation in the future, but the model requires significant refinement before additional conclusions can be made. Nonetheless, this system may be valuable for the investigation of additional SCA7 pathogenic mechanisms, such as the effects of expanded ATXN7 on TFTC/STAGA composition and chromosome remodelling.

Recent studies have made noteworthy advances in the strategies for culturing retinal cells from iPSCs, such that three-dimensional structures containing functional photoreceptors, grown in vitro, have been described (Zhong et al., 2014). These complex structures contain an assortment of cells, and will therefore be valuable for physiological studies, whilst additional sorting or manipulation will be required for transcriptional experiments and therapeutic transplantation. Despite the difficulties associated with deriving, culturing and purifying retinal cells from iPSCs, it is anticipated that these cells will prove invaluable for interrogating
pathophysiology, testing the efficacy of drug and gene therapeutics, and perhaps acting as cell-replacement agents in the future (Tucker et al., 2014).

5.3.3 In vitro modelling of late-onset conditions

A major concern with the utilisation of iPSC-derived cells for in vitro modelling of late-onset degenerative conditions, is the issue of imitating the aging process in an artificial environment. Firstly, the reprogramming of adult somatic cells (even from older individuals) results in the reversal of many characteristics associated with older cells, such as telomere shortening, protein aggregation, DNA damage and impaired mitochondrial function (reviewed in Mahmoudi and Brunet, 2012). Moreover, many of these characteristics are maintained within the cells differentiated from the iPSCs. The results from iPSC differentiation studies have also implied that maturation of the differentiated cells does not occur more rapidly in vitro, and often mimics developmental timing (Brennand, 2013). These processes are likely to account for the lack of significant disease-associated phenotypes in various iPSC-derived models of PD, AD and ALS (reviewed in Liu et al., 2012).

In order to induce "aging in a dish", established strategies used in other cell-based models may be employed with some efficacy. These strategies include serial passaging and/or long-term culture, or the application of cellular stressors. Whilst serial passaging is not possible in terminally differentiated post-mitotic cells such as photoreceptors, the iPSCs may be initially differentiated into a progenitor-type cells such as neural stem cells, which can then be cultured and expanded as needed before being differentiated into the mature cell type. This was demonstrated by the HD iPSC Consortium, who studied the effects of HD in patient-derived neural stem cells (HD Consortium, 2012). Several reports of the production of retinal precursors and progenitors from pluripotent stem cells exist (Table 15, pg 90), but it is unclear how amenable these cells would be to long-term passage and culture.

As an alternative to long-term culture, the application of in vitro stressors may be capable of precipitating age-related phenotypes in differentiated cells. Treatment of iPSC-derived neurons with the proteosome inhibitor MG132 resulted in the formation of aggregates in HD patient-derived cells, but not control cells (Jeon et
al., 2012). Other examples of potential stressors included electrical stimulation, or treatment with neurotoxins such as hydrogen peroxide or 6-hydroxydopamine (Nguyen et al., 2011).

Perhaps the most novel method of inducing in vitro aging was proposed recently by Miller et al., (2013). In an attempt to recapitulate aging in PD iPSC-derived cells, the authors used synthetic mRNA to overexpress the progerin protein in the cells. Progerin is a shortened form of lamin A, formed as a result of mutations in the LMNA gene. Progerin production is associated with the development of a rare genetic disorder called Hutchinson-Gilford progeria syndrome, which is characterised by premature aging and early death. The overexpression of progerin within PD iPSC-derived fibroblasts and dopaminergic neurons resulted in an improved recapitulation of the disease, since the cells exhibited several associated phenotypes such as dendrite degeneration, mitochondrial swelling and inclusion body formation (Miller et al., 2013). It is anticipated that future studies will identify advanced methods to produce in vitro models and methods that are more sensitive, effective and valuable, for the investigation of neurodegenerative diseases and therapeutic interventions.

5.4 Conclusion
This project employed a three-pronged, multi-disciplinary approach to investigate SCA7 in southern Africa. It is likely that this study will provide short-, medium- and long-term benefits to SCA7 patients and their families, both locally and internationally. As a result of the updated protocol for molecular testing, three individuals who would have otherwise received an inconclusive result have been given a confirmed diagnosis of SCA7, which may impact their clinical management and family planning. This has also highlighted the need to develop similar confirmatory tests for the other types of SCA. Whilst the epidemiological investigation of the polyglutamine SCAs in southern Africa has no immediate benefit or consequences to patients, it has highlighted the need to explore the prevalence of these diseases on the African continent, since there is a significant paucity in this area of research. Finally, the iPSC-based investigation has provided the first description of an in vitro study of SCA7 patient-derived retinal cells. Although these
experiments did not provide any obvious insights into SCA7 pathogenesis, they have laid the foundation for future investigations, and highlighted the areas which require improvement. This disease-modelling approach is becoming increasingly popular for inherited retinal degenerative studies, and it is expected that SCA7 patients will ultimately benefit in the future.

The work presented here demonstrates the importance of communication and collaboration between clinicians, medical scientists and research laboratories. The early work pioneered by Professors Beighton and Bryer within the Divisions of Human Genetics and Neurology at the University of Cape Town led to the establishment of the dedicated Neurogenetics clinic at Groote Schuur Hospital, where many study participants have been recruited. It was recognised that molecular diagnoses would be beneficial for these patients and their families, therefore the NHLS laboratory at Groote Schuur Hospital initiated a confirmatory testing service. The observation of the unique ethnic distribution within the SCA7 patient group in the early clinical studies lead to research projects to examine whether a founder haplotype existed in the cohort. After the discovery of the SA SCA7-associated haplotype, the SNP that formed part of the haplotype was used as a target for a prospective allele-specific RNAi-based therapy. Through a continued relationship with the NHLS diagnostic laboratory, the haplotype study has now been extended to incorporate families from other African countries, including Namibia, Zambia and Nigeria. Additionally, small projects undertaken within the research laboratory have optimised additional molecular confirmatory tests, including the TP PCR assay for SCA7 as described here. This test has been successfully transferred from the research laboratory to the NHLS diagnostic laboratory, where it is used routinely to confirm or refute cases of apparent homozygosity. Finally, the unique biological resource presented by the SCA7 patient cohort has been used to create iPSC lines which may be used in in vitro studies to better understand the underlying pathological mechanisms of the disease, and to test future therapies. Whilst a investigation into iPSC-derived retinal cells was described here, an additional study within the Division of Human Genetics is exploring the creation of SCA7 iPSC-derived neuronal cells. It is anticipated that these continuous interactions between
doctors, diagnostic laboratories and researchers may ultimately lead to better management, counselling, care and outcomes for SCA7 patients on the African continent.
Appendices

Appendix 1- Ethics

A1.1 Example of patient information sheet and consent form (haplotyping studies)

INFORMATION SHEET
FOR SCA7 AFFECTED PATIENTS

Research project: An investigation of the SCA7 mutation in African patients

Introduction
You are invited to take part in a research project into the biological mechanisms behind Spinocerebellar ataxia type 7 (SCA7). You have been selected to participate because you have SCA7, and you may have a other family members with SCA7. We would like to ask you to donate a small amount of blood from your body. You are free to agree or refuse to participate in this study – it’s up to you. If you decide to participate but change your mind later on, you can let us know and we will destroy the sample.

What is this project about?
We are studying a disease called Spinocerebellar ataxia type 7 (also called SCA7). If someone has SCA7, some of their brain cells and their eye cells die. This makes it difficult for these patients to control how they move around, and they can also become blind.

SCA7 is caused by a mutation (or mistake) in the DNA. Our laboratory has discovered that all South African SCA7 patients got the mutation from a single common ancestor that lived many years ago. We would now like to test whether SCA7 patients from other African countries got the mutation from the same common ancestor.

What would we like from you?
We have contacted you because you are affected with SCA7. We will ask for a blood or saliva sample from you. Your doctor will take the blood from a vein in your arm, or you will be asked to spit into a special tube.

What will happen to your sample?
After taking your blood, we will isolate your DNA in the laboratory. We will do some tests on your DNA to check if it looks similar to DNA from South African SCA7 patients. We will only use your DNA for research into SCA7. Your sample will be identified by a unique code. Information about the code will be kept in a secure location and access limited to research study personnel.

Benefits of the Study
This study is purely for research purposes, and offers no direct benefit to you or your family, at this stage. However, in the long term, your participation in this study may allow us to find out more about the cause of SCA7, particularly due to the familial aspect of the disease. This may help us to develop a way to treat SCA7 and other similar diseases in the future.

Important points to understand
• Your DNA may be kept in storage in the laboratory for many years. Your cells will only be used for SCA7 research. Future studies will be approved by the appropriate research ethics committee.
Your decision to participate is voluntary. You have the right to refuse to participate.
If you first agree to participate and then change your mind, you are free to withdraw your consent and discontinue your participation at any time.
You will not receive any direct benefits from this study.

If you have any questions regarding this study, please do not hesitate to contact us at any time at the numbers listed below.

**Professor Jacquie Greenberg**
Division of Human Genetics,
Suite N3.14 Werbner & Beit North Building,
Level 3, HDMM, Faculty of Health Sciences, UCT
Office: 021-4066299
Email Address: jacquie.greenberg@uct.ac.za

**Professor Marc Blockman,**
Chair, UCT Human Research Ethics Committee,
GSH, Faculty of Health Sciences,
Office: 021-4064492
Email address: marc.blockman@uct.ac.za

Professor Blockman may be contacted by research subjects to discuss patients' rights.
Professor Greenberg is the Principal Investigator on this project.

**PATIENT CONSENT FORM**
I hereby agree to participate in this study, and I give my voluntary and informed consent to donate a blood sample to be used as a source of biological material for SCA7 research purposes only.

The details of this study have been explained to me in a language that I understand and my questions have been answered by __________________________ DATE: __________

Patient Signature: __________________________

Witnessed Consent: __________________________

Date: __________________________
Please complete the following:

Surname:__________________________________________
First Name(s):____________________________________
Contact Address: _________________________________ Town: _____________________________
Tel: _____________________________________________
Cell: ____________________________________________
Date of Birth: Year:_________ Month:______________ Day: ___________________________
Sex: M / F
Ethnic origin: Mother: _______________ Father: _______________
Home language (mother tongue):________________________
Doctor's name, address and phone number:___________________________________________

At what age were you first diagnosed with SCA?

At what age did you first notice your symptoms? (eg difficulty walking, poor vision etc)

Do you have any other family members with SCA?  □ Yes  □ No
If yes, what is your relationship with this person / these people? (eg Mother, Brother etc). A pedigree can be
drawn on the other side of the page if necessary.
A1.2 Example of patient information sheet and consent form (iPSC studies)

Information Sheet
For Affected Patient Skin Samples

Research project: An Induced Pluripotent Stem Cell-derived model of retinal degeneration associated with SCA7
(UCT HREC REF 454/2011)

Introduction
You are invited to take part in a research project into the biological mechanisms behind Spinocerebellar ataxia type 7 (SCA7). You have been selected to participate because you have SCA7. We would like to ask you to donate a small piece of skin from your body. You are free to agree or refuse to participate in this study – it’s up to you. If you decide to participate but change your mind later on, you can let us know and we will destroy the samples and cell lines created from your sample.

What is this project about?
Your body is made of many tiny building blocks, called cells. There are many different types of cells, and they each look different and perform different functions in your body. Your skin cells look very different to your hair cells, and these look very different to your blood cells. Scientists can grow cells in the laboratory. The cells can divide to make more cells, so many cells can be grown from a small sample. There is a special type of cell, called stem cells. Stem cells are special because they can become any other type of cell that is found in your body, if they are grown in the right way. If they are grown in one way they can become skin cells, but if they are grown in another way they can become brain cells. This means that scientists can use stem cells to grow many different types of cells in the laboratory. If they are grown in the right way, stem cells can survive indefinitely (for many years).

We are studying a disease called Spinocerebellar ataxia type 7 (also called SCA7). If someone has SCA7, some of their brain cells and their eye cells die. This makes it difficult for these patients to control how they move around, and they can also become blind. We would like to understand why these cells die, but we can’t study them in the laboratory because it is dangerous to take brain cells or eye cells from patients. We would like to take skin cells from SCA7 patients and treat them in a special way, so that they turn into stem cells. We can then use these stem cells to grow brain cells and eye cells. We will then compare these cells to brain cells and eye cells made from stem cells from people without SCA7. We hope that by studying these cells we will be able to better understand how the disease works, and to potentially develop therapies for SCA7.

What would we like from you?
We have contacted you because you are affected with SCA7. We will ask for a skin sample from you. The skin sample is obtained by a process called a “punch biopsy”. In this procedure, a small area of skin on the thigh or arm is thoroughly cleaned and injected with a local anesthetic, or an anesthetic cream is applied to the skin. When the skin is numb, a small round blade of four millimeters in diameter is pressed into the skin, creating a circular cut approximately four millimeters deep. This round piece of skin is then removed. Pressure is applied to stop bleeding and the resulting hole in the skin covered with a sterile bandage. The anesthetic may sting for several seconds during the injection; afterwards, the punch biopsy should cause no discomfort. The biopsy takes about ten minutes
to perform, including time for cleaning and preparation. The wound usually heals within three days. A punch biopsy will result in minor scarring at the biopsy site. We will only ask once for a punch biopsy. The consultation and biopsy procedure should not take more than an hour. We will give you the contact details of the doctor doing the biopsy, in case you have any concerns after the biopsy has been done.

This is the approximate size of the biopsy: ○

What will happen to your sample?
After taking the biopsy from your skin, we will use it to grow skin cells (called fibroblasts) in the laboratory. We will then create cell lines from these cells—they are like copies of your cells, but they can only survive in a bottle with feeding liquid. We can use these cells to create eye and brain cells that live indefinitely (for many years). We will only use these cell lines for research into SCA7. We work together with people at the University of Oxford in the United Kingdom. As part of this project, it is possible that your cells will be sent to those collaborators.

Your sample will be identified by a unique code. Information about the code will be kept in a secure location and access limited to research study personnel.

Important points to understand
- Your cells may be kept for many years and may be used by researchers at the University of Cape Town, or at the University of Oxford (UK). Your cells will only be used for SCA7 research. Future studies will be approved by the appropriate research ethics committee.
- Your decision to participate is voluntary. You have the right to refuse to participate. This will not affect your treatment now or in the future.
- If you first agree to participate and then change your mind, you are free to withdraw your consent and discontinue your participation at any time.
- You will not receive any benefits from this study.
- We will reimburse participants R150 for travel and other expenses incurred.

If you have any questions regarding this study, please do not hesitate to contact us at any time at the numbers listed below

Professor Jacquie Greenberg
Division of Human Genetics,
Suite N3.14 Wernher & Beit North Building,
Level 3, IDMM, Faculty of Health Sciences, UCT
Office: 021-4066299
Email Address: jacquie.greenberg@uct.ac.za

Professor Mare Blockman,
Chair, UCT Human Research Ethics Committee,
GSH, Faculty of Health Sciences,
Office: 021-4066492
Email address: mare.blockman@uct.ac.za

Professor Blockman may be contacted by research subjects to discuss patient/participants' rights.
Professor Greenberg is the Principal Investigator on this project.

CONSENT FORM

I hereby agree to participate in this study, and I give my voluntary and informed consent to donate a sample of skin to be used as a continuous source of biological material for SCA7 research purposes only.

The details of this study have been explained to me in a language that I understand and my questions have been answered by ___________________________ DATE: __________

Patient Signature: ___________________________

Witnessed Consent: __________________________

Date: __________________________
Please complete the following:

Surname: ___________________________________________
First Name(s): ______________________________________
Contact Address: ________________________ Town: _____________
Tel: ____________________________
Cell: __________________________
Date of Birth: Year: __________ Month: __________ Day: __________
Sex: M / F
Ethnic origin: Mother: ________________ Father: ________________
Doctor’s name, address and phone number: _______________________

At what age were you first diagnosed with SCA? _______________________
At what age did you first notice your symptoms? (eg difficulty walking, poor vision etc) ________________

Do you have any other family members with SCA? □ Yes □ No
If yes, what is your relationship with this person / these people? (eg Mother, Brother etc). A pedigree can be drawn on the other side of the page if necessary.
# Appendix 2 – Primer sequences

## A2.1 Genotyping primer sequences

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<th>Primer</th>
<th>Sequence (5' to 3')*</th>
<th>Reference (if applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATXN7 CAG Repeat Genotyping</td>
<td>Atxn7(CAG)n F</td>
<td>HEX - ATTGTAGGAGCGGGAAGAATG</td>
<td>Ms Fiona Baine (personal communication)</td>
</tr>
<tr>
<td></td>
<td>Atxn7(CAG)n R</td>
<td>CACGACATCTTGAGAC</td>
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</tr>
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<td>Atxn7 CAG RNA F</td>
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</tr>
<tr>
<td></td>
<td>SCA7 TP F</td>
<td>TACGACATCCCAGTTTGAAGCGAGCACAGCAGCA</td>
<td>Majounie et al., 2007</td>
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<td>SCA7 TP R1</td>
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<td></td>
<td>Seq ATXN7 F</td>
<td>GGAGTGGAAACGAAAGC</td>
<td>Sequencing of multiplex primer binding sites</td>
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<td></td>
<td>Seq ATXN7 R</td>
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### ATXN7 Haplotype markers

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<tr>
<td>AC1 FORWARD</td>
<td>CTGTGCTTGGAGACAGGAAT</td>
<td></td>
<td>(Greenberg et al., 2006)</td>
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<tr>
<td>AC1 REVERSE</td>
<td>TCTGAGACCCCCATCATTT</td>
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<td></td>
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<tr>
<td>SNP FORWARD</td>
<td>AAGGGTTCGTCACCTGACACT</td>
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<tr>
<td>SNP REVERSE</td>
<td>GCTGACATCTCATTCTCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3S1287 FORWARD</td>
<td>ATACACAAAACAAGCAGCTATGGT</td>
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<tr>
<td>D3S1287 REVERSE</td>
<td>GAGGTGATCTTGGCCTT</td>
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### SCA Multiplex PCR

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<th>Reference (if applicable)</th>
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<tr>
<td>ATXN1 FORWARD</td>
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<td>Dorschner et al., 2002</td>
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<tr>
<td>ATXN1 REVERSE</td>
<td>FAM-gcggccccaaagggtcagtCAAGATTGGCAGTGCTG</td>
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<td>ATXN2 FORWARD</td>
<td>aaaagggttcagtGAGCCCCACCTCATTTC</td>
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<tr>
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<td>HEX-aaaaagggttcagtGGCCGCTTGGACATTGG</td>
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<tr>
<td>ATXN3 FORWARD</td>
<td>NED-gcggccccaaagggtcagtCCAGTGACCTTTGATTCGAA</td>
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<tr>
<td>ATXN3 REVERSE</td>
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<td>ATXN6 FORWARD</td>
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<td>gcggccccaaagggttccagtCACGACTGTCCAGCAGATCC</td>
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</table>

*Lowercase letters in primer sequences indicate non-binding tails to adjust amplicon sizes.

### SCA duplex PCR (SCA12 and SCA17)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<td>Holmes et al., 1999</td>
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<td>SCA17 REVERSE</td>
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A2.2  qPCR primer sequences

### qPCR (Reference Genes)

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Dr Sally Cowley, personal communication

(Boucherie et al., 2013)

http://primerdepot.nci.nih.gov/
### qPCR (Transcriptional Dysregulation Genes)

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## Appendix 3 – Antibodies used for immunocytochemistry

### A3.1 Primary antibodies

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<th>Supplier</th>
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### A3.2 Secondary antibodies

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Appendix 4 – Molecular reagents and protocols

A4.1 Agarose gel electrophoresis

3 grams of Seakem LE agarose (Lonza) was weighed and added to 100ml of 1x Tris Boric acid Ethylenediaminetetraacetic acid (EDTA) solution (89mM Tris, 89mM Boric acid, 2mM EDTA) in a glass flask. The solution was heated until dissolved, and ethidium bromide solution (Sigma Aldrich) was added to achieve a final concentration of 10mg/ml. When cooled, the agarose solution was poured into a gel electrophoresis tray to set.

5µl of PCR product was mixed with 3µl of 1x loading dye (Fermentas) and added to the agarose gel. The GeneRuler 100bp DNA ladder Plus (Fermentas) was electophoresed alongside PCR products to allow for approximate size determination. Electrophoresis was performed at 160V for approximately 40 minutes. PCR products were visualised under UV light using the UVIPro UVIGold transilluminator (UVitec Limited). The UVIPro (version 12.3) software was used to capture and edit images.

A4.2 GeneRuler 100bp DNA ladder Plus (Fermentas)
A4.3 Allele bins for SCA7 haplotype markers

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Appendix 5 – Cell culture medium and preparation of additives

All culture medium was filtered through a 0.22µm filter prior to use.

A5.1 Standard tissue culture medium

445ml Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich)
50ml Foetal calf serum (FCS, PALL Laboratories)
5ml 100x Antibiotic antimycotic solution (Sigma Aldrich)

Stored at 4°C.

A5.2 MEF culture medium

50ml FCS
5ml 100x Non-essential amino acids (Sigma Aldrich)
5ml 100x Glutamax (Invitrogen)
0.5ml 1000x β-mercaptoethanol (Sigma)
5ml 100x Antibiotic antimycotic solution (Sigma Aldrich)
434.5ml DMEM (Sigma Aldrich)

Stored at 4°C.

A5.3 iPSC culture medium

400ml Knockout DMEM (Invitrogen)
100ml Knockout serum replacement (Invitrogen)
5ml 100x Non-essential amino acids (Sigma Aldrich)
2.5ml 100x Glutamax (Invitrogen)
3.5µl 1000x β-mercaptoethanol (Sigma)
5ml 100x Antibiotic antimycotic solution (Sigma Aldrich)
500µl Basic fibroblast growth factor (bFGF, 10µg/ml, Miltenyi)

Working aliquots of 40ml were stored at -20°C.

A5.4 Preparation of MEF conditioned medium

Thaw inactivated MEFs into a 25cm$^2$ or 75cm$^2$ culture flask in MEF medium. The following day, replace MEF medium with iPSC culture medium without bFGF. After 24 hours, remove medium and replace with new iPSC medium. Store conditioned medium at 4°C. Repeat the procedure every day for up to 7 days. Before use, add bFGF to conditioned medium and filter through a 0.22µm filter.
A5.5  Embryoid body culture medium (EB medium)

86.9ml Knockout DMEM (Invitrogen)
1ml  100x Glutamax (Invitrogen)
1ml  100x Non-essential amino acids (Sigma Aldrich)
0.1ml  1000x β-mercaptoethanol (Sigma)
10ml  FCS (PALL Laboratories)
1ml  100x Antibiotic antimycotic solution (Sigma Aldrich)

Stored at 4°C.

A5.6  Mesoderm differentiation medium

500µl  50mM Ascorbic acid solution (Sigma Aldrich)
50ml  Embryoid body culture medium

Stored at 4°C.

A5.7  Ectoderm differentiation medium

48ml  Neural basal medium (Invitrogen)
48ml  DMEM F12 (Invitrogen)
0.5ml  N2 supplement (Invitrogen)
1ml  B27 supplement (Invitrogen)
1ml  100x Glutamax (Invitrogen)
1ml  100x Antibiotic antimycotic solution (Sigma Aldrich)

Stored at 4°C.

A5.8  1000x β-Mercaptoethanol preparation

Dilute 69.8µl of β-Mercaptoethanol (14.3M, Sigma Aldrich) in 10ml of 1x PBS.

Stored at 4°C.

A5.9  Basic fibroblast growth factor preparation

Reconstitute lyophilised powder (Miltenyi) in sterile distilled water to 100µg/ml (10000x) and store at -80°C in small aliquots. Dilute aliquots to 1000x with 0.1% bovine serum albumin (Miltenyi) in 1x PBS and freeze at -20°C in 40µl aliquots (short term storage). Add aliquot to 40ml iPSC culture medium and filter (0.22µm) prior to use.
A5.10 Acidic fibroblast growth factor preparation
Reconstitute 25µg vial of aFGF (R&D Systems) in sterile 1x PBS containing 0.1% BSA to give 100µg/ml (dissolve contents of vial in 250µl PBS). Store at -80°C. Use 5µl per 10ml of culture medium to give 50ng/ml.

A5.11 Y-27632 preparation
Dissolve 1mg vial of Y-27632 (Sigma Aldrich) in 2.96ml sterile water to give a 1mM stock. Aliquots stored at -20°C. Use 10µl per 10ml of culture medium to give a final concentration of 10µM.

A5.12 Preparation of retinoic acid solution
Prepare a 100mM solution of all-trans Retinoic acid (Sigma Aldrich) in DMSO (Sigma Aldrich) by diluting 50mg in 1.67ml. Store at -80°C. Before use, dilute 100mM to 100µM in culture medium (use 10µl stocker per 10ml medium). Use 10µl per 10ml of culture medium to give a final concentration of 100nM.

A5.13 Preparation of taurine solution
Prepare a 50mM taurine solution (Sigma Aldrich) in sterile H2O and filter with a 0.22µm filter (dissolve 0.5g in 80ml H2O). Store at -80°C. Use 20µl per 10ml of culture medium for a final concentration of 100µM.

A5.14 Preparation of sonic hedgehog solution
Reconstitute 25µg vial of recombinant sonic hedgehog (R&D Systems) at 10µg/ml in sterile 1x PBS containing 0.1% BSA (dissolve in 2.5ml). Store at -80°C. Use 60µl per 10ml of culture medium to give a 3nM concentration.
Appendix 6 – Cell culture protocols

All cells were grown in a 37°C incubator with 5% carbon dioxide (CO₂). Many of the protocols described here were kindly provided by the following individuals: Janine Scholefield, Lauren Watson, Robea Ballo and Sally Cowley.

A6.1 Skin biopsies and dermal fibroblast isolation

Skin biopsies were taken from the inner forearm. The area was wiped with a sterile swab before administration of a local anesthetic. A 4 millimetre sterile circular biopsy punch (Miltex) was used to biopsy a section of skin, which was placed in a sterile container containing standard tissue culture medium (pg 177). Skin biopsies were transported at room temperature or 4°C and processed within 48 hours.

Dermal fibroblasts were isolated using a modified protocol based on that described by Freshney (Freshney and Freshney, 1994). The skin biopsy was placed in a 35mm sterile tissue culture dish in a drop of standard culture medium and finely sliced using two sterile scalpel blades. Tissue fragments were divided into six 35mm dishes and covered with a flamed coverslip. Thereafter 2.5ml of culture medium was added to each dish, and dishes were placed in a 37°C incubator with 5% carbon dioxide (CO₂). The culture medium was changed every 3 to 4 days. When cells reached confluence, the coverslip was flipped into a fresh 35mm dish with 2.5ml culture medium, and each dish was incubated as previously described until confluence was reached. Fibroblasts in confluent dishes were trypsinised (passaged) into 25cm² tissue culture flasks, whereafter they were passaged and used for further experiments, or frozen and stored in liquid nitrogen as necessary (pg 181).

A6.2 Trypsinisation (passaging) of adherent cells (fibroblasts)

1. Aspirate medium from dish/flask.
2. Rinse once with 1x phosphate buffered saline (PBS).
3. Add enough pre-warmed trypsin/EDTA solution (Sigma-Aldrich, Germany) to cover the surface of the dish/flask.
4. Incubate the dish at 37°C for 5 minutes.
5. Add an equal volume of culture medium to activate the trypsin solution, and gently triturate the cells.
6. Transfer cell solution to a 15ml conical tube and centrifuge at 1500 rpm for 5 minutes.
7. Aspirate off the supernatant, and resuspend the cell pellet in a small amount of culture medium.
8. Split cells into the required number of dishes and add the appropriate volume of culture medium.

A6.3 Freezing of cells (fibroblasts)
1. Trypsinise cells as described, and resuspend the desired number of cells in 1ml cold freezing medium (DMEM containing 20% FCS and 10% Dimethyl Sulfoxide) in a cryovial.
2. Place cryovials in a freezing container (Nalgene) at -80°C overnight.
3. Transfer cryovials to liquid nitrogen storage.

A6.4 Thawing of cells (fibroblasts)
1. Retrieve cryovial from liquid nitrogen storage, thaw rapidly by swirling in a 37°C waterbath, without allowing the cap to submerge.
2. Add thawed cells to 10ml pre-warmed culture medium in a 15ml conical tube.
3. Centrifuge at 15 000 rpm for 5 minutes, aspirate off medium.
4. Resuspend cell pellet in fresh culture medium, add to appropriate cell culture dish and place in 37°C incubator.

A6.5 Preparation of mouse embryonic fibroblast feeder cells (MEFs)
Mouse embryonic fibroblasts (MEFs) were used as feeder cells during the maintenance and culture of iPSCs. Embryos were dissected from female mice between embryonic days 12.5 and 14.5. Heads, limbs and internal organs were removed and the trunk was washed in DMEM and transferred to a 10cm tissue culture dish, followed by mincing to a fine consistency with a surgical blade. The resulting homogenate was transferred into a 0.05% trypsin solution and pulled through a 16 or 18 gauge needle to further disaggregate the solution. Thereafter
the cells were incubated at 37°C in a rocking incubator for 20 to 30 minutes. Approximately 20ml of standard culture medium was added to the tube, and any floating tissue or cartilage was removed. Cells were centrifuged at 1500 rpm for 5 minutes, followed by removal of the resultant supernatant. The pellet was washed with culture medium to remove excess trypsin, followed by an additional centrifugation step. Cells were added to a 10cm tissue culture dish previously coated in a 0.1% gelatin solution, with an appropriate volume of culture medium. Cells were grown, passaged or frozen as necessary, and inactivated by passage 5 to be used as feeder cells.

A6.6 Inactivation of MEFs
MEFs to be used as feeder cells were inactivated by incubation with Mitomycin C. Treatment of cells with Mitomycin C results in DNA cross-linking and cell cycle arrest. Treated cells are suitable as feeders since they are mitotically inactive, but continue to produce the necessary factors to support stem cell growth. To inactivate MEF feeder cells (iMEFs), flasks were incubated with a 10µg/ml Mitomycin C solution (Sigma Aldrich) at 37°C for 2 to 3 hours, followed by three washes with 1x PBS to remove excess Mitomycin C. Thereafter cells were trypsinised and frozen at appropriate concentrations for future use.

A6.7 Preparation of MEF feeders for iPSCs
MEF feeders were plated one day before iPSCs. Wells were coated with a 0.1% gelatin solution (see following section). For culture in a 6-well culture plate (35cm wells), approximately $0.3 \times 10^6$ iMEFs were thawed and added to each well in MEF culture medium. The cells were incubated overnight at 37°C. The following day, each well was washed three times with 1x PBS solution to remove floating cells and traces of MEF medium, and replaced with fresh iPSC culture medium.

A6.8 Preparation of gelatin-treated dishes
A 0.1% sterile gelatin solution was prepared by adding 0.5g of gelatin powder (Sigma Aldrich) to 500ml of sterile distilled water. The solution was autoclaved and stored at 4°C.
Prior to use, an appropriate volume of gelatin solution (1ml per 35cm dish, 4ml per 10cm dish) was added to each dish, followed by incubation at 37°C for approximately 30 minutes. Excess gelatin was removed and plates were dried at room temperature for 20 minutes before use.

**A6.9 Preparation of Matrigel coated dishes**

1. Prepare aliquots of huES-qualified Matrigel (Stem Cell Technologies) according to the dilution factor provided on the certificate of analysis and keep frozen at -80°C. Keep Matrigel on ice at all times to avoid gelling.
2. Thaw one aliquot of Matrigel on ice (may be done overnight in a 4°C fridge).
3. Dispense 25ml of cold DMEM F12 (Sigma Aldrich) into a 50ml conical tube on ice.
4. Add Matrigel to cold medium and mix well.
5. Use the diluted Matrigel to coat tissue culture plasticware (1ml per 35cm² well).
6. Swirl the dish to spread Matrigel solution across the surface.
7. Incubate at room temperature for at least one hour.
8. Remove excess Matrigel solution using a serological pipette or by aspiration.
9. Immediately add an appropriate volume of culture medium.

**A6.10 Preparation of poly-D-lysine, fibronectin and laminin-coated dishes**

1. Coat surface with 1ml of poly-D-lysine solution (0.1mg/ml, Sigma Aldrich) per 25cm². Rock gently to ensure even coating of culture surface.
2. After 5 minutes, remove solution by aspiration and thoroughly rinse surface with sterile tissue culture grade water.
3. Allow to dry at least two hours.
4. Mix 800µl of 60µg/ml laminin (BD Biosciences) and 8µl of 1mg/ml fibronectin (Sigma Aldrich).
5. Apply 500µl mixture per well of a 6-well plate.
6. Incubate the slide at 37°C overnight.
7. Add 500µl of PBS to each well of the coated slide. Slides containing PBS can be store in a humidified incubator for several days.
8. Before use, aspirate the PBS from the wells.
A6.11 Reprogramming of dermal fibroblasts

Reprogramming of dermal fibroblasts into iPSCs was achieved through the introduction of Sendai virus vectors (SeVdp) carrying OCT4, SOX2, KLF4 and c-MYC (Nishimura et al., 2011). Low passage human dermal fibroblasts (below P5) were prepared in 6 well plates in varying quantities (0.75 x 10^5, 1 x 10^5, 1.5 x 10^5 or 2 x 10^5) in standard cell culture medium. The following day, the well with cells at a 80 to 90% confluency was chosen for infection. The medium was replaced with culture medium lacking antibiotic, and 50µl of titred SeVdp (a kind gift from Dr Mahito Nakanishi) was added dropwise to the well. The cells were left to stand at room temperature for 2 hours, whereafter the plate was transferred to the incubator and cultured overnight at 37°C. Inactivated MEFs were thawed onto gelatinised plates in MEF culture medium and allowed to settle overnight. Full class II safety precautions were taken during the entire infection procedure.

The following day, MEF culture medium was removed from the iMEF wells and replaced with standard culture medium. Viral medium was removed from infected fibroblast wells, followed by a wash with 1x PBS. Infected cells were incubated with 0.5ml of TrypLE solution (Invitrogen) for 5 minutes at 37°C. Cells were rinsed from the dish with 1ml culture medium, counted, and transferred to a 15ml conical tube. Tubes were centrifuged for 3 minutes at 1000 rpm. The medium supernatant was removed and the pellet was resuspended in culture medium to a concentration of 8 000 – 16 000 cells/ml. One millilitre of the cell solution was added to each well of iMEFs and incubated overnight.

On the third day, the medium was removed from the infected cells and wells were rinsed with warm knockout DMEM (Invitrogen). Medium was replaced with 2ml of warm iPSC culture medium (Appendix 5 – Cell culture medium and preparation of additives, pg 177). The medium in each well was replaced with iPSC culture medium every second day until the 10th day post-infection, whereafter it was replaced with MEF conditioned medium (Appendix 5 – Cell culture medium and preparation of additives, pg 177).
From day 15 to 20, the wells were closely monitored for the presence of early iPSC colonies. Whilst the MEF feeder cells and plated infected fibroblasts exhibit a typical fibroblastic morphology (elongated), iPSC colonies show many tightly packed cells, often with a distinct "edge" to the colony. These colonies were examined under a microscope and picked using a sterile needle. Each colony (or "clone") was placed in a separate well containing fresh iMEFs (plated the previous day) in iPSC culture medium with 10µM of Y-27632 dihydrochloride (Rho kinase inhibitor, Sigma Aldrich) and left to settle for 48 hours. Thereafter half of the culture medium was changed every 24 hours.

iPSC colonies were grown on iMEFs for up to 7 days, or until they had reached a sufficient size for passaging. Depending on the downstream application, colonies were passaged manually (by dissecting each colony into multiple patches, using a sterile needle) or enzymatically using TrypLE (Invitrogen) onto fresh iMEFs, with the addition of 10µM Y-27632 dihydrochloride. Numerous patches/colonies were grown in each well, with passaging occurring every 5 to 7 days. Excess iPSC colonies were frozen for later use and thawed as needed (Appendix 6 – Cell culture protocols, pg 187).

A6.12 Transition of iPSCs from iMEF feeders to feeder-free culture conditions

It takes 3 passages to eliminate all iMEFs and for the iPSCs to be fully adapted to mTeSR medium.

1. Begin with one well of a 6-well plate of iPSCs that have been cultured for 6 days on iMEFs.
2. Passage the cells, as described in A6.11, and transfer all the cells to one matrigel-coated well.
3. Feed as per A6.11.
4. The cells should become confluent in a few days, and can then be split 1:2
5. The next (third) passage, a few days later, is 1:4
6. The cells are now considered adapted, and can be passaged routinely.
A6.13 Maintenance of iPSCs on Matrigel (Corning)

1. Warm TrypLE (Life Technologies) to 37°C in a water bath.
2. Aspirate medium from the well of iPSCs and wash with 1ml of 1x PBS.
3. Add 1ml TrypLE.
4. Incubate at 37°C for 5 minutes, until the cells start to lift off the plate.
5. Wash the cells off the plate and add to a 15ml conical tube containing 9ml of 1x PBS.
6. Centrifuge at 1500 rpm for 5 minutes.
7. Discard supernatant and resuspend cells in an appropriate volume of mTESR medium (depending on ratio of split).
8. Add 2ml of cells in mTESR medium to each Matrigel-coated well.
9. Add Y-27632 dihydrochloride (Sigma-Aldrich) to a final concentration of 10µM.
10. Incubate at 37°C, 5% CO2.
11. Change mTESR medium every day (2ml of pre-warmed medium).
12. Cells may be passaged when they become confluent.

A6.14 Preparation of iPSCs for Karyotype analysis

(Adapted from Barcelona Stem Cell Bank protocol)

1. Add 2µl colcemid (Life Technologies) per ml of culture medium to the line to be karyotyped. Leave for 45 minutes at 37°C in the CO2 incubator.
2. Wash twice with 1x PBS.
3. Add 2ml warm 0.25% Trypsin, place flask in 37°C incubator for 5 minutes. Use a 1ml serological pipette to break the cell into a single cell suspension. Inactivate trypsin by adding 8ml of culture medium. Collect suspension in a 15ml tube.
4. Centrifuge at 1200 rpm for 5 minutes at room temperature.
5. Aspirate supernatant and wash with 5ml of 1x PBS.
6. Centrifuge at 1200 rpm for 5 minutes at room temperature.
7. Aspirate supernatant. Resuspend the pellet in 2ml of hypotonic solution at 37°C (KCl 0.075M) drop by drop, shaking the tube at the same time in a
vortex. Keep vortexing and add 8ml of hypotonic solution. Leave for 10 minutes in a 37°C water bath.

8. Add 1ml of Carnoy fixative (3:1 solution of methanol and acetic acid) drop by drop while shaking the tube in a vortex.

9. Centrifuge at 1800 rpm for 10 minutes at room temperature. Remove supernatant and resuspend the pellet in 2ml of cold Carnoy fixative while vortexing. Keep vortexing and add an additional 8ml of cold Carnoy fixative.

10. Store solution at -20°C until it can be processed.

11. Prepare slides, stain and analyse using routine procedures for Cytogenetics laboratory.

A6.15 Preparation of poly(2-hydroxyethyl methacrylate) (poly-HEMA) coated dishes

1. Prepare a 120mg/ml poly-HEMA (Sigma Aldrich) solution in 95% ethanol, and leave to stir overnight.
2. Filter solution using a 0.22µm syringe filter.
3. Coat wells with poly-HEMA solution and leave to evaporate (3 hours at room temperature, or overnight at 37°C).
4. Rehydrate dish with culture medium for 15 minutes.
5. Aspirate medium, and add cells in an appropriate volume of culture medium.

A6.16 Freezing manually passaged iPSCs

1. Cut colonies into patches as for normal subculturing (approximately 100 patches).
2. Add patches to culture medium in a 15ml centrifuge tube, centrifuge at 1200 rpm for 5 minutes.
3. Remove supernatant and resuspend cell pellet in 0.5ml ice cold iPSC culture medium.
4. Add an equal volume of 2x freezing medium (20% DMSO [Sigma Aldrich], 60% FCS [PAA Laboratories], 20% Knockout DMEM [Invitrogen]).
5. Transfer the 1ml solution to a labelled cryovial in a precooled freezing container (Nalgene).
6. Place freezing container at -80°C overnight, thereafter transfer cryovials to liquid nitrogen storage tank.

**A6.17 Freezing enzymatically passaged iPSCs**

1. Wash well of iPSCs with 1x PBS.
2. Add 1ml TrypLE (Invitrogen) prewarmed to 37°C.
3. Incubate at 37°C until cells are lifting (approximately 5 minutes).
4. Flush well with an equal volume of 1x PBS, triturate to remove clumps.
5. Remove an aliquot for counting, centrifuge remaining cells at 1200 rpm for 5 minutes.
6. Resuspend cells at 2x 10^6/ml in cold iPSC culture medium.
7. Add an equal volume of 2x iPSC freezing medium (concentration 1 x 10^6).
8. Freeze in 1ml aliquots as described above.

**A6.18 Thawing manually passaged iPSCs**

1. Prepare a well of iMEFs (plated the day before thawing of iPSCs) in iPSC culture medium.
2. Thaw cryovial of iPSCs in a 37°C waterbath until half the ice has melted.
3. Transfer to tube with 10ml 1x PBS.
4. Centrifuge at 1200 rpm for 5 minutes.
5. Remove supernatant, gently resuspend patches in iPSC culture medium and centrifuge again.
6. Remove supernatant, resuspend patches in 0.5ml of medium from the well of iMEFs.
7. Add Y-27632 dihydrochloride solution (Sigma Aldrich) to a final concentration of 10µM.
8. Incubate at 37°C overnight.
9. The following day, add 1ml iPSC culture medium.
10. Thereafter, feed as normal (50% medium change per day).
A6.19 Thawing enzymatically passaged iPSCs

1. If plating onto iMEFs, prepare a well the previous day. Before thawing iPSCs, change iMEF culture medium to iPSC culture medium. If thawing onto Matrigel, coat well with Matrigel at least 1 hour in advance.

2. Thaw cryovial of iPSCs in a 37°C waterbath until half the ice has melted.

3. Transfer to tube with 10ml 1x PBS.

4. Centrifuge at 1000 rpm for 5 minutes.

5. Resuspend cell pellet in 1ml of iPSC culture medium and add to well with iMEFs or Matrigel.

6. Add Y-27632 dihydrochloride solution (Sigma Aldrich) to a final concentration of 10µM.

7. Incubate at 37°C overnight.

8. The following day, add 1ml iPSC culture medium.
Appendix 7 - Differentiation of iPSCs into retinal cells
(from Boucherie et al., 2013)

A7.1 Media formulations

**Neural Differentiation Medium**
475ml DMEM F12 (Sigma Aldrich)
5ml 100x Glutamax (Invitrogen)
5ml 100x N2 (Invitrogen)
10ml 50x B27 (Invitrogen)
5ml 100x Antibiotic antimycotic solution (Sigma Aldrich)

**Photoreceptor Differentiation Medium**
100ml Neural Differentiation Medium
500µl Retinoic acid (100µM stock, Sigma Aldrich)
1ml Taurine (1mM stock, Sigma Aldrich)
100µl bFGF (10µg/ml stock, Miltenyi)
50µl aFGF (100µg/ml stock, R&D Systems)
600µl SHH (500nM stock, R&D Systems)

A7.2 Protocol
Culture iPSCs as a monlayer on Matrigel and passage routinely (enzymatically) until the required number of wells are obtained (at least 3 wells of a 6-well plate).

Day 0

1. Dissociate iPSCs using TrypLE (Invitrogen). Detach cells with a cell scraper in mTESR. Break colonies by gentle pipetting and replate at a dilution of 1:3-1:4 in Neural Differentiation Medium with 10µM Y-27632 on 1% (v/v) Matrigel-coated wells.
2. Incubate for 1 hour at 37°C.
3. Aspirate medium and cover attached cells in 2% (v/v) Matrigel solution, diluted in Neural Differentiation Medium. Incubate the dish overnight at 37°C in a humidified atmosphere of 5% CO₂.
Day 1

1. Remove medium and replace with Neural Differentiation Medium (containing no Matrigel). Incubate the dish at 37 °C in a humidified atmosphere of 5% CO₂.

Days 3, 5, 7, 9

1. Remove medium and replace with Neural Differentiation Medium. Incubate the dish at 37 °C in a humidified atmosphere of 5% CO₂.

Days 10-30

1. Remove medium and replace with Photoreceptor Differentiation Medium every second day. Incubate the dish at 37 °C in a humidified atmosphere of 5% CO₂.

At the end of the culture period, store cell pellets at -80°C for later use (RNA extraction), or fixed for immunocytochemistry.
Appendix 8 – Immunocytochemistry

1. Culture cells in the appropriate medium, in culture dishes/wells containing coverslips.
2. Wash three times with 1x PBS.
3. Incubate with 4% paraformaldehyde (PFA) solution (Sigma Aldrich) at room temperature for 30 minutes.
4. Aspirate PFA, wash three times with 1x PBS.
5. Add blocking solution (5% FBS, 0.01% Triton-X (Sigma Aldrich) in 1x PBS) for one hour.
6. Add primary antibody (diluted in blocking solution), incubate overnight at 4°C.
7. The following day, wash three times with 1x PBS.
8. Add secondary antibody (diluted in blocking solution), incubate at room temperature for 2 hours, in the dark.
9. Wash three times with 1x PBS.
10. For nuclear staining, add Hoechst solution (0.5µg/ml, Sigma Aldrich), incubate at room temperature for 5 minutes.
11. Wash three times with 1x PBS.
12. Mount coverslip onto slide with a small amount of Mowiol mounting fluid (Sigma Aldrich), allow to dry at room temperature (covered) for at least an hour (preferably overnight).
13. Visualise with fluorescent microscope.

All slides were visualised with the Zeiss Axiovert 200M inverted fluorescence microscope (using AxioVision 4.8 software) or the Zeiss Axiovert 200M LSM 510 Meta confocal microscope (using ZEN 2009) software.
Appendix 9 – Allele distribution graphs
Distribution of allele sizes amongst tested individuals at the SCA1, 2, 3, 6 and 7 loci.

**SCA1 normal alleles**

![Graph showing allele size distribution for SCA1 normal alleles](image)

- Number of alleles
- Allele size (CAG repeats)
- n=1159

**SCA1 expanded alleles**

![Graph showing allele size distribution for SCA1 expanded alleles](image)

- Number of alleles
- Allele size (CAG repeats)
- n=52
SCA2 normal alleles

Number of alleles vs Allele size (CAG repeats)

n=84

SCA2 expanded alleles

Number of alleles vs Allele size (CAG repeats)

n=36
SCA3 normal alleles

SCA3 expanded alleles

n=1107

n=4
SCA6 normal alleles

Number of alleles

Allele size (CAG repeats)

n=1068

SCA6 expanded alleles

Number of alleles

Allele size (CAG repeats)

n=6
SCA7 normal alleles

SCA7 expanded alleles

n=92

n=42
Appendix 10 – iPSC karyotype reports

65.3.1 (P1a) iPSCs

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**LABORATORY REPORT**

**Specimen** Stem Cell Collection

**Tests ordered** Hid Chr

**BLOOD CHROMOSOME ANALYSIS**

Chromosome analysis of 15 metaphase cells revealed the karyotype 46,XY, consistent with a normal male pattern.

No gross structural abnormality was detected on Giemsa banded metaphases. Chromosomal changes which may be clinically significant such as subtle rearrangements or micro-deletions may not be detected in some metaphase spreads.

The chromosome band resolution is approximately 400 g-bands.

This test was performed at NHLS Cytogenetics, C21, New GSH
Tel: 021 404 4509

Information regarding Genetic Services is available at Tel: 021 404 6304 / 021 404 5235

Authorised by: T Ruppelt Medical Technologist Test(s): Hid Chr

--- End of Laboratory Report ---

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213.2.2 (P2a) iPSCs

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Ref: R-14

Labno: ECG0021918 (05/02/2014)

Patient: 213.2.2

Age(Sex): DoB Not stated

Ref Dr: DR GREENBERG

DIV OF HUMAN GENETICS, LEVEL 3
IIDMM WERNER & BEIT NORTH
UCT, ANZIO ROAD
OBSERVATORY
7925

Taken: 0 Date Rcv'd 27/11/13 15:30

Report: 10/02/14 14:09

FAX: 021 406 6826

LABORATORY REPORT

Specimen: Stem Cell Collection
Tests ordered: Bld Chr

BLOOD CHROMOSOME ANALYSIS

Chromosome analysis of 10 metaphase cells revealed the karyotype: 46,XX consistent with a normal female pattern.

No gross structural abnormality was detected on Giemsa banded metaphases. Chromosomal changes which may be clinically significant such as subtle rearrangements or micro-deletions may not be detected in some metaphase spreads.

The chromosome band resolution is approximately 500g-bands.

This test was performed at NHLS Cytogenetics, C21, New GSH
Tel: 021 404 4909

Information regarding Genetic Services is available at
Tel: 021 406 6204 / 021 404 6235

Authorised by: T Ruppelt Medical Technologist Test(s): Bld Chr

--- End of Laboratory Report ---
213.2.5 (P2b) iPSCs

--- End of Laboratory Report ---
213.3.5 (C1a) iPSCs

--- End of Laboratory Report ---
**LABORATORY REPORT**

**Specimen**
Stem Cell Collection

**Tests ordered**
Bld Chr

**BLOOD CHROMOSOME ANALYSIS**

Chromosomal analysis of 10 metaphase cells revealed the Karyotype: 46,XX consistent with a normal female pattern.

No gross structural abnormality was detected on Giemsa banded metaphases. Chromosomal changes which may be clinically significant such as subtle rearrangements or micro-deletions may not be detected in some metaphase spreads.

The chromosome band resolution is approximately 400g-bands.

This test was performed at NHLB Cytogenetics, C21, New USB
Tel: 021 404 4899

Information regarding Genetic Services is available at
Tel: 021 406 6236 / 021 404 6235

Authorised by: T Ruppelt Medical Technologist Test(s): Bld Chr

--- End of Laboratory Report ---
Appendix 11 - Transcriptional changes in SCA7 patient-derived retinal cells, compared to unaffected control cells

**Experiment 1**

![Graph showing transcriptional changes for Experiment 1]

**Experiment 2**

![Graph showing transcriptional changes for Experiment 2]
Experiment 3 - Retinal genes

Experiment 3 - Other genes
Appendix 12 - Transcriptional changes in SCA7 patient-derived fibroblasts, compared to unaffected control cells

Experiment 1

Experiment 2

Experiment 3 (Family 926)
Appendix 13 - Transcriptional changes in SCA7 patient-derived iPSCs, compared to unaffected control cells

Experiment 1

Experiment 2

Experiment 3 (all iPSCs)
References


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