Developing and characterizing cellular models for neurodegenerative diseases.

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Developing and characterizing cellular models for neurodegenerative diseases

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Supervisor: Prof. Sanbing Shen
Contents
Acknowledgements........................................................................................................ v
Declaration .................................................................................................................. x
Abbreviations .............................................................................................................. xi
Summary ...................................................................................................................... xv
Chapter 1: General Introduction ................................................................................. 1
  1.1 Classification, Symptoms and Clinical Course of Amyotrophic Lateral Sclerosis .. 2
  1.2. Neuropathology .................................................................................................. 3
  1.3 Genetics of ALS .................................................................................................... 4
    1.3.1 SOD1 ........................................................................................................... 4
    1.3.2 TDP-43 ....................................................................................................... 6
    1.3.3 FUS ............................................................................................................. 6
    1.3.4 C9ORF72 .................................................................................................... 8
  1.4 Molecular mechanisms of ALS ............................................................................ 13
    1.4.1 Autophagy ................................................................................................... 15
    1.4.2 Neuronal Autophagy and Neurodevelopment ............................................. 18
    1.4.3 Autophagy in ALS ....................................................................................... 19
    1.4.4 Autophagy – an exciting therapeutic target in ALS .................................... 21
    1.4.5 Astrocyte mediated toxicity in ALS ............................................................ 22
  1.5 Cellular models of ALS ....................................................................................... 24
    1.5.1 Dermal Fibroblasts .................................................................................... 24
    1.5.2 Induced Pluripotent Stem Cells (iPSCs) ................................................................ 26
    1.5.3 Direct Cellular Reprogramming ................................................................. 27
  1.6 Current Challenges ............................................................................................. 31
  1.7 Hypotheses of Thesis ......................................................................................... 33
Chapter 2: Materials & Methods ............................................................................... 34
  2.1 Fibroblast biopsy & cell culture ......................................................................... 35
  2.2 Fibroblast cell treatments ................................................................................... 35
  2.3 Coating Dishes ................................................................................................... 36
  2.4 iPSC Culture ...................................................................................................... 36
  2.5 iPSC Differentiation ........................................................................................... 36
  2.6 Motor Neuron Differentiation ............................................................................. 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7 Astrocyte Differentiation</td>
<td>37</td>
</tr>
<tr>
<td>2.8 Conditioned medium</td>
<td>37</td>
</tr>
<tr>
<td>2.9 Protein extraction</td>
<td>38</td>
</tr>
<tr>
<td>2.10 Western Blot – Buffer Recipes</td>
<td>38</td>
</tr>
<tr>
<td>2.11 Western Blot Analysis</td>
<td>38</td>
</tr>
<tr>
<td>2.12 RNA Extraction &amp; cDNA Synthesis</td>
<td>39</td>
</tr>
<tr>
<td>2.13 RT-PCR</td>
<td>39</td>
</tr>
<tr>
<td>2.14 Immunocytochemistry</td>
<td>42</td>
</tr>
<tr>
<td>2.15 Flow Cytometry</td>
<td>44</td>
</tr>
<tr>
<td>2.16 Molecular Cloning</td>
<td>44</td>
</tr>
<tr>
<td>2.17 Mouse Embryonic Fibroblast (MEF) Harvest</td>
<td>46</td>
</tr>
<tr>
<td>2.18 Virus Production</td>
<td>47</td>
</tr>
<tr>
<td>2.19 Cell Transduction &amp; Reprogramming</td>
<td>48</td>
</tr>
<tr>
<td>2.20 Differentiation of iNSCs</td>
<td>51</td>
</tr>
<tr>
<td>2.21 Statistical analyses</td>
<td>52</td>
</tr>
<tr>
<td>Chapter 3: Amyotrophic Lateral Sclerosis Patient Fibroblasts Demonstrate</td>
<td></td>
</tr>
<tr>
<td>Impaired Autophagy and Increased Sensitivity to Oxidative Stress</td>
<td></td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>54</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>55</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>58</td>
</tr>
<tr>
<td>3.3.1 Generation of fibroblast cultures</td>
<td>58</td>
</tr>
<tr>
<td>3.3.2 ALS-related proteins are expressed in dermal fibroblasts</td>
<td>58</td>
</tr>
<tr>
<td>3.3.3 C9ORF72 may be implicated in the autophagy process</td>
<td>68</td>
</tr>
<tr>
<td>3.3.4 Autophagy regulates some ALS related proteins</td>
<td>68</td>
</tr>
<tr>
<td>3.3.5 ALS patient fibroblasts show impaired initiation of autophagy</td>
<td>75</td>
</tr>
<tr>
<td>3.3.6 ALS patients demonstrate normal levels of phagophore associated proteins</td>
<td>75</td>
</tr>
<tr>
<td>3.3.7 ALS patient fibroblasts demonstrate decreased autophagic flux</td>
<td>78</td>
</tr>
<tr>
<td>3.3.8 ALS patients demonstrate increased levels of LAMP-1, a lysosomal marker</td>
<td>78</td>
</tr>
<tr>
<td>3.3.9 Fibroblasts respond to autophagic induction via Rapamycin</td>
<td>83</td>
</tr>
<tr>
<td>3.3.10 Rapamycin regulates ATG12</td>
<td>86</td>
</tr>
<tr>
<td>3.3.11 Autophagy and Apoptosis</td>
<td>88</td>
</tr>
<tr>
<td>3.3.12 H₂O₂ induces apoptosis in fibroblasts</td>
<td>89</td>
</tr>
</tbody>
</table>
5.3.5 Changes in morphology ................................................................. 166
5.3.6 Neurosphere Generation – Monolayer Vs. Suspension Culture .......... 168
5.3.7 Culturing of reprogrammed spheres.................................................. 176
5.3.8 Reprogrammed cells express markers of neural stem cells .............. 179
5.3.9 Investigating the regional identity of iNSCs ....................................... 181
5.3.10 iNSCs are capable astroglial differentiation...................................... 186
5.3.11 iNSCs can generate oligodendrocyte precursor cells ................... 189
5.3.12 iNSCs can generate neurons ............................................................ 191
5.3.13 iNSCs can generate GABAergic neurons ....................................... 195
5.3.14 iNSCs can generate Glutamatergic neurons, but not TH positive neurons 198
5.4 Discussion......................................................................................... 199

Chapter 6 ............................................................................................. 203
6.1 General Discussion ........................................................................... Error! Bookmark not defined.
6.2 Patient fibroblasts are a useful tool to study autophagic mechanisms .... Error! Bookmark not defined.
6.3 Patient iPSC derived astrocytes differentially regulate the autophagy process ................................................................................ Error! Bookmark not defined.
6.4 Derivation of the NSC for potential replacement therapy of ALS and disease modelling ................................................................. Error! Bookmark not defined.
6.5 Conclusions and Future Perspectives ............................................. 222

REFERENCES ...................................................................................... 223
# Table of Figures

Figure 1.1: Pathological mechanisms reported in ALS........................................14
Figure 1.2: ALS-related proteins may be implicated in the autophagy pathway.16
Figure 2.1. An overview of the reprogramming strategy used to generate
induced neural stem cells .............................................................................50
Figure 3.1. Culture of fibroblasts from biopsies. .................................................59
Figure 3.2. C9ORF72 is expressed in both the nucleus and the cytoplasm of
dermal fibroblasts. .........................................................................................60
Figure 3.3. SOD1 is expressed in both the nucleus and the cytoplasm of dermal
fibroblasts. .................................................................................................63
Figure 3.4. C9ORF72 co-localizes with SOD1....................................................64
Figure 3.5. TDP-43 expression in patient and control fibroblasts......................68
Figure 3.6. FUS expression in ALS patient and control fibroblasts...............67
Figure 3.7. C9ORF72 co-localizes with ULK1, a component of the autophagy
initiation complex. .........................................................................................69
Figure 3.8. C9ORF72 may occasionally localize with p62 in cytoplasmic puncta
in fibroblasts. .................................................................................................70
Figure 3.9. Autophagy does not regulate the expression of SOD1 or C9ORF72. 72
Figure 3.10. Autophagy regulates the expression of TDP-43. ..........................73
Figure 3.11. Inhibition and activation of autophagy decrease and increase FUS
expression respectively. ...............................................................................74
Figure 3.12. ALS patient fibroblast demonstrated impaired autophagic initiation. ..........................76
Figure 3.13. Patient and control fibroblasts express similar levels of proteins
associated with autophagosome formation..................................................77
Figure 3.14. ALS patients demonstrate increased basal p62, indicative of
impaired autophagic flux...............................................................................79
Figure 3.15. Patient fibroblasts demonstrate increased numbers of p62 positive
puncta...........................................................................................................81
Figure 3.16. Patient fibroblasts demonstrate increased staining for LAMP-1, a
lysosomal marker.........................................................................................82
Figure 3.17. Fibroblasts respond to autophagic induction via Rapamycin........85
Figure 3.18. Patients demonstrate decreased availability of free ATG12........87
Figure 3.19. 1mM H2O2 induces apoptosis in dermal fibroblasts......................90
Figure 3.20. 1mM H2O2 induces apoptosis in fibroblasts.................................91
Figure 3.21. Patient fibroblasts are more sensitive to apoptotic induction via
H2O2.............................................................................................................93
Figure 3.22. Pre-treatment with Rapamycin does not ameliorate increased
sensitivity to apoptosis. .................................................................94
Figure 4.1. iPSCs resemble ESCs and are positive for alkaline phosphatase
staining. ..................................................................................108
Figure 4.2. Immunocytochemistry analysis demonstrates expression of
pluripotency markers in control and patient iPSCs. ..........................110
Figure 4.3. iPSCs express pluripotency markers as determined by RT-PCR. .110
Figure 4.4. Patient and control iPSCs are capable of differentiation towards
ectoderm lineage. ......................................................................112
Figure 4.5. Patient and control iPSCs are capable of differentiation towards
endoderm lineage. ......................................................................113
Figure 4.6. Patient and control iPSCs are capable of differentiation towards
mesoderm lineage. ......................................................................114
Figure 4.7. iPSCs generate small rounded cells, typical of neural progenitors.
.................................................................................................115
Figure 4.8. Differentiating cells express NESTIN and PAX6, markers of neural
stem cells ..................................................................................119
Figure 4.9. Differentiating cells express OLIG2, a marker of motor neuron
progenitor cells. ........................................................................119
Figure 4.10. iPSCs differentiated for 14 days express high levels of Ki67, a
marker of proliferative cells. .....................................................120
Figure 4.11. MNPs derived from iPSCs express NESTIN, a marker of neural
stem cells. ..................................................................................122
Figure 4.12. Generation of neurons from MNPs. .................................124
Figure 4.13. Differentiated neurons express MNX1, a marker of motor neurons.
.................................................................................................125
Figure 4.14. MNPs are capable of astrocyte differentiation. ..................127
Figure 4.15. Astrocytes express GFAP by western blot analysis ..............128
Figure 4.16. Astrocyte conditioned medium from ALS patients decreases
viability of both patient and control motor neurons..........................130
Figure 4.17. Expression of autophagy pathway components among the
astrocytes derived from the control, ALS and FXS iPSCs. .................132
Figure 4.18. Densitometry of autophagy related proteins in ALS and Fragile X
patient iPSC derived astrocytes ..................................................133
Figure 4.19. Patient and control astrocytes express similar levels of ALS-related
proteins ........................................................................................135
Figure 4.20. Astrocyte conditioned medium supports the growth of HEK293T
cells .........................................................................................137
Figure 4.22. Astrocyte conditioned medium induces autophagy in HEK293T
cells .........................................................................................141
Figure 4.23. Astrocyte conditioned medium induces autophagy in HEK293T cells. ................................................................. 142
Figure 4.24. Patient ACM results in increased accumulation of p62 positive puncta. .......................................................... 143
Figure 4.25. Patient astrocyte conditioned medium increases levels of SOD1 expression. .................................................. 145
Figure 4.26. Autophagy inducers modulate p62 puncta in HEK293T cells treated with ACM. .................................................. 147
Figure 4.27. Hypothesized mechanism of action. ................................................. 148
Figure 5.1. Generation of high titre retroviruses and lentiviruses in HEK293T cells. ................................................................. 160
Figure 5.2. Induction of neurosphere morphology from human dermal fibroblasts. ................................................................. 162
Figure 5.3. Cells proliferating from induced neurospheres express NESTIN, a marker of neural stem cells.............................. 163
Figure 5.4. iNSCs can generate both neurons and astrocytes. ..................... 164
Figure 5.5. Morphology of MEF-derived cells at day 8 of monolayer reprogramming with different sets of factors.......................... 167
Figure 5.6. Morphology of MEF-derived cells at day 18 of reprogramming in monolayer culture. ............................................. 169
Figure 5.7. Morphology MEF-derived cells at day 18 of reprogramming in suspension culture............................................... 171
Figure 5.8. Morphology of MEF-derived at day 27 of reprogramming in monolayer culture. ..................................................... 173
Figure 5.9. Morphology MEF-derived cells at day 27 of reprogramming in suspension culture............................................... 175
Figure 5.10. Mean number of neurospheres obtained using different combinations of reprogramming factors.......................... 177
Figure 5.11. Morphology of cells in monolayer culture at passage 3. ............ 178
Figure 5.12. Expression of stem cell markers in reprogrammed cells........... 180
Figure 5.13. Expression of rostral NSC patterning markers Foxg1, Emx2 and Nkx2.1 in reprogrammed cells. ............................... 182
Figure 5.14. Expression of hindbrain markers Nkx6.1 and Hoxb4 in reprogrammed cells.......................................................... 183
Figure 5.15. Expression of ventral stem cell markers Olig2 and Hes5, and midbrain marker Lmx1a................................................. 184
Figure 5.16. Analysis of Olig2 positive cells by immunocytochemistry. .......... 185
Figure 5.17. Astrocyte differentiation of reprogrammed cells at three weeks. 187
Figure 5.18. Differentiation of reprogrammed cells towards astrocytes for 5 weeks. ................................................................ 188
Figure 5.19. Differentiation of reprogrammed cells towards oligodendrocytes for 3 weeks. .................................................................190
Figure 5.20. Differentiation of reprogrammed cells towards Dcx positive neurons for 3 weeks. .................................................................193
Figure 5.21. Differentiation of reprogrammed cells towards Map2 positive neurons for 3 weeks. .................................................................194
Figure 5.22. Differentiation of reprogrammed cells towards GABAergic neurons for 3 weeks. .................................................................197
Figure 5.23. Differentiation of reprogrammed cells towards GABAergic neurons for 5 weeks. .................................................................197
Figure 5.24. Differentiation of reprogrammed cells towards Glutamatergic neurons for 3 weeks. .................................................................199
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Finally, I would like to thank my family for their unending support through the years. Without it I could not have come this far.
Declaration

This thesis describes work that I undertook between 2011 and 2015 at the Regenerative Medicine Institute, National University of Ireland, Galway. This work was supervised and mentored by Professor Sanbing Shen.

I declare that the results presented herein are from original experimental work which has been carried out by me for the purpose of this thesis. The work described within this thesis has not been submitted for degree, diploma or other qualification at any other university.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
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<tr>
<td>ACM</td>
<td>Astrocyte Conditioned Medium</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha Fetal Protein</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
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<tr>
<td>APS</td>
<td>Ammonium Per Sulfate</td>
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<tr>
<td>ASM</td>
<td>Alpha Smooth Muscle Actin</td>
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<tr>
<td>ATG</td>
<td>Autophagy-related gene</td>
</tr>
<tr>
<td>BAFA1</td>
<td>Bafilomycin A1</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>C9orf72</td>
<td>Chromosome 9, Open Reading Frame 72</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline Acetyl Transferase</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone Mediated Autophagy</td>
</tr>
<tr>
<td>DPR</td>
<td>Dipeptide Repeat</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid Body</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>fALS</td>
<td>Familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FTLD</td>
<td>Frontal Temporal Lobar Dementia</td>
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<tr>
<td>FUS</td>
<td>Fused in Sarcoma</td>
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<tr>
<td>FXS</td>
<td>Fragile X Syndrome</td>
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<td>GABA</td>
<td>Gamma Amino butyric Acid</td>
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<tr>
<td>GDNF</td>
<td>Glial Derived Neurotrophic Factor</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent protein</td>
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<tr>
<td>IGF2</td>
<td>Insulin-like Growth Factor 2</td>
</tr>
<tr>
<td>iN</td>
<td>Induced Neuron</td>
</tr>
<tr>
<td>iNSC</td>
<td>Induced Neural Stem Cell</td>
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<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<tr>
<td>LIR</td>
<td>LC3 Interacting Region</td>
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<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<tr>
<td>MND</td>
<td>Motor Neuron Disease</td>
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<tr>
<td>MNP</td>
<td>Motor Neuron Progenitor</td>
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<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<tr>
<td>mTORC1</td>
<td>Mammalian Target of Rapamycin Complex 1</td>
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<tr>
<td>NEAA</td>
<td>Non Essential Amino Acid</td>
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<tr>
<td>NIM</td>
<td>Neural Induction Media</td>
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<td>NPC</td>
<td>Neural Progenitor Cell</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>NPC</td>
<td>Neural Progenitor Cell</td>
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<td>NSC</td>
<td>Neural Stem Cell</td>
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<td>OKSM</td>
<td>OCT4, KLF4, SOX2, C-MYC</td>
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<tr>
<td>OPTN1</td>
<td>Optineurin</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PI3P</td>
<td>Phosphatidyl-inositol-3-phosphate</td>
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<tr>
<td>PLS</td>
<td>Primary Lateral Sclerosis</td>
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<td>PMA</td>
<td>Progressive Muscular Atrophy</td>
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<tr>
<td>PSC</td>
<td>Pluripotent Stem Cell</td>
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<td>Pur</td>
<td>Purmorphamine</td>
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<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RAN</td>
<td>Repeat Associated Non-ATG (Translation)</td>
</tr>
<tr>
<td>RARβ</td>
<td>Retinoic Acid Receptor β</td>
</tr>
<tr>
<td>RAPA</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>sALS</td>
<td>Sporadic Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>SBF</td>
<td>SOX2, BRN2, FOXG1</td>
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SBFI  SOX2, BRN2, FOXG1, IGF2
SBFR  SOX2, BRN2, FOXG1, RARβΔ384
SEM   Standard Error of the Mean
Shh   Sonic Hedgehog
SMA   Smooth Muscle Actin
SMN   Survival of Motor Neuron
SOD1  Superoxide Dismutase 1
TDP-43 TAR DNA-binding protein 43
TGFβ  Transforming Growth Factor β
tuj1  Beta-III Tubulin
UBD   Ubiquitin Binding Domain
UBQLN2 Ubiquilin 2
ULK1  Unc-like Kinase 1
UPS   Ubiquitin-Proteosome System
Summary

The focus of this work was to develop and validate in vitro cell culture models of neurodegenerative diseases, with a focus on Amyotrophic Lateral Sclerosis (ALS). ALS is a devastating neurodegenerative disease characterized by the progressive loss of motor neurons, eventually leading to paralysis and death. A hallmark of ALS, as well as other neurodegenerative diseases, is the accumulation of wild-type and mutant proteins into cytoplasmic aggregates. Autophagy is a homeostatic process required for the regulation and recycling of long-lived and misfolded proteins. As such, we focused on investigating the role of autophagy in ALS.

Using ALS patient and control dermal fibroblasts, we began by investigating whether ALS-related proteins are regulated by the autophagy pathway, and whether patient cells would demonstrate any impairments of the pathway. Our primary findings demonstrated that ALS patient fibroblasts demonstrate both impaired autophagic initiation and impaired autophagic flux, that is, the turnover of targeted proteins. These cells are additionally more sensitive to oxidative stress induced apoptosis, possibly due to impaired autophagic mechanisms.

We next investigated the role astrocytes may play in the autophagy pathway in the setting of ALS. For this, induced pluripotent stem cells were generated from three ALS patients and three healthy controls. These were characterized and differentiated to motor neurons and astrocytes, where we demonstrated that patient astrocytes can induce cytotoxicity in both healthy and control motor neurons, as previously reported. We hypothesized that this cytotoxicity may be mediated through impairments of the autophagy pathway. ALS patient and control iPSC derived astrocytes demonstrate similar levels of autophagy components. However, when we examined levels of mTOR, a protein involved in the regulation of many cellular processes, we show that it is greatly increased in patients relative to controls. We further show that conditioned medium from both ALS patient and control astrocytes induces autophagy in HEK293T cells via upregulation of pro-autophagic proteins BECLIN-1 and ATG12. Interestingly,
LC3B, a protein required for the selective degradation of p62 and other cargo, is also upregulated, but only in response to control conditioned medium. Patient conditioned medium further induces an increase in p62 puncta accumulation in cells, indicating impaired autophagic flux. Whether increased mTOR expression is responsible for impaired activation of LC3B is unclear, but these data suggest the possibility that patient astrocytes may induce accumulation of autophagy and p62-selective proteins by dysregulating the balance between autophagosome formation and turnover.

We additionally aimed to develop novel methods for the direct induction of neural stem cells from dermal fibroblasts. Building on work published by other groups, we investigated whether overexpression of IGF2, a growth factor highly upregulated in hippocampal neural stem cells, or RARBΔ384, could improve reprogramming efficiency. We successfully generated and characterized neural stem cells using combinations of SOX2/BRN2/FOXG1, SOX2/BRN2/FOXG1/IGF2 and SOX2/BRN2/FOXG1/RARBΔ384. RT-PCR analysis demonstrated that these populations retain a ventral hindbrain regional identity. These cells were capable of generating oligodendrocyte progenitor cells, mature astrocytes and glutamatergic and GABAergic neurons, indicating tri-lineage differentiation potential. However, they did not generate motor neurons or dopaminergic neurons. RARBΔ384 increased the numbers of GABAergic neurons obtained at three weeks of differentiation, while IGF2 was shown to increase survival of GABAergic neurons over a 5 week period. Despite not showing propensity for motor neuron generation, these cells may be useful for the rapid generation of astrocytes for disease modeling in ALS, avoiding the need for the generation of iPSCs.
Chapter 1

General Introduction
1.1 Classification, Symptoms and Clinical Course of Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), also known as motor neuron disease (MND) and Lou Gehrig’s disease, is a devastating neurodegenerative disease characterized by the progressive loss of motor neurons (Chiò et al. 2013; Kiernan et al. 2011). Early symptoms typically include muscle weakness and atrophy which eventually progresses to full paralysis, and subsequently death.

Although a relatively rare disease, ALS is the third most common neurodegenerative disease, following Alzheimer's disease and Parkinson’s disease (Renton et al. 2014). Mean incidences in Europe and North America are 2.8 and 1.8/100,000 population respectively. Prevalence rates currently lie at 5.4/100,000 in Europe and 3.4/100,000 in North America (Chiò et al. 2013). The disease appears to affect men slightly more than women at a ratio of 1.4:1 (Logroscino et al. 2010). Survival rates vary widely in patients. Mortality rates in the twelve months following diagnosis range from 22-34% (Millul et al. 2005; del Aguila et al. 2003). In the majority of cases median survival lies between 2-4 years following onset (Chiò et al. 2009; del Aguila et al. 2003; Gordon et al. 2012). The age of onset varies with subtypes of disease; juvenile forms of the disease affecting individuals younger than 25 years account for ~1% of ALS cases, with early adult-onset cases (individuals younger than 45 years old) account for a further 10% of all cases (Turner et al. 2012; Logroscino et al. 2010). The remaining cases occur in patients over 45 years of age.

A total of eight clinical phenotypes have been identified in ALS patients, being classified depending on where symptoms originate (classic, bulbar, flail arm, flail leg, pyramidal, respiratory, pure lower motor neuron, pure upper motor (Chiò et al. 2011). Different phenotypes may be indicative of progression with lower limb onset associated with higher median survival rates relative to upper limb onset and bulbar phenotypes (median survival of 39, 27 and 25 months respectively) (Mandrioli et al. 2006). However, regardless of phenotype, the disease inexorably progresses to affect the entire body.
Currently there is no cure for ALS. The only FDA approved therapeutic treatment for ALS is the drug Riluzole, a reputed glutamate blocker (Rowland & Shneider 2001). However, this drug only extends lifespan by 2-3 months, with no discernible improvements in muscle function or strength. Therefore, there is an urgent need for the development of novel and efficacious therapeutics as well as the need to identify the precise mechanisms leading to neuronal degeneration.

1.2. Neuropathology

Although the molecular mechanisms underlying the development of ALS remain to be fully elucidated, post mortem analyses and animal models have allowed the identification of neuropathological hallmarks of ALS. The major pathological feature of ALS is the degeneration of both upper and lower motor neurons (Perry et al. 2010). Further to this, populations of inter neurons within the motor cortex and spinal cord are also known to degenerate (Hossaini et al. 2011; Morrison et al. 1998), in addition to widespread reactive gliosis (Barbeito et al. 2004).

The most common pathological hallmark of ALS is the presence of proteinaceous inclusions containing SOD1, TDP-43 or FUS (Matus et al. 2013). Further, nearly all cases of ALS, sporadic and familial, contain TDP-43 inclusions, with and without mutations in this gene (Lee et al. 2011). Interestingly, the only exceptions identified are patients with SOD1 mutations – these are characteristically devoid of TDP-43 pathology, suggesting the possibility of alternative pathogenic mechanisms (Mackenzie et al. 2007). The events leading to the formation of these inclusions remain widely debated and further research is required to elucidate whether they are a source of cellular toxicity, or simply a symptom of another underlying pathogenic mechanism.

Up to 15% of all ALS patients may also experience frontal temporal lobar degeneration (FTLD) and approximately 30-50% of patients with ALS will suffer
mild cognitive deficits (Dormann & Haass 2013; Liscic et al. 2008). Despite a clear clinical overlap, mutations in certain genes such as SOD1 give rise to pure ALS phenotypes (Mackenzie et al. 2010). Mutations within the C9ORF72 gene on the other hand are responsible for a large proportion of both ALS and FTLD cases (Xiao et al. 2015).

1.3 Genetics of ALS
Familial ALS cases make up approximately 10% of all ALS cases, and the key susceptibility genes identified so far include SOD1, TDP-43, FUS, and C9ORF72.

1.3.1 SOD1
ALS can be caused by mutations in a number of genes (Table 1.1). The first genetic mutation attributed to ALS was identified in the SOD1 gene (Deng et al. 1993). It is estimated that mutations in the SOD1 gene account for between 12-24% of all familial ALS (fALS) cases (Andersen 2006). SOD1 is a ubiquitously expressed antioxidant enzyme which acts to protect cells from toxic free radical superoxides by converting them to water and hydrogen peroxide (Reddi & Culotta 2013). In previous decades the vast majority of research on ALS has focused on animals with a full copy of the human SOD1 cDNA sequence containing a G93A mutation (Acevedo-Arozena et al. 2011). Additionally, there is a wide variety of other SOD1 transgenic models of ALS also available, each demonstrating variable pathology regarding time of onset and death (Bruijn et al. 1997; Jonsson et al. 2006). Over 150 mutations have been identified within this gene alone, most resulting in the misfolding and aggregation of the protein (Saccon et al. 2013; Andersen et al. 2003).
<table>
<thead>
<tr>
<th>GENE</th>
<th>Role in Autophagy</th>
<th>Reference</th>
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<tr>
<td>C9ORF72</td>
<td>May regulate endosomal trafficking</td>
<td>(Farg et al. 2014)</td>
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<tr>
<td>SOD1</td>
<td>Regulated by autophagy, may regulate BECLIN-1 expression</td>
<td>(Li et al. 2015; Nassif et al. 2014a)</td>
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<td>FUS</td>
<td>FUS stress granules regulated by autophagy</td>
<td>(Ryu et al. 2014a)</td>
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<tr>
<td>TDP-43</td>
<td>Regulated by autophagy</td>
<td>(Wang et al. 2015)</td>
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<tr>
<td>SQSTM1/p62</td>
<td>Autophagy receptor</td>
<td>(Chen et al. 2014)</td>
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<td>OPTINEURIN</td>
<td>Autophagy receptor</td>
<td>(Maruyama et al. 2010)</td>
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<td>UBIQUILIN 2</td>
<td>Autophagy receptor</td>
<td>(Williams et al. 2012)</td>
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<tr>
<td>DYNEIN</td>
<td>Regulates transport of late endosomes</td>
<td>(Ravikumar et al. 2005)</td>
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<tr>
<td>CHMP2B</td>
<td>Regulates endosome formation</td>
<td>(Cox et al. 2010; Menzies et al. 2015)</td>
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<tr>
<td>ALS2</td>
<td>Regulates endolysosomal trafficking</td>
<td>(Lai et al. 2009)</td>
</tr>
<tr>
<td>SIGMAR1</td>
<td>Regulates autophagosome maturation</td>
<td>(Menzies et al. 2015)</td>
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<tr>
<td>FIG 4</td>
<td>Mutations result in accumulation of p62/LC3</td>
<td>(Ferguson et al. 2009)</td>
</tr>
<tr>
<td>DCTN1</td>
<td>Regulates transport of autophagosomes</td>
<td>(Ikenaka et al. 2013)</td>
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Table 1.1. List of genes associated with the development of ALS. The most common genes related to the development of ALS may be implicated in the autophagy pathway.

The mechanisms by which SOD1 mutations cause ALS remain to be elucidated. The majority of research points to a toxic gain of function as knockout mice do not develop ALS pathology (Saccon et al. 2013). It has been shown in both cell culture and animal models that mutant SOD1 proteins have the propensity to form aggregations within cells (Bruijn et al. 1998). Aberrant aggregations have also been demonstrated in wild type SOD1 (Chattopadhyay et al. 2008; Bosco
et al. 2010; Forsberg et al. 2010; Synofzik et al. 2012). This may be indicative of a unifying mechanism underlying sporadic and mutation-specific ALS.

1.3.2 TDP-43
TDP-43 is an RNA binding protein with diverse cellular functions including transcriptional regulation, alternative splicing and RNA stabilization (Buratti & Baralle 2001; Ou et al. 1995). Mutations within this gene account for approximately 4% of fALS cases, in addition to a small number of sporadic ALS cases (Renton et al. 2014). The majority of mutations within the TDP-43 gene are located within the C-terminal region (Lagier-Tourenne et al. 2010). TDP-43 is intrinsically prone to aggregation, and mutations within the gene act to accelerate aggregation. TDP-43 is the primary component in proteinaceous inclusions in ALS (Neumann et al. 2006; Arai et al. 2006). However, patients and animals with mutations in the SOD1 gene are characteristically devoid of TDP-43 pathology (Mackenzie et al. 2007).

TDP-43 is known to be recruited to stress granules where it interacts with several proteins associated with the regulation and formation of stress granules (McDonald et al. 2011). It has been suggested that increased or prolonged incorporation into stress granules may result in the formation of pathological stress granules, insensitive to degradation (Parker et al. 2012). Oxidative stress has been shown to decrease the solubility of TDP-43 by inducing inter and intramolecular disulfide crosslinks (Cohen et al. 2011), another mechanism by which TDP-43 may accumulate within cells.

1.3.3 FUS
Fused in Sarcoma (FUS, also known as TLS) is a member of the TET family of proteins and plays diverse roles in numerous cellular processes including DNA/RNA binding, transcriptional regulation (Law et al. 2006), DNA damage repair (Mastrocola et al. 2013), splicing and RNA transport (Sama et al. 2014). FUS shares a high sequence homology to the TDP-43 gene. Indeed the identification of TDP-43 as a culprit in ALS led to the subsequent identification of mutations within the FUS gene in a subset of patients (Kwiatkowski et al. 2009).
Mutations in the *FUS* gene have been implicated in the development of both ALS and FTLD (Neumann et al. 2009; Vance et al. 2009). Mutations in *FUS* may account for between 4-6% of fALS cases and between 0.7-1.8% of sALS cases (Kwiatkowski et al. 2009; Vance et al. 2009).

*FUS* contains an N-terminal transcriptional activation domain, followed by DNA/RNA binding domains. The C-terminal region encodes a nuclear localization signal, and the majority of FUS is typically located within the nucleus. The majority of mutations in the *FUS* gene are located within the coding sequence for the nuclear localization domain, resulting in mislocalization of the protein to the cytoplasm (Vance et al. 2009; Kwiatkowski et al. 2009). Mutations which cause the most severe impairment in nuclear localization result in earlier onset of disease with more rapid progression (Dormann & Haass 2011). Additionally, several mutations have been identified within the 3′ untranslated region of the gene, leading to overexpression of the protein (Sabatelli et al. 2013a). This also results in mislocalization of the protein to the cytoplasm.

Under stress conditions, including oxidative stress, the cytoplasmic FUS becomes incorporated into stress granules, a pathological hallmark of both ALS and FTLD (Lenzi et al. 2015). Whether these granules are consequence of prolonged stress or induced toxic effects within the cells remains to be established. Interestingly, cytoplasmic inclusions positive for FUS are often observed in ALS and FTLD patients even in the absence of mutations within the gene (Munoz et al. 2009). However it has been demonstrated that FUS positive inclusions from ALS and FTLD patients are differentially composed. For example, FUS-positive aggregations in FTLD patient also contain other TET family proteins such as EWS and TAF15, which are not present in ALS cases (Neumann et al. 2011; Davidson et al. 2013). Further, inclusions in ALS-FUS patients also contain highly methylated FUS which are not observed in FTLD patients (Dormann et al. 2012). These studies indicate that although both
diseases share many similar clinical properties, they likely arise from divergent pathomechanisms.

It has also been suggested that the formation of stress granules may be cytoprotective by sequestering pro-apoptotic proteins (Arimoto et al. 2008). It has been hypothesized that stress granule formation is a precursor stage to the formation of pathological inclusions (Wolozin 2012). However, the mechanisms which lead to the subsequent formation of pathological insoluble inclusions remain unknown. It may be due to high concentrations of FUS within the cytoplasm (Kato et al. 2012), the presence of other aggregation prone proteins (Gilks et al. 2004) or other yet unknown mechanisms. It has also been hypothesized that impaired turnover of cytoplasmic proteins by autophagy and/or proteosome degradation pathways may result in the build of FUS aggregations as autophagy is known to regulate FUS positive stress granules (Ryu et al. 2014a).

1.3.4 C9ORF72
Most recently, a GGGGCC hexanucleotide repeat expansion in the C9ORF72 gene has been identified to be responsible for up to 50% of all fALS and some sALS cases (DeJesus-Hernandez et al. 2011). Over 90% of Europeans have 2-10 copies of the GGGGCC hexanucleotide repeat expansion located in the first intron of the gene, while patients typically show over several hundred, or even thousands, of the repeats (Renton et al. 2011). The lower limit at which the number of repeats becomes pathogenic remains unclear; several studies have indentified between 20 and a few hundred repeats in both healthy and patient samples (Buchman et al. 2013; Beck et al. 2013).

The prevalence of this mutation varies widely among different populations. Up to 61% of fALS cases in a Finnish population can be explained by this mutation in the C9ORF72 gene (Majounie et al. 2012), but as few as 3.4% of fALS patients in Japan carry the repeat expansion (Konno et al. 2012). This mutation accounts for 9% of all Irish ALS cases (Byrne et al. 2012).
It had previously been observed that ALS is often associated with Frontotemporal Dementia (FTD), either in the same family, or, indeed, in the same patient. However, it was only with the identification of the C9ORF72 mutation that a molecular link between the two was identified (Renton et al. 2011). The repeat expansion in this gene has also been attributed to the development of many other neurological disorders including Alzheimer’s disease, Huntington’s disease phenocopies, primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA), albeit at much lower incidences (Van Rheenen et al. 2012; Harms et al. 2013; Hensman et al. 2014;). There is also preliminary evidence of C9ORF72 repeat expansions being present in cases of schizophrenia, bipolar disorder and other disorders (Meisler et al. 2013; Galimberti et al. 2014). However, these cases are rare and may simply be due to chance as repeat expansions in this gene are also observed in a small percentage of healthy controls (Majounie et al. 2012; Renton et al. 2011).

To date, little is known about the function of the C9ORF72 protein. Both loss of function and toxic gain of function mechanisms have been proposed (Gendron et al. 2014). Ablation of C9ORF72 in C. elegans results in degeneration of GABAergic motor neurons and increased sensitivity to environmental stresses (Therrien et al. 2013). More recently however, it has been demonstrated that knockout of C9ORF72 in mice is insufficient to induce motor neuron degeneration; the primary effect observed was a significant decrease in body weight (Koppers et al 2015).

Unlike other ALS-related proteins (TDP-43, SOD1, FUS), C9ORF72 does not form cytoplasmic inclusions itself. However, the repeat expansion results in the formation of dipeptide repeats (DPRs) by repeat associated non-ATG (RAN) translation (Ash et al. 2013). These dipeptides form foci which sequester many proteins, notably RNA binding proteins, and have been shown to be cytotoxic in various models of neurodegeneration (Mizielinska et al. 2014; Wen et al. 2014; May et al. 2014). Cellular and Drosophila models have relied on overexpressing DPRs by cDNA constructs (Wen et al. 2014; Yamakawa et al. 2015).
Interestingly however, a more recent study using histological analyses of patient spinal cords post mortem infrequently detected these dipeptide repeats (Gomez-Deza et al. 2015). The most obvious pathological feature observed was TDP-43 positive inclusions. As such the authors suggest that DPRs are not the toxic cause of motor neuron degeneration.

Another pathological feature identified in C9ORF72 repeat expansion patients is the formation of RNA foci arising from expanded repeat sequences (Almeida et al. 2013). C9ORF72 RNA is capable of recruiting proteins to RNA foci (Rogelj 2014). Among these proteins are RNA-binding proteins and RNA export factors (Lee et al. 2013; Cooper-Knock et al. 2014). The recruitment of these proteins to RNA foci may disrupt their function, thus leading to pathological features of ALS. Further studies will be warranted to further investigate the role of C9ORF72 in neurodegeneration.

1.3.5 Autophagy Receptor Proteins
It was initially presumed that autophagy was a non-selective process. However, several proteins have recently been identified to bind specific cargo and subsequently recruit this cargo to autophagosomes (Majcher et al. 2015). These autophagy receptor proteins include p62, optineurin (OPTN1) and ubiquilin-2 (UBQLN2) and mutations in each of these proteins has been implicated in the pathogenesis of ALS (Ceballos-Diaz et al. 2015; Fecto et al. 2011; Wong & Holzbaur 2014).

1.3.6 p62
p62 is a critical regulator of several cellular processes, including autophagy. It contains a ubiquitin binding domain (UBD) for the selective targeting of ubiquitinated proteins, as well as an LC3-interacting region (LIR) domain, for direct incorporation of cargo to autophagosomes (Majcher et al. 2015). ALS patients presenting with mutations in the p62 gene display p62 positive inclusions in motor neurons, with a concomitant increase in TDP-43, possibly suggesting defective autophagy and regulation of TDP-43 (Teyssou et al. 2013).
Mutations in the p62 gene were first associated with Paget’s disease, with the majority of patients presenting with mutations in the C-terminal UBA domain (Rea et al. 2014). However, some mutations have been identified as common to ALS and Paget’s disease patients. Reasons as to why some patients develop ALS, while others develop Paget’s disease remain unclear, but are possibly due to influence of other genomic loci. Interestingly, mutations located in the LIR have been identified in ALS patients, suggesting a direct link between autophagy and ALS pathogenesis (Chen et al. 2014). With further relevance to ALS, p62 has also been shown to contain a domain specific for the binding of SOD1 to LC3 by a ubiquitin independent mechanism (Gal et al. 2009). However, mutations within this region of the protein have not been identified (Majcher et al. 2015).

Interestingly, knockdown of p62 in zebrafish results in the locomotor dysfunction, and activation of autophagy ameliorates this phenotype (Lattante et al. 2014). However, whether decreased protein or loss of function is responsible for phenotype is unclear.

1.3.7 Optineurin
Optineurin also contains a UBD and LIR, and plays a role in selective autophagy (Majcher et al. 2015). Mutations in this gene were first identified in a small Japanese cohort, which demonstrated inclusions positive for optineurin with TDP43 and ubiquitin (sALS), as well as SOD1 (fALS) (Maruyama et al. 2010). Interestingly, unlike other ALS-related genetic mutations, mutations in OPTN1 have not been identified in FTLD patients (Rollinson et al. 2012). Conversely, mutations have been associated with another neurodegenerative disease – primary open-angle glaucoma – and this has been specifically attributed to impaired autophagy (Chalasani et al. 2014). Mutations associated with the development of ALS include those within the UBD. It has been demonstrated that this protein may play a major role in a specific type of autophagy.
responsible for the turnover of damaged mitochondria (i.e. mitophagy) (Wong & Holzbaur 2014).

Similar to p62, knockdown of optineurin in zebrafish results in motor dysfunction (Korac et al. 2013). These results are similar to a zebrafish model expressing a mutant form of the SOD1 protein. However, whether decreased protein, loss of function or toxic gain of function are responsible for disease pathogenesis requires further investigations.

1.3.8 Ubiquilin-2
Like p62 and optineurin, ubiquilin-2 contains an ubiquitin binding domain which can specifically target ubiquitinated proteins. Mutations within the UBQLN2 gene have been demonstrated in both sALS, fALS and ALS-FTLD patients (Zhang et al. 2014). Interestingly, even in the absence of mutations, cytoplasmic inclusions in ALS patients are often shown to contain ubiquilin-2 which are often also positive for TDP-43 (Williams et al. 2012; Deng et al. 2011). It has been demonstrated that ubiquilin-2 binds to TDP-43 and over expression can lead to a decrease in the levels of TDP-43 protein (Cassel & Reitz 2013). To date the mutations identified within the ubiquilin-2 gene are located within a PXX region rich in proline residues, which is required for protein-protein interactions (Williams et al. 2012).

Each of these proteins contains an ubiquitin binding domain, suggesting selective degradation of ubiquitinated proteins. Further, both p62 and optineurin contain LC3-interacting domains, by which they directly target proteins to the developing autophagosomes. Ubiquilin-2 also recruits proteins directly to autophagosomes, though lacking an established LC3-interacting domain the mechanism by which this occurs remains unclear. However, it has been demonstrated that its ubiquitin binding domain is essential for this interaction (N’Diaye et al. 2009).
1.3.9 Other ALS Associated Mutations
Including those discussed above, mutations in a wide number of other genes have been identified. However, these are much less widely studied, particularly in relation to the autophagy pathway. Both CHMP2B and SIGMAR1 may regulate autophagosome maturation (Menzies et al. 2015; Cox et al. 2010). Further, mutations in proteins associated with endosomal trafficking have also been reported including ALS2 and DYNEIN (Lai et al. 2009; Ravikumar et al. 2005). Additionally, mutations have been reported in SETX and VAPB, though the underlying molecular deregulations have not been reported (Kenna et al. 2013).

1.4 Molecular mechanisms of ALS
Despite mutations in many genes being identified as causative of ALS through decades of research, the underlying molecular mechanisms of pathogenesis remain unknown (Wang et al. 2015). However, perturbations in many cellular processes have been identified including oxidative stress (Pollari et al. 2014), glutamate excitotoxicity, protein misfolding and aggregation, autophagy (Chen et al. 2012; Otomo et al. 2012), proteosome function, apoptosis, endoplasmic reticulum induced stress (Walker et al. 2013), mitochondrial damage, RNA metabolism, vesicle trafficking, neuroinflammation (Consilvio et al. 2004) and release of toxic factors by glial cells (Haidet-Phillips et al. 2011). It remains to be elucidated which of these may be causative of ALS and which are merely symptoms of other underlying molecular mechanisms. However, the identification of protein aggregates in all ALS cases provides surmounting evidence to suggest a role for impaired proteostasis in disease pathology. Figure 1 illustrates many of the mechanisms perturbed in ALS (Vucic et al. 2014).
Figure 1.1: Pathological mechanisms reported in ALS (Vucic et al. 2014).
1.4.1 Autophagy

Cellular homeostasis relies on a constant balance between the synthesis of new proteins, and the recycling of long-lived proteins to maintain sufficient amino acid supply. Many cellular processes, such as proliferation and differentiation, are regulated by modulating the balance between these two mechanisms (Nedelsky et al. 2008).

Two main systems in the cell are responsible for the degradation of unwanted proteins; the ubiquitin-proteosome system (UPS) and autophagic mechanisms. The UPS is predominantly associated with the regulation of short lived proteins, whereas the autophagy pathways are required for the turnover of long-lived and misfolded proteins by lysosome.

Autophagy is a cellular mechanism involved in degradation and clearance of proteins (Chen et al. 2012; Otomo et al. 2012). There are three distinct types of autophagy; Chaperone mediated autophagy (CMA), microautophagy and macroautophagy. Macroautophagy (henceforth referred to simply as autophagy) is the most important and widely studied regarding neuronal homeostasis and function (Rami 2009). Both over activation and under activation of this pathway can be detrimental to cell survival. The autophagy pathway can be divided into several stages; initiation, elongation and degradation, each of which has been reported to be deregulated in various neurodegenerative disorders.

Autophagy is regulated by the mammalian Target of Rapamycin complex 1 (mTORC1), which comprises mTOR, and other signaling proteins including RAPTOR, DEPTOR and PRSA40. This complex plays important roles in many cellular processes including proliferation and protein synthesis (Kim et al. 2011a). An overview of the autophagy process is depicted in Figure 2, indicating where ALS related proteins SOD1, TDP-43, p62, Ubiquilin2, optineurin, CHMP2B and SIGMAR1 may affect the pathway. Although there is evidence of other proteins implicated in impaired autophagy (C9ORF72 and FUS), the exact mechanisms by which mutations in these proteins induce autophagic dysfunction is unclear.
Figure 1.2: ALS-related proteins may be implicated in the autophagy pathway. mTOR regulates autophagy initiation by regulating the phosphorylation status of ULK1, a component of the autophagy initiation complex. This complex promotes autophagy by activating VPS34 and BECLIN-1 (which may be regulated by SOD1 (Nassif et al. 2014). Cargo is recruited into the developing autophagosome by LC3-II and autophagy receptor proteins including p62, optineurin and Ubiquilin 2, with mutations in each identified in ALS patients (Chen et al. 2014; Maruyama et al. 2010; Williams et al. 2012). Autophagosome maturation is regulated by many proteins, including CHMP2B and SIGMAR1 of which mutations in ALS patients have also been identified (Cox et al. 2010). Finally, fusion of the autophagosome to the lysosome allows degradation of contents within, which often include ALS related proteins such as SOD1 and TDP-43 (Wang et al. 2015; Li et al. 2015).
The autophagy initiation complex comprises ULK1, ULK2, ATG13, ATG101 and the adapter protein FIP200 (Alers et al. 2012). Together this complex regulates autophagy initiation via signals from the mTOR complex (mTORC1), AMPK signaling and the p53 pathway (Füllgrabe et al. 2014; Alers et al. 2012). mTOR negatively regulates the phosphorylation status of ULK proteins at serine 555 through p70S6K, thereby negatively regulating autophagy (Kim et al. 2011a). However, under conditions of nutrient starvation, mTOR is inhibited, thus dephosphorylating p70S6K, and allowing the activation of the initiation complex.

Conversely, AMPK signaling positively modulates the phosphorylation of alternative ULK1 phosphorylation sites to induce initiation of autophagy. Further, p53 may either activate or inhibit initiation, depending on its own activation status (Morselli et al. 2011; Füllgrabe et al. 2014). As such autophagy is a complex process regulated by a wide variety of cellular signals and pathways.

The initiation complex acts to recruit autophagy related gene (ATG) encoded proteins to the site of autophagosome formation and phosphorylate BECLIN-1 (Russell et al. 2013). This promotes activity of the autophagosome formation complex comprising BECLIN-1, VPS34, PI3KCIII and ATG14L, which acts to fine tune the autophagy process (Abrahamsen et al. 2012). During membrane nucleation phosphatidyl-inositol-3-phosphate (PI3P) is generated and accumulates, allowing the recruitment of PI3P binding proteins (Shibutani & Yoshimori 2014). These include WIPI1 and WIPI2, which are essential for the recruitment of ATG16L1 to the forming autophagosome (Dooley et al. 2014).

For membrane elongation two sets of proteins are required. Firstly, ATG7 acts on ATG12 and ATG10 resulting in the conjugation of ATG5-ATG12. This then further binds to ATG16L (Shpilka et al. 2012). This complex is dependent on previously accumulated PI3P and WIPI proteins to target membranes. In parallel, LC3 is cleaved by ATG4 to generate LC3-I and subsequently further processed by ATG3 and ATG7 to form LC3-phosphatidylethanolamine (PE), or simply, LC3-II (Weidberg et al. 2011). This complex binds to the forming autophagosome membrane and plays a role in recruiting cargo targeted for
degradation by p62, which identifies proteins targeted for degradation via ubiquitin-binding domains (Rogov et al. 2014). This cargo is subsequently targeted to autophagosomes for degradation by the interaction of p62 and LC3.

Completed autophagosomes are transported along microtubules by dynein proteins to the lysosomal rich perinuclear region (Ravikumar et al. 2005). Here SNARE proteins regulate the fusion of the autophagosome to the lysosome resulting in the degradation of internalized cargo (Fader et al. 2009; Furuta & Amano 2010).

1.4.2 Neuronal Autophagy and Neurodevelopment
While autophagy is an essential process for most cell types, neurons may be particularly sensitive to accumulation of toxic proteins (Lee et al. 2013). Unlike other cells, neurons are post-mitotic and cannot undergo cell division as a means to dilute out toxic cellular waste. For this reason, neuronal cells are more sensitive to impairments of proteostasis and require tight regulation of autophagic and proteosomal degradation pathways to maintain homeostasis. Impaired proteostasis machinery is typically observed in an age-related manner, and may help explain why the majority of neurodegenerative diseases associated with impaired proteostasis display a late onset (Hindle 2010; Ben-Zvi et al. 2009). Ablation of either Atg5 or Atg7, key proteins involved in autophagy, specifically in neurons of mice results in neuronal degeneration and motor dysfunction, demonstrating its requirement for cellular survival (Hara et al. 2006; Komatsu et al. 2006). Interestingly, it has been shown that axons are more susceptible to degeneration mediated by impaired autophagy and many studies have demonstrated that autophagy is essential for axonal growth and homeostasis (Coupé et al. 2012; Komatsu et al. 2007). It is also likely that autophagy regulates synapse development at neuromuscular junctions, as well as presynaptic structure and function in dopaminergic neurons (Shen & Ganetzky 2010; Hernandez et al. 2012).
Autophagy is also essential in neuronal differentiation. Inhibition of autophagy impairs the differentiation of neuroblastoma cells, as well as neural progenitor cells (Zhao et al. 2010). Further, a recent study has demonstrated that sonic hedgehog (Shh), a widely studied morphogen in neurodevelopment, induces autophagy in hippocampal neurons. This suggests an important role for autophagy in neurodevelopment. As such, dysfunction of the autophagy pathway during development is likely to be detrimental.

Fragile X syndrome is the most common genetic disease within the spectrum of autism disorders (Zalfa et al. 2003; Lee et al. 2013). It is caused by a CGG trinucleotide expansion in the FMR1 gene. The translated protein, FMRP, acts to negatively regulate synaptic mRNA translation. Fmr1 knockout mice show increased mTOR signaling, an effect also observed in human patients (Sharma et al. 2010; Hoeffer et al. 2012). Evidence has suggested a correlation between deregulated mTOR signaling and impaired dendritic spine morphogenesis (Nimchinsky et al. 2001; Comery et al. 1997). This exemplifies the essential role of autophagic balance in neurodevelopmental processes.

1.4.3 Autophagy in ALS

Impairments of the autophagy pathway have been implicated in a wide variety of neurodegenerative disorders, including Parkinson’s disease, Alzheimer’s disease and ALS. Aggregations of toxic proteins are a hallmark of ALS. However, debate remains as to whether these are cytotoxic culprits in disease pathogenesis, or whether they may be cytoprotective by sequestering otherwise toxic misfolded proteins (Gal et al. 2007). It may be possible that protein aggregations occur due to failure of autophagy mechanisms to recycle these proteins. Alternatively, it has been suggested that aggregation of toxic misfolded proteins impairs autophagy by sequestering proteins which are essential for normal function of the pathway.

Initial evidence of a role for autophagy in the pathogenesis of ALS came from animal models of ALS which demonstrated increased levels of LC3-II, the autophagosome associated form of the protein (Morimoto et al. 2007).
However, interpreting levels of autophagy related proteins is a convoluted matter – both increasing levels of autophagy and impairments of autophagic flux can each lead to the increase of certain proteins, including LC3-II and p62. As such, Morimoto et al could not definitively state whether their results were due to increased formation of autophagosomes, or as a result of impaired turnover, and thus increased buildup of autophagosomes. The increase in autophagic vesicles was confirmed in patient lumbar spinal cords; Immunohistochemical analysis indicated the presence of LC3 and p62 in patient spinal motor neurons, with more obvious features present in degrading neurons (Sasaki 2011). No autophagic features were observed in control samples.

It remains unclear as to whether autophagy is increased or decreased in the setting of ALS. Inhibition of the UPS has been shown to induce autophagy by a compensatory mechanism (Korolchuk et al. 2010). Mutant SOD1 directly impairs the UPS, resulting in similar activation of the autophagy pathway (Crippa et al. 2010). Indeed, increased autophagy as indicated by increased LC3-II accumulation has been reported in ALS mtSOD1 mice (Li et al. 2008). Additionally, it has been shown that mtSOD1 transgenic mice, haploinsufficient of Beclin-1, show an increase in lifespan (Nassif et al. 2014). This may suggest a pathogenic role for increased Beclin-1 in the pathogenesis of ALS.

It may also be possible that an imbalance between various autophagy pathway components results in pathological effects. Increased generation of autophagosomes, without concomitant signals for their turnover, may explain the aggregation of many proteins so often observed in ALS.

The most convincing evidence for a role of autophagy in the pathogenesis of ALS may be attributed to the basal function of ALS-related genes. Nearly all of the genes associated with ALS may be implicated somehow in the autophagy pathway (Table 1.1). Autophagy has previously been shown to regulate the turnover of TDP-43 by p62 (Brady et al. 2011). Further, FUS positive inclusions identified in patients are typically positive for p62, suggesting a similar interaction (Deng et al. 2010). SOD1, or more specifically, mutated SOD1, may
interact with BECLIN-1-BCL2L1 complex, thus regulating the autophagy process (Nassif et al. 2014). p62, itself a major component of the autophagy-lysosome system, is mutated in a number of ALS cases (Teyssou et al. 2013). And C9ORF72 may interact with LC3B, suggesting a possible role in the autophagy process (Farg et al. 2014).

Additionally, mutant SOD1, FUS and TDP-43, often identified in pathological inclusions, can inhibit autophagic flux and decrease clearance of unwanted proteins (Damme et al. 2014). Interestingly, it has been demonstrated that disease mechanisms may be recapitulated in iPSCs in the absence of protein aggregations, providing evidence that protein aggregations are merely a symptom, rather than causative of ALS (Kiskinis et al. 2014; Chen et al. 2014; Wainger et al. 2014).

1.4.4 Autophagy – a potential therapeutic target in ALS

Many studies have shown that increasing autophagy is beneficial in ALS. Increased autophagy via HSPB8 results in increased solubility and clearance of mutant SOD1, with no effect on wild-type protein (Crippa et al. 2010). A similar response was observed in mice deficient of XBP-1 – the consequential increase in autophagy reduced accumulation of mutant SOD1 inclusions in spinal cord (Hetz et al. 2009). Further, overexpression of FYVE, an autophagy related protein, regulates the turnover of mutant SOD1, TDP-43 and C-terminal fragments of TDP-43, TDP-25 (Han et al. 2014).

Pharmacological activators of the autophagy pathway have also been shown to be beneficial in several studies. These include Rapamycin (Staats et al. 2013), Trehalose (Zhang et al. 2014), Lithium (Fornai et al. 2008) and Valproate (Wang et al. 2015), suggesting that modulation of the autophagy pathway may represent an efficacious target in therapeutic settings. However, the molecular targets by which these activators act to ameliorate phenotype is unclear.

Conversely, another group has shown that lithium had no beneficial effect on survival in two other strains of SOD1 transgenic mice (Pizzasegola et al. 2009).
Additionally, a separate study indicated that Rapamycin was detrimental to survival in a specific SOD1 transgenic model (Zhang et al. 2011).

Each of the studies which demonstrate a positive effect of autophagic induction on the amelioration of pathology has utilized mtSOD1 animals. Surmounting evidence suggests that mutations in SOD1 give rise to ALS with divergent pathological mechanisms (Mackenzie et al. 2007). If mutations in other ALS related genes differentially impair autophagy, activation of the autophagy pathway may prove ineffective or detrimental (Yamamoto & Yue 2014). Further studies in non-SOD1 patients and animal models are required. In this sense, I studied autophagy in ALS patient-derived cells with or without GGGGCC expansion on C9ORF72.

1.4.5 Astrocyte mediated toxicity in ALS
Degeneration of motor neurons is the primary characteristic associated with ALS. However, prolonged neuroinflammation is also observed in patients and animal models (Barbeito et al. 2010). It is becoming increasingly evident that non-neuronal cells may play a significant part in many neurological diseases. Early studies identified that diminished expression of mutant SOD1 in astrocytes greatly delayed disease progression (Yamanaka et al. 2008). More convincingly, motor neurons derived from ESCs were selectively sensitive to toxicity induced by astrocytes harboring ALS related mutations (Di Giorgio et al. 2007; Nagai et al. 2007; Ilieva et al. 2009). Conversely, these toxic effects are not observed on other neuronal populations (GABAergic neurons and interneurons), nor do other cell types (fibroblasts or cortical neurons) produce the same secreted toxic effects. More recently, direct conversion of patient fibroblasts to neural stem cells and subsequent differentiation to astrocytes demonstrated that astrocytes secrete factors which are directly toxic to motor neurons, in a cell contact independent manner (Meyer et al. 2014a). This conclusively demonstrates that ALS is not a disease solely concerning motor neurons, but rather likely involves interplay between many cell types. Several recent publications have focused on
identifying the molecular mechanism by which astrocytes may induce cytotoxicity of motor neurons.

Several studies have attempted to identify the soluble factors which mediate motor neuron cell death. A 2008 paper identified prostaglandin D2 as a potential culprit, but blocking of this protein only offered modest improvements in motor neuron survival (Di Giorgio et al. 2008). Marchetto et al additionally identified activated inflammatory responses and oxidative stress in response to patient astrocytes (Marchetto et al. 2008). Further changes in NFkB, Akt and JNK signaling have been reported, but these studies failed to identify specific factors responsible (Haidet-Phillips et al. 2011).

More recent evidence demonstrates that astrocytes mediate motor neuron death via oxidative stress (Rojas et al. 2014). This study demonstrated that cell death is mediated through c-Abl, a tyrosine kinase, which induces apoptosis. Inhibition of this kinase or treatment with antioxidants was sufficient to prevent cell death. Whether these mechanisms can be recapitulated in sporadic ALS and fALS associated with other mutations will be interesting to validate.

Another report has indicated that increased α2-Na/K ATPase/α-adducin complex in SOD1 mice may be responsible for inducing motor neuron cell death (Gallardo et al. 2014). Both pharmacological and genetic disruption of Na+ K+ ATPase was protective in response to mtSOD1 astrocyte conditioned medium. Na+ K+ ATPase has been shown to regulate autosis, a mechanism of cell death triggered by increased BECLIN-1, starvation and hypoxia-ischemia (Liu et al. 2013). Gallardo et al do not suggest a link between autophagy and their mechanism of cell death. In fact, little has been reported regarding astrocytes and autophagy. One study published by Perucho et al demonstrated that glial cell conditioned medium induced the autophagy pathway in a mouse model of Huntington’s disease (Perucho et al. 2013). As such, it will be interesting to see how astrocytes may mediate the autophagy pathway, and whether this is different between ALS patient and control astrocytes.
1.4.6 Microglia & ALS
In addition to the astrocytes playing a role in the pathogenesis of ALS, many studies have also implicated a role of microglia in neurodegeneration (Gerber et al. 2012). Microglia support both neurons and astrocytes, while also retaining immunological functions (Brites & Vaz 2014). It has been demonstrated mutant SOD1 activates microglia, which subsequently produce factors which decrease motor neuron viability (Roberts et al. 2013). However, the identity of these factors are currently unknown, but likely do not include TNF-α, nitric oxide or super anion radicals (Brites & Vaz 2014; Roberts et al. 2013).

1.5 Cellular models of ALS
Animal models have been invaluable for investigating potential causes of ALS. However, these models are limited by their specificity to mutations in particular genes. As such, the mechanisms responsible for sporadic ALS or caused by relatively rare mutations cannot be easily explored. Furthermore, results obtained in animal models are not always relevant to human pathology (Kissel et al. 2011). While primary human patient neural tissue is ideal for examining neuropathology it is difficult to obtain, and usually only available post mortem. To overcome these difficulties many cellular models of ALS have been developed and are widely used in research.

1.5.1 Dermal Fibroblasts
Dermal fibroblasts are readily accessible from patients and allow the derivation of cell populations which are capable of long term propagation and expansion. Research in the fields of Parkinson’s disease (Auburger et al. 2012), Huntington’s disease (Erie et al. 2015), Alzheimer’s disease (Corlier et al. 2015) and ALS have all shown fibroblasts to recapitulate some of the molecular aspects of disease progression.

In the case of ALS, many studies have shown, via histological analyses, that skin from ALS patients demonstrate increased levels of many ALS related proteins including TDP-43, FUS and VCP, as well as markers associated with neuroinflammation, TNF-α and IL-6 (Suzuki et al. 2010; Oketa et al. 2013;
Ishikawa et al. 2012; Fukazawa et al. 2013; Ono et al. 2001). Interestingly, increased levels of LC3, a key autophagy protein, have also been reported in skin samples from patients (Wang et al. 2015). The authors suggest that LC3 may be useful as a biomarker of ALS, but fail to investigate the cause or effect of increased LC3 in these samples. Together these studies validate the potential for non-neural tissues to recapitulate some disease mechanisms of ALS, and the possibility of identifying novel biomarkers using patient derived skin samples.

More recent studies have focused on the culture of these cells, and subsequent analysis using various methodologies. Mislocalization of ALS-related proteins is often observed in patient cells such as motor neurons. These findings have been successfully recapitulated in fibroblasts from patients regarding TDP-43, FUS and U1 snRNP (Sabatelli et al. 2015; Yamazaki et al. 2012; Yu et al. 2015; Sabatelli et al. 2013; Schwartz et al. 2014). Conversely, another study has suggested that localization of FUS (and SMN) is not altered in patient fibroblasts (Kariya et al. 2014). Differences observed may be due to different patient cohorts, but at present it is unlikely that FUS alone will act as a suitable biomarker for all ALS cases.

RNA-seq analysis has implicated differences in many biological process in patient fibroblasts including hypoxic response, RNA processing and metabolic deregulation (Raman et al. 2014). Mitochondrial dysfunction has been widely implicated in the pathogenesis of ALS (Xie et al. 2015), and several publications have reported mitochondrial dysfunction in ALS patient derived fibroblasts (Allen et al. 2015; Kirk et al. 2014a; Bannwarth et al. 2014; Allen et al. 2014). It will be interesting to investigate the molecular cues leading to metabolic imbalance, and at which stage of disease progression do they occur.

The possibility for drug screening is another potential use for patient derived fibroblasts. In one such study, screening of small compounds was performed to identify molecules which may regulate C9ORF72; bromodomain inhibitors were capable of increasing the expression of non-expanded C9ORF72, without increasing expression of the expanded form of the gene, thus possibly
overcoming issues proposed by haploinsufficiency mechanisms (Zeier et al. 2015). Another report detailed the effect of valproate as a possible therapeutic agent on dermal fibroblasts, used to increase levels of reportedly decreased SMN, but this was only performed for one patient sample (Tremolizzo et al. 2014).

With the exception of the identification of increased LC3 in skin samples from ALS patients, no study has yet investigated autophagic mechanisms in dermal fibroblasts. The first chapter of this thesis will focus on investigating the expression of ALS related genes in dermal fibroblasts from ALS patients and controls, as well as autophagic mechanisms.

1.5.2 Induced Pluripotent Stem Cells (iPSCs)

In 2006 Takahashi & Yamanaka first showed that it was possible to take a somatic cell and de-differentiate it to a pluripotent state, similar to an embryonic stem cell (ESC) (Takahashi & Yamanaka 2006). One year later, this groundbreaking technology was applied to human cells, opening up an exciting new field of research (Takahashi et al. 2007). These cells, capable of unlimited self-renewal, maintain the potential to generate cells of all three germ layers. As cellular models of “disease in a dish”, they have allowed unprecedented advancements in our understanding of disease mechanisms in both familial and sporadic ALS cases.

ALS may be one of the most highly investigated diseases using iPSC technology (Liu Y 2015). Several studies have utilized ESCs and genetic manipulation methodologies to successfully recapitulate neurodegeneration observed in ALS (Karumbayaram et al. 2009). However, genetic manipulation of PSCs only allows investigation into mechanisms associated with a small number of ALS cases, the majority having no known genetic mutation. As such, the generation of iPSCs from fALS patients harbouring various different causative mutations, as well as sALS patients, has allowed identification2of various
disease mechanisms, and the potential to identify novel therapeutic targets. iPSCs have been used to identify defects in various mechanisms including membrane hyperexcitability, neurofilament aggregation, impaired nucleocytoplasmic transport and intranuclear aggregation of TDP-43 (Wainger et al. 2014; Chen et al. 2014; Burkhardt et al. 2013; Zhang et al. 2015).

Further improvements in methodologies may arise from the generation of iPSCs and subsequent genetic manipulation to establish lines which differ only by their genetic mutation. Such lines would demonstrate defective mechanisms specific for various ALS related mutations.

1.5.3 Direct Cellular Reprogramming

iPSC technology has been invaluable in furthering our understanding of disease mechanisms. However, it has its own drawbacks. The potential for iPSCs to generate teratomas is a key obstacle in the development of cellular transplantation therapies. Working with iPSCs can often be time consuming and labor intensive, particularly with newly generated cell lines. Additionally, techniques for differentiating iPSCs are often difficult and lengthy. Furthermore, work with iPSCs is tremendously costly relative to other cell types (Beers et al. 2015).

To overcome some of the challenges with iPSCs, an increasing focus has been directed towards the direct reprogramming methodologies; that is the direct conversion of fibroblasts (or other somatic cells) to cells of interest including blood cells, hepatocytes (Huang et al. 2011), cardiomyocytes (Ieda et al. 2010) and various cells of the neural lineage (Wapinski et al. 2013; Xue et al. 2013; Ambasudhan et al. 2011; Kalani & Martirosyan 2012; Thier et al. 2012).

The generation of functional neurons directly from fibroblasts has been demonstrated (Son et al. 2011; Pfisterer et al. 2011; Caiazzo et al. 2011). Although these may be a useful tool for more rapid evaluation of disease mechanisms and/or drug screening, the efficiencies at which functional neurons
are generated are relatively low. In addition, as they are post-mitotic, these cells cannot be propagated for further studies, thus limiting their use for repeated studies.

Alternative strategies have focused on the generation of induced neural stem cells (iNSCs), directly from somatic cells (Table 1.2). The first successful report of this utilized the canonical reprogramming factors (OCT4, SOX2, KLF4 and C-MYC) to begin reprogramming towards a pluripotent state, and directing differentiation at a partially reprogrammed state towards neural stem cells (Kim et al. 2011). The authors were unable to determine whether the reprogrammed cells were converted directly to neural stem cells, or whether they passed through a pluripotent state temporarily. Subsequent research indeed determined that these cells likely passed through a pluripotent state, thus retaining the potential for residual, undifferentiated cells to form teratomas (Bar-Nur et al. 2015).

Subsequent approaches investigated the potential of lineage specific transcription factors for the direct generation of specific cell types, avoiding the use of pluripotency factors and the potential of indirectly inducing iPSCs (Lujan et al. 2012). Different publications demonstrate varying success. Kim et al showed that their reprogrammed cells were only capable of retaining neural stem cell phenotype for five passages (Kim et al. 2011). Others showed the generation of lines which were capable of long term propagation (Xi et al. 2013; Thier et al. 2012), but these were limited to studies using rodent cells. Ring et al. demonstrated the successful generation of iNPCs (induced neural progenitor cells) from human cells using only SOX2 over expression, but these required the use of mouse feeder layers which is not ideal. Possibly the most successful methodology produced to date has been by Mirakhorli et al (2015) which utilized protein transduction methods and small molecules in human fibroblasts, avoiding the integration of viral vectors.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Factors</th>
<th>Starting Cell Type</th>
<th>Self Renewal Potential</th>
<th>Neuronal Differentiation</th>
<th>Astrocyte/Oligodendrocyte Differentiation</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Kim 2011</td>
<td>OCT4, SOX2, KLF4, C-MYC</td>
<td>MEFs</td>
<td>3-5 passages</td>
<td>TUJ, GABA, TH</td>
<td>GFAP</td>
<td>Poor Self renewal</td>
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<tr>
<td>Lujan 2012</td>
<td>SOX2, BRN2, FOXG1</td>
<td>MEFs</td>
<td>Up to 12 passages tested</td>
<td>TUJ, MAP2</td>
<td>GFAP</td>
<td>Limited to MEFs, neuronal sub types not tested</td>
</tr>
<tr>
<td>Thier 2012</td>
<td>SOX2, C-MYC, KLF4 + Limited OCT4</td>
<td>MEFs</td>
<td>&gt;50 passages</td>
<td>TUJ, NeuN, GABA</td>
<td>GFAP, O4, PLP</td>
<td>Limited to MEFs</td>
</tr>
<tr>
<td>Han 2012</td>
<td>SOX2, C-MYC, KLF4, E47, BRN4</td>
<td>Adult Mouse Fibroblasts</td>
<td>&gt;130 passages</td>
<td>TUJ, Dcx</td>
<td>GFAP, S100B, O4, NG2, Olig2</td>
<td>Limited to mouse cells, neuronal sub types not tested</td>
</tr>
<tr>
<td>Ring 2012</td>
<td>SOX2</td>
<td>Human Fibroblasts</td>
<td>&gt; 40 passages</td>
<td>TUJ, VGLUT, GABA</td>
<td>GFAP, OLIG2</td>
<td>Requires Feeder Layer</td>
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<tr>
<td>Corti 2012</td>
<td>OCT4, SOX2, or NANOOG</td>
<td>Human Astrocytes</td>
<td>N.D.</td>
<td>TIJ, MAP2</td>
<td>GFAP, O4</td>
<td>Demonstrated in astrocytes only, neuronal sub types not tested</td>
</tr>
<tr>
<td>Sheng 2012</td>
<td>Pax6, Ngn2, Hes1, Id1, Ascl1, Brn2, c-Myc, Klf4</td>
<td>Sertoli Cells</td>
<td>&gt;25 passages</td>
<td>NeuN, TH, GABA, ChAT, PITX3</td>
<td>GFAP, O4</td>
<td>Demonstrated in sertoli only</td>
</tr>
<tr>
<td>Tian et al 2013</td>
<td>Sox2, Bmi1 and c-Myc, Brn2, TLX</td>
<td>Adult Mouse Fibroblasts</td>
<td>N.D.</td>
<td>TUJ1, MAP2, GAD67, TH</td>
<td>GFAP, O4</td>
<td>Limited to mouse cells</td>
</tr>
<tr>
<td>Xi 2013</td>
<td>OCT4, SOX2,</td>
<td>Rat</td>
<td>&gt;60</td>
<td>TUJ, MAP2, GFAP, Rip</td>
<td></td>
<td>Limited to rat cells</td>
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<tr>
<td>Year</td>
<td>Method</td>
<td>Cell Type</td>
<td>Passage Range</td>
<td>Markers</td>
<td>Additional Information</td>
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<tr>
<td>Su 2013</td>
<td>C-MYC, KLF4</td>
<td>Fibroblasts</td>
<td>Up to 10</td>
<td>GABA, TH, TUJ, NeuN, TH, GABA, VGLUT</td>
<td>Limited to mouse cells</td>
<td></td>
</tr>
<tr>
<td>Mitchell 2014</td>
<td>OCT4</td>
<td>Human Fibroblasts</td>
<td>N.D.</td>
<td>TUJ, MAP2, GFAP, O4</td>
<td>Self renewal undetermined, neuronal sub types not tested</td>
<td></td>
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<tr>
<td>Zou 2014</td>
<td>Sox2, c-Myc, and Brn2 or Brn4</td>
<td>Human Fibroblasts</td>
<td>&gt;20 passages</td>
<td>TUJ, MAP2, DCX, -</td>
<td>Neuronal differentiation only, neuronal sub types not tested</td>
<td></td>
</tr>
<tr>
<td>Cheng 2014</td>
<td>Small Molecules, hypoxia</td>
<td>Mouse cells, Human urinary cells</td>
<td>Up to 13</td>
<td>TUJ, MAP2, Neun, GFAP, OLIG2, MBP</td>
<td>Limited characterization of human iNPCs</td>
<td></td>
</tr>
<tr>
<td>Yu 2015</td>
<td>SOX2, HMGAA2</td>
<td>Human Fibroblasts</td>
<td>&gt;40 passages</td>
<td>DCX, MAP2, α-internein, NF, TH, ChAT, GFAP, O4</td>
<td>Requires Feeder Layer</td>
<td></td>
</tr>
<tr>
<td>Mirakhori 2015</td>
<td>TAT-SOX2 (protein)</td>
<td>Human Fibroblasts</td>
<td>Up to 20</td>
<td>TUJ, MAP2, GABA, TH, GFAP, O4</td>
<td>Extended duration of reprogramming</td>
<td></td>
</tr>
<tr>
<td>Lee 2015</td>
<td>OCT4</td>
<td>Cord blood, Blood progenitors</td>
<td>N.D.</td>
<td>TUJ, BRN3A, ISL1, TH, GFAP, O4</td>
<td>Self renewal undetermined</td>
<td></td>
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Table 1.2. Details of previously published methods to generate neural stem cells from somatic cell types.
Despite large successes in reprogramming methodologies, the majority of research fails to examine the regional identity of stem cells generated by different methodologies. During development early stage neural stem cells retain the ability to generate various types of neuronal cells. However, in the presence of FGF and EGF this potential is lost and neural stem cells typically become restricted to specific neuronal lineages (Elkabetz et al. 2008). Several publications report the generation of iNSCs and characterize using pan neural stem cell markers (NESTIN/PAX6) and differentiate to neurons which express pan neuronal markers (such as TUJ1) (Lujan et al. 2012; Han et al. 2011; Corti et al. 2012). It is unclear which neuronal subtypes these cells may subsequently generate. Other publications demonstrate differentiation towards various neuronal subtypes including GABAergic, Glutamatergic, dopaminergic and motor neurons. However, the efficiencies at which these individual cell types may be generated remain unclear (Lee et al. 2015; Su et al. 2013; Cheng et al. 2014; Xi et al. 2013).

A recent report has demonstrated that induced neural stem cells derived from ALS patients successfully generate astrocytes which recapitulate disease mechanisms in vitro (Meyer et al. 2014a). However, the generation of ALS patient motor neuron populations from induced neural stem cells remains to be seen. In Chapter 3 of this thesis we investigate the potential of two novel reprogramming factors, IGF2 and RARβΔ384, to aid the generation of induced neural stem cells from mouse embryonic fibroblasts.

1.6 Current Challenges
One of the fundamental issues underlying ALS research is the focus on mtSOD1 transgenic animals. The vast majority of in vivo and in vitro research over the last two decades has focused on mutations in the SOD1 gene, primarily due to the availability of animal models with well established disease pathology. However, SOD1 mutations account for only a small percentage of ALS cases. Recently it has been shown that SOD1 patients display clinically distinct
phenotype with the absence of TDP-43 pathology, possibly suggesting alternative mechanisms of pathogenesis to sporadic cases and cases attributed to other mutations. This also highlights the importance to investigate non-SOD1 ALS with a hope they may be applicable to large proportion of the ALS patients.

Disease modeling using patient specific cells will allow identification of pathomechanisms associated with specific mutations, as well as mechanisms also responsible for the development of sporadic ALS. However, identifying subtle differences between patients and controls may be difficult and require the use of large numbers of cell lines, a costly situation which may not be feasible in many laboratories.

Research will further be aided with the recently developed animal models containing C9ORF72 deletions and repeat expansions as this mutation accounts for an increasing proportion of ALS cases.

Another issue arises in the reliability of iPSCs to model disease. Many methodologies for generation of these cells rely on integrative vectors which may cause insertional mutagenesis. A study by Gore et al. demonstrated that somatic mutations may occur during the reprogramming process (Gore et al. 2011). The consequence of these mutations is unknown, but caution should be taken when analyzing potential disease phenotypes, particularly when utilizing small numbers of cell lines. Furthermore, the reliability of a single iPSC clone to recapitulate disease of an individual is unknown. The use of more than one clone per donor may overcome this obstacle, as well as the use of pooled clones from the same donor to include a source of heterogeneity in cell populations.

Many labs also utilize many different differentiation techniques, giving rise to varying yields of specific cell types. This can also make it troublesome to compare results between research groups. Standardization of differentiation protocols which yield homogenous cell populations will be highly advantageous to advancement of research. In addition to this, the generation of motor neurons
in mixed cell populations also containing glial cells may confound analyses on motor neurons as these cell types will directly modulate the phenotype of the motor neurons. It is even unclear, if the motor neuron cells are isolated via FACS methods whether the initial culture of cells will affect the neurons themselves.

The high cost of iPSC generation, characterization and differentiation is another obstacle in ALS research. However, with advancements in methodologies and technologies it is likely that the costs will decline over time. For example novel methodologies for the direct induction of specific cell types may avoid the need for the generation of iPSCs and high costs associated therein (Thier et al. 2012; Xue et al. 2013).

1.7 Hypotheses of Thesis
The overarching aim of this thesis is to generate and characterize cellular models of neurodegenerative disease, with a focus on ALS. Our hypotheses are as follows:

Hypothesis 1: ALS patient fibroblasts recapitulate impaired autophagy observed in ALS neuronal cells and tissues.

Hypothesis 2: ALS-iPSC derived astrocytes induce motor neuron death by disrupting the balance between components of the autophagy pathway.

Hypothesis 3: Combinations of novel transcription factors may be used to directly reprogram fibroblasts to neural stem cells with unique regional identities.
Chapter 2

Materials & Methods
2.1 Fibroblast biopsy & cell culture

Patient fibroblast samples from ALS patients were obtained from Prof. Orla Hardiman and Dr. Alice Vadja, Trinity College Dublin, as listed in Table 1.1. For age-matched control samples, 3mm punch biopsies were obtained from three healthy volunteers (ALSC001, ALSC002 and ALSC003). Wells of a 6-well plate were scored using a scalpel blade to create the roughness of surface on culture plate. Each biopsy was dissected into three equally sized pieces, each of which were transferred into a well of a 6-well plate, and pressed firmly into marks scored into the well to ensure the adherence of biopsy to the culture dish. Biopsies were allowed to dry for approximately five minutes to improve adherence to the dish. 2 mls of fibroblast growth medium was then added slowly along the wall of culture well in order not to disturb the adherence of biopsies, and culture dishes were incubated at 37°C, in 5% CO₂ until fibroblasts began to emerge. The culture medium were then gently refreshed every second day without disturbance of adherence of the biopsy. Fibroblast culture medium was comprised of DMEM-GlutaMax (Gibco) supplemented with 10% FBS (Thermo Fisher) and 1% Penicillin/Streptomycin (Gibco).

When cells became densely packed surrounding the tissue and had filled approximately 50% of the well, the cells were washed with phosphate buffered saline (PBS) and passaged using 0.05% Trypsin-EDTA (Gibco) into a new well of a 6-well plate. Fibroblast cultures were subsequently passaged at a ratio of 1:3 when cells reached 80-90% confluency.

2.2 Fibroblast cell treatments

To investigate autophagy, cells were treated with Rapamycin (Sigma Cat# R8781) at various concentrations or 100nM Bafilomycin A1 (Sigma Cat# B1793) for 24 hours after which protein or RNA was harvested. To induce apoptosis cells were treated with H₂O₂ (Sigma Cat# 216763) at various concentrations for 5 hours after which protein was harvested.
2.3 Coating Dishes

For various applications cell culture dishes were coated with Geltrex (1:100 dilution, Life Technologies A1413301), Gelatin (0.1% in PBS, Sigma Cat# G9391) or Fibronectin (6µg/ml, Millipore Cat# FC010) for 1 hour at 37°C, in 5% CO₂. Coating was then removed and replaced with DMEM. Plates were used immediately or stored at 4°C until use. Geltrex was re-used up to three times.

2.4 iPSC Culture

iPSCs were maintained in Pluristem medium (Millipore Cat# SCM130) in 6-well dishes coated with Geltrex. Cells which were obviously differentiating, based on changes in morphology, were manually removed using a p20 pipette under a microscope inside the cell culture hood. The iPS cells were passaged by manually cutting colonies into small squares, scraping from the dish using a p20 pipette and splitting between new dishes. This was done once every 6-7 days, splitting cells at a ratio depending on confluency (from 1:1 up to 1:6).

2.5 iPSC Differentiation

The iPSC differentiation was carried out as described by Du et al 2015 (Du et al. 2015). To begin differentiation of iPSCs, one confluent well of iPSCs was split into six new wells. The following day medium was changed to neural induction medium (NIM) containing: DMEM/F12 (Gibco) and Neurobasal (Gibco) mediums (1:1 ratio), 1% Penicillin/Streptomycin, 0.5x N2 (Gibco Cat# 17502-048), 0.5x B27 (Gibco Cat# 17504-044), 0.1mM Ascorbic Acid (Sigma A4403-100mg) and 1% Glutamax (Gibco Cat# 35050061). This basal medium was supplemented with 1µM SB (Sigma Cat# S4317-5mg), 3µM CHIR (Sigma Cat# SML1-46-25mg) and 100nM LDN (Sigma Cat# SML0559). The medium was renewed every 2 days.

After 6 days the cells were split at a ratio of 1:6 using Accutase (Sigma Cat# A6964) and seeded into dishes coated with Geltrex. CHIR concentration was reduced to 1µM, and 0.5µM Purmorphamine and 0.1µM Retinoic acid were also added. This was then referred to as MNP Medium. The medium was
replenished every 2 days. After a further 6 days motor neuron progenitor cells (MNPs) were established.

2.6 Motor Neuron Differentiation
To differentiate towards motor neurons, MNPs were dissociated using Accutase and cultured in suspension to form neurospheres in the absence of CHIR, SB or LDN. Additionally, Purmorphamine concentration was decreased to 0.1μM and Retinoic acid concentration increased to 0.5μM to drive neuronal differentiation. This medium was then referred to as MN medium, i.e. motor neuron medium. After 6 days in suspension spheres were enzymatically dissociated with Accutase and seeded into 6-well dishes coated with Geltrex. Alternatively, spheres were seeded directly onto Geltrex coated plates and neurons allowed to grow out. The medium was replenished every 2 days.

To validate motor neuron phenotype, cells were fixed using as described above and immunocytochemistry was performed using anti-MNX1, a marker of motor neurons.

2.7 Astrocyte Differentiation
To differentiate towards astrocytes MNPs were split at a ratio of 1:10 onto dishes coated with Geltrex in MNP medium. The following day medium was changed to DMEM supplemented with 10% FBS and 0.3% N2. Cells were passaged at 80-90% confluency using 0.05% Trypsin/EDTA. Cells were split at a ratio of 1:4 and seeded onto dishes coated with Fibronectin. Medium was replenished every 2-3 days. Cells were maintained in culture up to passage 4. To validate cells as astrocytes, both immunocytochemistry and western blot analyses were performed to detect GFAP, a marker of astrocytes.

2.8 Conditioned medium
For collecting conditioned medium astrocytes were grown in T-175 flasks coated with Fibronectin until confluent. Cells were then washed three times in PBS and fresh medium added to flasks. Conditioned medium was collected after 7 days
as previously reported (Nagai et al. 2007). This was then centrifuged at 400g for 5 minutes to remove cell debris and stored at -80°C until use. For co-culture experiments with motor neurons, motor neuron medium was used to produce conditioned medium. For experiments using astrocyte conditioned medium with HEK293T cells, astrocytes were cultured in DMEM with 10% FBS to produce conditioned medium.

2.9 Protein extraction

Cells were trypsinzed, washed with PBS and centrifuged at 400g for 5 minutes. Cell pellets were resuspended in protein extraction buffer comprising RIPA buffer (Sigma, Cat# R0278), sodium orthovanadate (0.2mM) and protease inhibitors (Santa Cruz, sc-29130). Volume of buffer used was relative to the size of tissue culture plastic in which cells were grown. For cells in a 6-well plate 100µl of buffer was used. Cells in buffer were vortexed briefly and placed on ice. Cells were vortexed every 10 minutes for 30 minutes, after which the solution was centrifuged for 10 minutes at 10,000g to remove cellular debris. The supernatant was transferred to a new tube and stored at -80°C until use. Protein was quantified using BCA kit from Thermofisher (Cat# 23225). 10µg of protein was used for western blot analysis.

2.10 Western Blot Analysis

10µg of protein was diluted to 20µl with H₂O and supplemented with 5µl of 5X loading dye. The samples were boiled for 5 minutes in a heating block set to 100°C, and were then centrifuged briefly to collect total volume and 25µl was loaded into each well of a 12% SDS-acrylamide gel, alongside a coloured protein ladder (NEB Cat# P7712S). Gels were run for approximately 2.5 hours at 100 volts. Gels were stopped when bromophenol blue indicator ran off the bottom of the gel.

Proteins were then transferred onto PVDF membrane (Amersham, product code: 10600023) using wet transfer method. Proteins were transferred for 70 minutes at 100 volts, with the tank kept in an ice box to prevent overheating. Blots were then blocked in 5% Bovine Serum Albumin (Sigma Cat# A2153) for 1
hour on a shaker at 45rpm. Primary antibodies were then incubated on blots as indicated in Table 2.3 at 4°C overnight.

The following day blots were washed three times for five minutes with TBS-T and incubated with secondary antibody (Cell Signaling Technologies, #7074S and #7076S) for one hour at room temperature. Blots were then washed three times for 5 minutes at room temperature with TBS-T, and bands detected using chemiluminescence detection reagent (Millipore Cat# WBKLS0500). Equal volumes of Reagent A and B were mixed and approximately 1ml of solution was used to cover each blot. Bands were detected using Alpha Innotech Gel Doc/Chemiluminescence system.

2.11 RNA Extraction & cDNA Synthesis
Cells were trypsinised, washed and centrifuged at 400g for 5 minutes. RNA was extracted using the Qiagen RNeasy Kit (Qiagen Cat# 74104) according to manufacturer’s instruction. RNA was eluted in a volume of 40µl of RNAse free water (supplied with kit) and quantified using the Nanodrop.

For synthesis of single strand cDNA, a total of 1µg of total RNA was used in a reaction with 4ul of ReadyScript cDNA Synthesis Mix (Sigma, RDRT-100RXN) and made to a final volume of 20µl. To synthesize cDNA the reaction was incubated as follows: 25°C for 5 minutes, 42°C for 30 minutes and then 85°C for 5 minutes. cDNA was stored at -20°C until use.

2.12 RT-PCR
For RT-PCR 5ng of cDNA was used in a 10ul reaction containing 0.4nM forward and reverse primers each, and 5µl of SYBFRfast (Applied Biosystems Cat# 4385612). Each sample was run in triplicate and normalized to expression of GAPDH. PCR cycle parameters: 95°C 20 seconds, followed by 40 cycles of [95°C 3 seconds, 60°C 30 seconds]. Primer sequences are listed in Table 2.2.
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2.13 Immunocytochemistry

For immunocytochemistry cells were washed twice with PBS and fixed in 4% paraformaldehyde (Santa Cruz, sc-281692) for 30 minutes at room temperature. Cells were then washed twice with PBS for 5 minutes each followed by two washes with 0.1% Tween 20 (Sigma P2287-100ml). Cells were then permeabilised for 30 minutes in 1% Triton X-100 (Sigma T8787-250ml). To prevent non-specific binding cells were then blocked with 0.1% BSA in PBS with 0.1% Tween 20 for 30 minutes at room temperature. Primary antibodies were then added and incubated overnight at 4°C. Cells were then washed three times with PBS for 5 minutes at room temperature, after which secondary antibodies were added at a dilution of 1:500 and incubated at room temperature, in the dark, rocking for one hour. Hoechst stain (Sigma, Cat# B2261) was also added, at a dilution of 1:5000 for the last ten minutes of this one hour incubation. Cells were then washed three times with PBS for five minutes at room temperature. Enough PBS to prevent cells from drying was then added to each well, after which they were imaged using an Olympus inverted fluorescence microscope or the Operetta Imaging system with Harmony image analysis software (Perkin Elmer). As an alternative to Hoechst staining, samples were sometimes treated with DAPI Fluoroshield (Sigma, F6057) after final wash steps, using just enough to prevent cells from drying. Antibodies used are detailed in Table 2.3. For quantification of staining, Harmony Image analysis was used. First, nuclei were identified using Hoechst/DAPI channel (Option: [Find Nuclei]). Settings were adjusted so that only whole nuclei were quantified (based on diameter and shape). Subsequently cytoplasmic stains were detected using alternative channels (wavelengths 488, 555 or 594) (Option: [Find Cytoplasm]). For the identification of intracellular puncta, the tool [Find Spots] was used. Once cellular regions were identified, staining intensities were quantified using the tool [Calculate Intensity Properties: Nuclei/Cytoplasm]. Settings were kept constant within each experiment. Cells to be analyzed within each experiment were grown, fixed, stained and analyzed at the same time.
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<td>Abcam – ab104899</td>
<td>Goat</td>
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</table>
### Table 2.3. Details of antibodies used in this thesis.

<table>
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<tr>
<th>Antibody Type</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Vendor</th>
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<tr>
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<td>CST, #7074S</td>
</tr>
<tr>
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<td>(Thermo Fisher)</td>
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<tr>
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<td>1:2000</td>
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</tr>
<tr>
<td>HRP</td>
<td>(Thermo Fisher)</td>
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<td>Secondary anti-mouse</td>
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<td>HRP</td>
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<tr>
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<td>1:2000</td>
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<td>CST, #7074S</td>
</tr>
<tr>
<td>Goat</td>
<td>(Thermo Fisher)</td>
<td></td>
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</tr>
</tbody>
</table>

### 2.14 Flow Cytometry

Flow cytometry was performed using BD Biosciences apoptosis detection kit (Cat# 559763). Briefly, cells were trypsinized and washed twice with PBS by resuspending cells and centrifuging at 400g for 5 minutes. Cells were then resuspended in supplied binding buffer at a concentration of $10^6$ cells per ml. To 100µl of this, 5µl of Annexin V and 7-AAD antibodies were added and gently vortexed and incubated for 15 minutes at room temperature. 400µl of 1X binding buffer was then added. The cells were mixed by gently vortexing and then sorted using the BD Accuri C6 Sampler flow cytometer. Cells expressing neither marker were considered healthy. Cells positive for Annexin V only were considered early apoptotic, double positive cells for both Annexin V and 7-AAD were considered late apoptotic, and cells positive only for 7-AAD were considered necrotic cells.

### 2.15 Molecular Cloning

Various lentiviral plasmids were generated for the overexpression factors for reprogramming towards neural stem cells. For details on plasmids obtained from Addgene see Appendix A.
All plasmid digestions were performed at 37°C for one hour. Digested plasmids were run on 1% agarose gels and desired fragments extracted using a scalpel blade. DNA was extracted from gel using Qiagen gel extraction kit (Cat# 28704), according to manufacturer’s instructions.

For subcloning, vector and insert DNA fragments were ligated together at a ratio of 1:3, vector:insert, using T7 ligase (NEB, Cat# M0318S), as per manufacturer’s instructions. This was kept at room temperature for one hour for ligation to occur.

Ligated DNA was subsequently transformed into DH5a competent cells (Bioline Cat# BIO-85027) by mixing 2ul of ligation reaction in 20ul of competent cells. This was placed at 42°C to allow DNA to enter bacterial cells. 1ml of LB media (Sigma, Cat# L3022) was then added and this was shaken at 37°C for 1 hour at 180rpm. This was then centrifuged at 600g for 5 minutes to pellet bacteria, which was then resuspended in 50ul LB media and spread onto LB-agar plates containing 100μg/ml Ampicillin (Sigma Cat# A9518). These were incubated overnight at 37°C. Successful ligation and transformations yielded bacterial clones which were picked using sterile tips and grown at 37°C, overnight, shaking at 180rpm, in 10mls of medium.

Minipreps were performed using Invitrogen miniprep kit, and clones validated by restriction digest using appropriate restriction enzymes. Clones determined to contain the correct sequence were further grown in 100mls of LB medium and harvested using Invitrogen Maxi prep kits. These plasmids were then used for the generation of lentiviruses and retroviruses.

OCT4, SOX2, KLF4 and C-MYC were subcloned from pMXs vectors using BamHI (Thermofisher, Cat# ER0051) and NotI (Thermofisher, Cat# ER0591) restriction enzymes, into pWPT-GFP lentiviral vector. BamHI and NotI flank the GFP sequence in the pWPT-GFP vector, thus removing the GFP sequence and replacing it with transgenes of interest.
FOXG1 and LIN28 were PCR amplified from pEB-C5 and IRES-GFP-FOXG1 plasmids from Addgene. Primers (listed in Table 2.1) were designed to incorporate BamHI and NotI sites on the forward and reverse primers respectively. PCR products were run on 1% agarose gels and extracted using Qiagen gel extraction kit. Extracted PCR products were first cloned into pGEM-T-Easy Vector (Promega, Cat# A1360) prior to being subcloned into pWPT vector. Similarly, IGF2 was PCR amplified from cDNA clone MGC:8683 IMAGE:2964584, and introduced to pWPT-GFP. RARBΔ384 was PCR amplified from a vector previously generated by Professor Shen. All PCR products, once cloned into pGEM-T-Easy vector were sent to MWG for DNA sequencing analysis. Correct sequence was confirmed by comparing to sequences available on the UCSC genome browser. BRN2 was synthesized by GenScript, with BamHI and Sall sites incorporated at 5’ and 3’ ends.

Transgenes were first cloned into pMXs-SOX2 retroviral vector using BamHI and NotI sites replacing SOX2 sequence. (BRN2 was cloned using Sall (Thermofisher, Cat# ER0642) instead of NotI, as the coding sequence for this gene contains a Sall restriction site). Later, all coding sequences were subcloned from pMXs vectors into pWPT-GFP lentiviral vector, replacing GFP sequence by BamHI-NotI sites (again using Sall for BRN2 instead of NotI).

2.16 Mouse Embryonic Fibroblast (MEF) Harvest

C57BL/6N mice were bred and, 12 days after the identification of plugs, pregnant females were sacrificed by cervical dislocation. Uterine horns were removed and placed in a dish of PBS. Each embryo was then separated from its placenta and embryonic sac and washed in PBS. To isolate fibroblastic tissue first the head was removed, followed by removal of all internal organs. The embryo was then turned over and the spinal cord region removed using a curved forceps. The remaining tissue was washed in PBS and then mechanically minced using a scalpel blade. Tissue was then further digested in trypsin/EDTA at 37°C for 15 minutes, tritutrating the cells every 5 minutes. An equal volume of fibroblast medium was then added to neutralize the trypsin and
cells were centrifuged for 5 minutes at 400g. The medium discarded and cell pellet was then resuspended in fresh medium and cells were seeded into T-175 flasks pre-coated with 0.1% gelatin. After 24 hours flasks were examined for fibroblast morphology. Any flasks which did not contain >95% fibroblastic cells were discarded. MEFs were then cultured similar to human fibroblasts, passaged at 80-90% confluency at a ratio of 1:3. Culture medium comprised DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin and was changed every 2-3 days.

2.17 HEK293T cell culture
HEK293T cells were maintained in DMEM high glucose (Gibco), supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were fed every other day and split at a ratio of 1:10 when cells became 80-90% confluent.

2.18 Virus Production
HEK293T cells were seeded at a density of 4.5 x10⁶ cells per 10cm dish. The following day cells were transfected with lentiviral or retroviral packaging vectors as indicated below, in addition to vectors containing transgenes, using JET PEI transfection reagent (Polyplus Transfections, Cat# 101-10N), as per manufacturers protocol.

<table>
<thead>
<tr>
<th>Lentivirus Production Mix (per 10cm dish)</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD2.G</td>
<td>9.5μg</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>4μg</td>
</tr>
<tr>
<td>psPAX2</td>
<td>4μg</td>
</tr>
<tr>
<td>Vector encoding Transgene (e.g. pWPT-GFP)</td>
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</tr>
</tbody>
</table>
### Retrovirus Production Mix (per 10cm dish)

<table>
<thead>
<tr>
<th>Vector Encoding Transgene (e.g. pMXs-GFP)</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4.5μg</td>
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<tr>
<td>pCMV-VSVG</td>
<td>0.5μg</td>
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</tbody>
</table>

The following day transfection efficiency was estimated by GFP transgene expression and medium was replenished. Virus containing supernatant was harvested 48 hours later and stored in 1ml aliquots at -80°C. Fresh medium was added to the transfected cells and a further harvest was performed 24 hours later (i.e. 72 hours). Again this was stored as 1ml aliquots in -80°C. HEK293T cells were then disposed of by incubating in Virkon for 30 minutes prior to discarding waste.

### 2.19 Cell Transduction & Reprogramming

An overview of the protocol used for cellular reprogramming is depicted in Figure 2.1. For viral transduction, MEFs were seeded at a density of 100,000 cells per well of a 6-well dish. The following day cells were washed with PBS and the medium replaced with fresh medium containing 200μl of virus containing supernatant encoding desired transgenes (e.g. GFP) and 8μg/ml of polybrene (Sigma, Cat#107689), in a total volume of 1ml. In the case that more than one transgene was utilized, up to four viruses were added simultaneously, each 200μl in volume. If more than four viruses were used they were split into two groups and transduced into cells on alternative days. Each transduction was repeated twice to improve transduction efficiency. Transduction efficiency was estimated by counting GFP positive cells 48 hours post transduction. In all cases GFP efficiency was 100% after two rounds of virus transduction. To determine
transduction with multiple viruses simultaneously did not lower transduction efficiency, cells were transduced with four and eight viruses at once, one of which being GFP. Again, transduction efficiencies were assessed at 48 hours following the second transduction. With four viruses GFP efficiency was observed to be 100%. However, with eight viruses, GFP expression was greatly limited. As such, transductions were limited to no more than four viruses simultaneously.
Figure 2.1. An overview of the reprogramming strategy used to generate induced neural stem cells from fibroblasts, and subsequent differentiation to astrocytes, neurons and oligodendrocytes.

Cells were maintained in fibroblast medium for 5 days, changing medium every second day, after which medium was switched to neural stem cell medium (NSC) comprising DMEM/F12 supplemented with 1% Non-essential Amino Acids (NEAA) (Gibco), 1X B27, 1X N2, 1X Glutamax, 1% Penicillin/Streptomycin. This was supplemented with 20μg/ml of Fibroblast Growth Factor (FGF) (Peprotech, Cat# 100-18C) and 20μg/ml Epidermal Growth Factor (EGF) (Sigma, Cat# E9644). Medium was replenished every 2 days.

At day 7 two wells of transduced cells from each condition were enzymatically dissociated using Accutase, centrifuged at 400g for 5 minutes, washed with PBS and resuspended in neural stem cell medium. These were cultured in 6-well plates without any coating to promote sphere formation. Medium was replenished by centrifuging cells at 400g for 5 minutes and resuspending in fresh medium. Remaining cells were maintained in monolayer culture.

Cells in monolayer were maintained on the same dishes for 28 days without splitting to observe the formation of colonies. At day 28 cells were blown into suspension using a P1000 pipette and cultured in suspension for 7 days. Sphere formation efficiency was estimated by counting neurosphere like formations at day 30. Medium was replenished by centrifuging cells at 400g for 5 minutes and resuspending cells in fresh medium.

At day 35 formed neurospheres were seeded into 6-well plates coated with Geltrex in NSC medium. Cells which proliferated from generated neurospheres were subsequently maintained in monolayer culture on Geltrex coated dishes. Medium was replenished every 2-3 days. Cells were passaged at 80% confluence using Accutase and split 1:3 into wells of a 6-well plate coated with Geltrex.
RT-PCR analysis for stem cell markers was used to confirm neural stem cell phenotype (Primers listed in Table 2.2).

2.20 Differentiation of iNSCs
To differentiate iNSCs towards astrocytes, neural stem cells were cultured in DMEM supplemented with 10% FBS and 0.3% N2 for three weeks and five weeks, changing medium every 2-3 days. Generation of astrocytes was confirmed by immunocytochemistry with anti-GFAP and ant-S100B, typical markers of astrocytes.

To differentiate iNSCs towards oligodendrocyte progenitor cells, neural stem cells were cultured in basal neural stem cell medium supplemented with T3 (Sigma Cat# D6397). Cells were maintained as such for 3 weeks, changing medium every 2-3 days. Immunocytochemistry was used to assess oligodendrocyte progenitor cell formation by staining with NG2, a marker of oligodendrocyte progenitor cells. NG2 is typically considered a marker of oligodendrocyte progenitor cells. However, this marker may also be expressed by other cells, such as macrophages (Moransard et al. 2011). Olig2 is considered another oligodendrocyte marker, but we have demonstrated this is also expressed in neural stem cells. For further validation of cellular phenotype, NG2 immunostaining should be coupled with other oligodendrocyte markers such as A2B5, GalC or MBP, which identify different stages of oligodendrocyte development.

To differentiate towards neurons neural stem cells were cultured in neural stem cell medium without FGF and EGF, but supplemented with retinoic acid (0.5µM) and Purmorphamine (0.1µM). Alternatively neural stem cell medium, without EGF and FGF, was supplemented with TGFβ (Peprotech, Cat# 100-36E) (10ng/ml) and FGF8 (R&D, Cat# 4745-F8) (100ng/ml) to attempt to generate dopaminergic neurons.
2.21 Statistical analyses

Graphpad Prism was used for all statistical analyses. For comparison of patient and control samples unpaired students T-tests were performed. For cells treated with drugs paired t-tests were performed to analyze samples with and without treatment. For the analysis of samples with more than two groups one-way Anova analysis was performed with Tukey post test to compare each group to every other group. *, ** and *** represent P<0.05, P<0.01 and P<0.001 respectively.
Chapter 3

Amyotrophic Lateral Sclerosis Patient Fibroblasts Demonstrate Impaired Autophagy and Increased Sensitivity to Oxidative Stress
3.1 Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive loss of both upper and lower motor neurons (Chiò et al. 2013). Mutations in C9ORF72, SOD1, TDP-43 and FUS genes together may account for up to 50% of familial ALS cases (Ling et al. 2013). However, genetics may only account for 10% of all ALS cases, with the remaining 90% having no known cause.

Recent evidence has suggested that dysfunction of the autophagy-lysosome pathway may be central to the pathogenesis of ALS, as well as many other neurological diseases (Wang et al. 2012). Activation of the autophagy pathway in mtSOD1 animal models has been shown to extend survival of the transgenic mice (Staats et al. 2013; X. Zhang et al. 2014), However, the molecular mechanisms underpinning impaired autophagy remain unclear.

Although the disease predominantly affects motor neurons, other neuronal subtypes such as interneurons have been shown to degenerate as well (Hossaini et al. 2011). It is unclear why motor neurons are more sensitive to degeneration than other cell types, and whether other cell types may also recapitulate disease mechanisms. Herein we aimed to investigate the expression of ALS related genes in dermal fibroblasts derived from sporadic ALS patients and patients harboring a G₄C₂ repeat expansion in the first intron of the C9ORF72 gene. We further investigated the expression of autophagy related genes to determine whether fibroblasts would recapitulate impaired autophagic mechanisms, and begin to elucidate the molecular targets responsible for impaired autophagy. We demonstrate that C9ORF72, TDP-43, and FUS may be implicated in the autophagy pathway, and that patient cells show impaired initiation of autophagy and decreased autophagic flux. Patient dermal fibroblasts are more susceptible to oxidative stress induced apoptosis by treatment with hydrogen peroxide, which cannot be ameliorated by Rapamycin. These data suggest that patient fibroblasts may be used as a disease model for investigation of disease mechanisms and for drug screening.
### 3.2 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive loss of both upper and lower motor neurons (Chiò et al. 2013). Initial symptoms include muscle weakness and atrophy which eventually progress to paralysis and death due to respiratory failure within 2-5 years of onset. Up to 10% of cases may be explained by known genetic mutations (Kiernan et al. 2011). Of these mutations, SOD1, TDP-43, FUS and C9ORF72 may account for up to 50% of familial ALS (fALS) cases (Ling et al. 2013). A G$_4$C$_2$ hexanucleotide repeat expansion in the first intron of the C9ORF72 gene has been identified as the most common cause of ALS to date (Yamakawa et al. 2015). Despite the identification of these causative genes, the molecular mechanisms underpinning ALS remain to be fully elucidated. Furthermore, up to 90% of ALS cases remain sporadic (sALS) with no known genetic links.

A hallmark of ALS pathology is the development of misfolded protein aggregates in motor neurons of the brain and spinal cord (Al-Chalabi et al. 2012). The compositions of these aggregates vary between patients, but typically include a variety of proteins implicated in the pathogenesis of ALS (Blokhuis et al. 2013). While mutated proteins are often causative of these aggregations, wild type proteins are also included within. Aggregations of TDP-43 are observed in nearly all cases of ALS, with the only notable exceptions being in the case of SOD1 mutations. These cases are characteristically devoid of TDP-43 pathology, which may suggest alternative mechanisms of neurodegeneration within this patient subset (Mackenzie et al. 2007). Although C9ORF72 itself does not form pathological aggregations, dipeptide repeats generated via non-ATG repeat associated translation may be included in these aggregations (May et al. 2014). These repeats have been shown to induce neurodegeneration in various models of ALS. However, recent evidence suggests that these repeats are rare in patient spinal cord and motor neurons, suggesting they are not the cause of degeneration in ALS (Gomez-Deza et al. 2015).
Autophagy is one of the key cellular mechanisms responsible for the regulation of misfolded and long lived proteins (Maruyama et al. 2014). mTOR regulates the kinase p70S6K which in turn regulates the phosphorylation status of ULK1 (Kim et al. 2011). ULK1 is a key component in the autophagy process, and forms the autophagy initiation complex with ATG13, ATG101 and FIP200 (Alers et al. 2012). This complex acts to recruit other autophagy-related proteins (ATG) to the site of autophagosome formation and to phosphorylate BECLIN-1 (Russell et al. 2013). The autophagosome formation complex (comprising BECLIN-1, VPS34, PI3KCIII and ATG14L) then regulates autophagosome formation. p62 (also known as SQSTM1) plays a central role in targeting cargo destined for degradation and recruits it to the autophagosome by interaction with LC3B (Maruyama et al. 2014). Degradation of targeted proteins occurs upon fusion with the lysosome, which expresses LAMP1/2, and p62 is itself degraded and recycled (Furuta & Amano 2010).

Several studies have implicated impaired autophagy in cellular and animal models of ALS (Sasaki 2011). Moreover, the majority of ALS related proteins may be implicated in the autophagy process itself (Table 3, references therein). As such, it stands to reason that impaired autophagy may play a central role in the generation of toxic protein aggregates, and the subsequent onset of neurodegeneration. Pharmacological activation of the autophagy pathway has been found to extend lifespan of ALS mouse models (Staats et al. 2013; Zhang et al. 2014). As such, a focus on elucidating the mechanisms by which impaired autophagy may lead to the development of ALS holds promise for the identification of novel therapeutic targets.

Patient fibroblasts are readily available for the generation of induced pluripotent stem cells (iPSCs). iPSCs allow investigations into disease mechanisms using patient specific and disease specific cells; i.e. motor neurons, astrocytes in the case of ALS. However, several recent publications have demonstrated that dermal fibroblasts may readily recapitulate certain aspects of ALS disease pathology – increased TDP43 expression and abnormal aggregate formation
and localization (Sabatelli et al. 2015), changes in bioenergetic markers (Kirk et al. 2014; Allen et al. 2015), impaired response to hypoxia and oxidative stress (Raman et al. 2014) and nuclear aggregation of FUS (Schwartz et al. 2014b) have all been reported. As autophagy is a homeostatic process which occurs in all cell types, we hypothesized that impaired autophagy may be recapitulated in dermal fibroblasts from ALS patients.

Herein we analyzed the expression of ALS and autophagy related proteins in ALS patient and age matched control fibroblasts. We demonstrate that C9ORF72, TDP-43, and FUS may be implicated in the autophagy pathway by co-immunostaining with autophagy related proteins and/or western blot analyses. We further demonstrate that patient cells show impaired initiation of autophagy and decreased autophagic flux as indicated by decreased p-ULK1 and increased p62 expression respectively. Patient dermal fibroblasts are more susceptible to oxidative stress induced apoptosis by treatment with hydrogen peroxide. While treatment with Rapamycin, an activator of the autophagy pathway, may induce initiation of autophagy, it does not ameliorate defects observed with autophagic flux or sensitivity to oxidative stress.

3.3 Methods

Fibroblasts from ALS patients and age matched controls were routinely cultured for the analysis of ALS and autophagy-related proteins by immunocytochemistry and western blot analyses. Standard microscopy in addition to automated imaging analysis using the Operetta high content imaging hardware coupled with Harmony image analysis software was used to identify protein co-localization, staining intensities and puncta formations. Autophagy was activated and inhibited with Rapamycin and Bafilomycin respectively, while apoptosis was induced using H$_2$O$_2$. Flow cytometry analysis to detect 7-AAD and Annexin V was used to determine cell viability. For full details on methodologies refer to Chapter 2.
3.4 Results

3.4.1 Generation of fibroblast cultures.
This work focused on the analysis of patient and control dermal fibroblast culture. Fibroblast cultures from ALS patients and two control lines were provided by Prof. Orla Hardiman, Trinity Biomedical Sciences Institute. Punch biopsies from three age matched controls were obtained in house and fibroblasts were allowed to proliferate from these. Figure 3.1A shows a biopsy in culture with cells emerging from within. Figure 3.1B shows typical fibroblast morphology after passaging the cells. Table 3.1 indicates details of control and patient fibroblasts. No obvious differences in morphology were observed between patient and control cells.

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Sex</th>
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<td>Control</td>
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<tr>
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<td>ALS31-001</td>
<td>57</td>
<td>Female</td>
<td>Patient</td>
<td>C9ORF72 Repeat Expansion</td>
</tr>
</tbody>
</table>

Table 3.1. Details of control and patient biopsy donors. Two patients tested positive for the C9ORF72 repeat expansion, with the remaining having no known genetic mutations.

3.4.2 ALS-related proteins are expressed in dermal fibroblasts
For fibroblasts to be useful for the study of molecular mechanisms of ALS we first needed to confirm the expression of ALS related proteins in these cells. To
this end cells from patients and controls were fixed and stained with antibodies for C9ORF72, SOD1, TDP-43 and FUS. For preliminary analyses of expression, mean intensities of staining were examined using the Operetta imaging system with Harmony imaging software. Expression of C9ORF72 was observed in both the nucleus and the cytoplasm with some cells often showing high perinuclear staining (Figure 3.2 A and C). No differences in expression between patient and control were observed (Figure 3.2E and F).

**Figure 3.1. Culture of fibroblasts from biopsies.** Cells were allowed to emerge from donor biopsies as shown in (A). When fibroblastic cells became compact and proliferated away from the biopsy they were passaged to generate cell populations as shown in (B). (C) and (D) show fibroblast images from ALS patient samples ALS17 and ALS21 respectively. Scale bars represent 200µm.
Figure 3.2. C9ORF72 is expressed in both the nucleus and the cytoplasm of dermal fibroblasts. Fibroblasts were grown under normal conditions and fixed for immunocytochemistry analysis for the expression of C9ORF72. Staining intensity was measured using the Operetta automated imaging hardware and Harmony image analysis software. Cells often demonstrate high
perinuclear staining. (A) C9ORF72 and expression in ALSC001 control fibroblasts with nuclei stained with Hoechst shown in (B). (C) C9ORF72 expression in ALS31-001 patient fibroblasts with nuclei stained with Hoechst shown in (D). (E/F) Quantification of staining intensity of nuclear and cytoplasmic C9ORF72 (Controls n=3, Patients n=4). T-test was used for statistical analyses. No significant differences between controls and patients were observed. Experiment was repeated twice. Error bars represent mean+/SEM. Scale bars represent 50µm.

We subsequently analyzed the expression of SOD1 in cells. Again, staining was observed in both the nucleus and the cytoplasm, with considerably higher expression evident in the nucleus (Figure 3.3A and C). Indeed, the data sheet provided with our antibody indicates predominantly nuclear localization. However, other antibodies available more readily detect cytoplasmic forms of this protein. The localization of SOD1 has been demonstrated to be disrupted in response to mutations within the SOD1 gene, with mutated isoforms of the protein accumulating in the cytoplasm of cells, significantly diminishing nuclear localization (Lee et al. 2015). For more in depth analysis of SOD1 in ALS patient samples, the use of additional antibodies may yield further insights. Analysis of intensities demonstrated that patient cells expressed higher levels of nuclear SOD1 with no difference in cytoplasmic expression observed (Figure 3.3 E and F). None of the patient samples used in this study harbor mutations in the SOD1 gene, suggesting that deregulation of this gene may be a common feature of sporadic and familial ALS.

Because both C9ORF72 and SOD1 were shown to stain in the nucleus, we investigated whether co-localization of these two proteins occurred (Figure 3.4). We observe an overlap in positive staining in large puncta within cells. There may also be more diffuse localization within the cytoplasm. Where we observe
high levels of C9ORF72 staining, there also appears to be increased staining of SOD1. However, SOD1 staining in the cytoplasm is faint.

We also analyzed the expressions of TDP-43 and FUS (Figures 3.4 and 3.5). Both were predominantly confined to the nucleus, and only low levels of expression were observed in the cytoplasm. No differences in protein localization between patients and control cells were observed (Figures 3.4E and F and Figure 3.5 E and F). Sabatelli et al (2015) recently demonstrated that TDP-43 is mislocalized to the cytoplasm in sALS and fALS patients, with the exception of mtSOD1 patients. Our results did not observe this same pathological feature, with sALS patient or C9ORF72 patient fibroblasts. We did not investigate co-localization of C9ORF72 with TDP-43 or FUS, as TDP-43 and FUS were predominantly expressed in nuclei (Fig. 3.4A and Fig3.5A).
Figure 3.3. **SOD1 is expressed in both the nucleus and the cytoplasm of dermal fibroblasts.** Fibroblasts were grown under normal conditions and fixed for immunocytochemistry analysis for the expression of SOD1. Staining intensity was measured using the Operetta automated imaging hardware and Harmony image analysis software. Significantly more expression is observed in the

Cytoplasmic SOD1 expression in control and patient fibroblasts

ALS patient fibroblasts demonstrate increased expression of nuclear SOD1
nucleus. (A) SOD1 expression in ALSC001 control fibroblasts, with nuclei stained with Hoechst shown in (B). (C) SOD1 expression in ALS31-001 patient fibroblasts with nuclei stained with Hoechst shown in (D). (E/F) Quantification of staining intensity of cytoplasmic and nuclear SOD1 (Controls n=3, Patients n=4). T-test was used for statistical analyses. Patient cells demonstrate significantly higher nuclear staining for SOD1 *p<0.05 was deemed statistically significant. Experiment was repeated twice. Error bars represent mean+/- SEM. Scale bars represent 50µm.

**Figure 3.4. C9ORF72 co-localizes with SOD1.** Cells were fixed and stained with antibodies for both C9ORF72 and SOD1. High expression of SOD1 occurs with high expression of C9ORF72 in puncta in fibroblasts, as indicated by a white arrow. Further, higher levels of cytoplasmic SOD1 typically demonstrate a similar staining pattern to C9ORF72. Representative images from ALSC001 fibroblasts are shown. (A) C9ORF72 staining. (B) SOD1 staining. (C) Hoechst
staining. (D) Merged image of C9ORF72, SOD1 and Hoechst staining. Experiment was repeated for three control and three patient fibroblast samples. Scale bars represent 50µm.

Figure 3.5. TDP-43 expression in patient and control fibroblasts. Fibroblasts were grown under normal conditions and fixed for immunocytochemistry analysis for the expression of TDP-43. Staining intensity was measured using
the Operetta automated imaging hardware and Harmony image analysis software. TDP-43 is expressed in the nucleus, with very low cytoplasmic staining. (A) TDP-43 expression in ALSC001 control fibroblasts, with Hoechst staining shown in (B). (C) TDP-43 expression in ALS31-001 patient fibroblasts, with Hoechst staining shown in (D). (E/F) Quantification of staining intensity of cytoplasmic and nuclear TDP-43 (Controls n=3, Patients n=4). T-test was used for statistical comparisons. No differences are observed between patients and controls. Experiment was repeated twice. Error bars represent mean +/-SEM. Scale bars represent 50µm.
Figure 3.6. FUS expression in ALS patient and control fibroblasts.

Fibroblasts were grown under normal conditions and fixed for immunocytochemistry analysis for the expression of FUS. Staining intensity was measured using the Operetta automated imaging hardware and Harmony image...
analysis software. FUS is expressed in the nucleus, with no obvious cytoplasmic staining. (A) FUS expression in ALSC001 control fibroblasts, with Hoechst staining in (B). (C) FUS expression in ALS31-001 patient fibroblasts, with Hoechst staining in (D). (D) Quantification of staining intensity of cytoplasmic and nuclear FUS (Controls n=3, Patients n=4). T-test was used for statistical analysis. No differences are observed between patients and controls. Experiment was repeated for three control samples and four patient samples. Error bars represent mean+/- SEM. Scale bars represent 50µm.

3.4.3 C9ORF72 may be implicated in the autophagy process
Little is known about the function of the C9ORF72 protein. A study by Farg et al suggested that it may play a role in endosomal trafficking and autophagy due to co-localization of the protein with Rab proteins and LC3B (Farg et al. 2014). We aimed to further implicate C9ORF72 in the autophagy process. As such we co-stained fibroblasts from patients and controls with C9ORF72 with p62, an autophagy receptor protein, and ULK1, a component of the autophagy initiation complex. We show that C9ORF72 sometimes stains puncta within cells, and these co-localize with ULK1 (Figure 3.7). Also we occasionally observe staining with p62, again in puncta (Figure 3.8). This further supports a role for C9ORF72 in the autophagic process as ULK1 is regulates the initiation of autophagy. However, the majority of p62 puncta do not stain positive for C9ORF72. This may suggest a temporal interaction between the two proteins.

3.4.4 Autophagy regulates some ALS related proteins
We next aimed to determine whether ALS proteins are differentially expressed via western blot analysis, and whether these proteins are regulated by autophagy. To this end fibroblasts were treated with Bafilomycin A1, a specific inhibitor of vacuolar type H\(^+\)-ATPase (V-ATPase) which prevents fusion of the autophagosome to the lysosome (Kawai et al. 2007). As such, autophagosomes containing proteins targeted for degradation via autophagy should be accumulated, leading to increased levels of protein. To confirm
efficacy of Bafilomycin A1, western blot analysis demonstrates increased accumulation of both p62 and LC3B-II, demonstrating decreased turnover of autophagosomes (Figure 3.14) (Zhang et al. 2013).

Figure 3.7. C9ORF72 co-localizes with ULK1, a component of the autophagy initiation complex. Fibroblasts were fixed and stained with antibodies for both C9ORF72 and ULK1. (A) C9ORF72 staining. (B) ULK1 staining. (C) Hoechst staining. (D) Merged image of C9ORF72, ULK1 and Hoechst staining. Immunocytochemistry demonstrates co-localization of C9ORF72 with ULK1 (examples indicated by white arrows. Images shown are from ALS17 fibroblasts, derived from a patient with no known mutation. Experiment was repeated with three control samples and four patient samples. Scale bars represent 50µm.
Figure 3.8. C9ORF72 may occasionally localize with p62 in cytoplasmic puncta in fibroblasts. (A) C9ORF72 immunocytochemistry analysis in fibroblasts. (B) p62 immunocytochemistry analysis in fibroblasts. (C) Hoechst staining of fibroblasts. (D) Merged imaged of C9ORF72, p62 and Hoechst staining. White arrows indicate small puncta positive for both p62 and C9ORF72. However, the majority of p62 puncta are not positive for C9ORF72. This may suggest a temporal interaction between the two proteins. Images shown are from ALSC001 fibroblasts, a healthy donor. Experiment was repeated for three control samples and four patient samples. Scale bars represent 50µm.
Our results showed that neither SOD1 nor C9ORF72 were differentially expressed in the presence of Bafilomycin A1, as determined by western blot analysis (Figure 3.9). This may suggest that these proteins are regulated by alternative protein degradation mechanisms such as the ubiquitin proteosome system (UPS). Alternatively, turnover of these proteins could occur at a slower rate and thus 24 hours is insufficient to notice any differences. Further, there were no differences in basal expression between patients and controls observed.

In contrast to SOD1 and C9ORF72, both TDP-43 and FUS responded to Bafilomycin A1 treatment. Significant increases in TDP-43 expression are observed in both patients and controls confirming that TDP-43 is regulated by autophagic mechanisms (Figure 3.10). Conversely, we observed a significant, and unexpected, decrease in FUS expression upon treatment with Bafilomycin A1 (Figure 3.11A and B). ALS is typically associated with the buildup and aggregation of disease related proteins. However this result would suggest that FUS itself plays a role in completion of the autophagy process, which is significantly diminished upon inhibition of fusion between the lysosome and autophagosome. To further investigate this we treated cells with Rapamycin to activate the autophagy pathway and mRNA levels of FUS (Figure 3.11C). We observed a significant increase in FUS mRNA. As such, we hypothesize that FUS plays a crucial role in the autophagy pathway.
Figure 3.9. Autophagy does not regulate the expression of SOD1 or C9ORF72. Patient and control fibroblasts were treated with 100nM Bafilomycin A1 (BafA1), to inhibit fusion of the autophagosome to the lysosome, for 24 hours after which protein was harvested. Untreated cells were used for comparison. (A) Western Blot analysis of fibroblasts from patients (n=3) and controls (n=4) treated with and without 100nM Bafilomycin A1 for 24 hours. (B) Densitometry of western blot analysis of C9ORF72 expression. (C) Densitometry of western blot analysis of SOD1 expression. Paired T-tests were used to identify changes in protein expression in response to BafA1. No statistical differences were observed. Experiment was repeated twice. Error bars represent mean +/- SEM.
Figure 3.10. Autophagy regulates the expression of TDP-43. Patient and control fibroblasts were treated with 100nM Bafilomycin A1 (BafA1), to inhibit fusion of the autophagosome to the lysosome, for 24 hours after which protein was harvested. Untreated cells were used for comparison. (A) Western Blot Analysis of TDP-43 expression, with and without Bafilomycin in patient (n=3) and control (n=4) cells. (B) Densitometry of western blot analysis. Paired T-test was used for statistical analysis. *p<0.05 and **p<0.01 were deemed statistically significant. Experiment was repeated twice. Error bars represent mean±/− SEM.
Figure 3.11. Inhibition and activation of autophagy decrease and increase FUS expression respectively. Patient and control fibroblasts were treated with 100nM Bafilomycin A1 (BafA1), to inhibit fusion of the autophagosome to the lysosome, for 24 hours after which protein was harvested. Untreated cells were used for comparison. Fibroblasts were also treated with 200nM Rapamycin to induce autophagy, after which RNA was harvested for RT-PCR analysis. (A) Western blot analysis of FUS expression in patient (n=3) and control (n=4) fibroblast with and without Bafilomycin A1 treatment. (B) Densitometry of western blot analysis. (C) RT-PCR of FUS expression in patient and control fibroblasts, with and without Rapamycin. Paired T-tests were used for statistical analyses. * $p<0.05$ and ** for $p<0.01$ were deemed statistically significant. Experiment was repeated twice. Error bars represent mean+/- SEM.
autophagy decreases FUS on the protein level, while activating autophagy increases FUS mRNA.

### 3.4.5 ALS patient fibroblasts show impaired initiation of autophagy

Our initial results suggest that the ALS related proteins C9ORF72, TDP-43 and FUS may each be implicated in the autophagy process, whether by co-immunostaining or western blot analysis and modulation of the pathway. As such we aimed to investigate whether patient fibroblasts would display impairments of the autophagy pathway. We began by analyzing expression of proteins involved in initiation of autophagy; ULK1 and ATG13 (Figure 3.12A). We observed no significant differences in the expression of total ULK1 (Figure 3.12C). Conversely, ATG13 was shown to be significantly decreased in patient cells (Figure 3.12D). We further analyzed the expression of phosphorylated ULK1 (Ser 555), the active form of the protein. This also demonstrated significantly decreased levels of expression in patient cells (Figure 3.12E). mTOR regulates the phosphorylation status of ULK1 through p70S6K. When mTOR is inhibited, p70S6K is dephosphorylated, allowing the phosphorylation of ULK1 at serine 555, thus initiating autophagy. As such, decreased levels of both ATG13 and pULK1 indicate that patient cells show a decreased propensity for autphagic initiation.

### 3.4.6 ALS patients demonstrate normal levels of phagophore associated proteins

We further investigated the expression of other proteins associated with the development of the autophagosome. No differences were observed in the levels of BECLIN-1, ATG3 or conjugated ATG12-ATG5, although there was a trend towards decreased BECLIN-1 (p=0.0506) (Figure 3.13). As such, it is possible that disease mechanisms do not directly impact autophagosome formation in dermal fibroblasts.
**Figure 3.12. ALS patient fibroblast demonstrated impaired autophagic initiation.** To assess levels of proteins associated with autophagic initiation protein was harvested from patient and control fibroblasts and analyzed by western blot. (A) Western blot analysis of ULK1 and ATG13, components of the autophagy initiation complex, in ALS patient (n=4) and control (n=4) fibroblasts. A lower band is observed on the blot with ATG13, but this is simply expression of another protein previously detected on the same blot. (B) Western blot analysis of pULK1 in ALS patient (n=6) and control (n=5) fibroblasts. (C-E) Densitometry analysis of western blots for ULK1, ATG13 and pULK1. T-tests were performed to compare differences in patient and control cells. * p<0.05 was deemed statistically significant. No differences in total ULK1 are observed, but decreased ATG13 and pULK1 are observed in patient cells. Experiment was repeated twice. Error bars represent mean+/-SEM.
Figure 3.13. Patient and control fibroblasts express similar levels of proteins associated with autophagosome formation. Analysis of proteins associated with autophagosome formation in patient (n=6) and control (n=5) fibroblasts. To determine whether patient fibroblasts show altered expression of autophagosome associated proteins, protein was harvested from patient (n=6) and control (n=5) fibroblasts and western blot analysis was performed. (A) Western blot analysis of BECLIN-1, ATG3 and ATG5-ATG12 in patient and control fibroblasts. (B-D) Densitometry analysis ATG3, ATG5-ATG12 and BECLIN-1 western blots. T-tests were used for statistical analysis. No significant differences were observed, though a trend towards decreased BECLIN-1 is observed in patient fibroblasts (p=0.0506). Experiment was repeated twice. Error bars represent mean+/− SEM.
3.4.7 ALS patient fibroblasts demonstrate decreased autophagic flux

We next investigated expressions of proteins associated with autophagic flux. Autophagic flux refers to the rate at which autophagosomes are degraded via fusion with the lysosome. Cells were treated with and without Bafilomycin A1 and levels of both p62 and LC3B were investigated. We observe significantly higher basal levels of the cargo receptor p62, suggestive of impaired turnover of autophagosomes as p62 itself is normally degraded during the autophagy process (Figure 3.14A and B). Conversely, no difference in basal LC3B was observed between patients and controls. Bafilomycin A1 treatment increased the levels of p62 and LC3B-II, the active form of the protein, by preventing their usual degradation by the autophagy process. As such we would suggest that increased levels of p62 are due to impaired autophagic flux.

To further investigate this we performed immunocytochemistry to detect p62 in control (Figure 3.15A) and patient cells (Figure 3.15C). Mean fluorescence intensity did not vary between patient and control cells (Figure 3.15F). However, a significant increase in p62 puncta was observed (Figure 3.12E). This may suggest impaired incorporation of cargo into autophagosomes, or impaired fusion with the lysosome, resulting in buildup of p62 positive autophagosomes. Regardless, it is highly suggestive of impaired protein turnover, and thus impaired autophagy.

3.4.8 ALS patients demonstrate increased levels of LAMP-1, a lysosomal marker.

The final stage of autophagy involves fusion of the autophagosome with the lysosome. As we observed increased p62 puncta indicating impaired autophagy, we aimed to investigate how this may affect levels of LAMP-1, a lysosomal marker. While no significant difference was observed by western blot analysis (Figure 3.16 E andG), immunocytochemistry indicated more intense staining of LAMP-1 in patient cells compared to controls (Figure 3.16 F). This indicates an
increase of lysosomes within patient cells, suggesting impaired fusion of the autophagosome with the lysosome and subsequent degradation.

Figure 3.14. ALS patients demonstrate increased basal p62, indicative of impaired autophagic flux. To investigate levels of autophagic flux in fibroblasts, patient and control fibroblasts were treated with and without 100nM Bafilomycin A1 for 24 hours to inhibit fusion of the lysosome to the autophagosome. Subsequently, protein was harvested and western blot analysis was performed. (A) Western blot analysis of p62 and LC3B, key proteins involved in autophagic flux, in patient (n=4) and control (n=3) fibroblasts, with and without Bafilomycin A1 treatment. (B) Densitometry of p62 western blot
analysis. Patient fibroblasts demonstrate increased basal p62, suggesting decreased autophagic flux. Bafilomycin A1 increases p62 expression due to decreased autophagic flux, as expected. (C) Densitometry of LC3B-II/LC3B-I western blot analysis. No difference in basal expression is observed between patient and control fibroblasts. Bafilomycin A1 treatment increases the ratio of LC3B-II/LC3B-I. No differences are observed between patient and control cells. * for \( p<0.05 \), ** for \( p<0.01 \), *** for \( p<0.001 \). Experiment was repeated twice. Error bars represent mean+/- SEM.
Patient fibroblasts demonstrate increased numbers of p62 puncta.

Patient and controls demonstrate similar mean intensity of p62 staining.

Figure 3.15. Patient fibroblasts demonstrate increased numbers of p62 positive puncta. To further investigate levels of p62 in fibroblasts, patient (n=4) and control (n=3) fibroblasts were fixed in paraformaldehyde and stained for p62 expression. p62 puncta were quantified using the Operetta automated imaging hardware and Harmony analysis software. (A) Images of ALSC001 fibroblasts stained with p62 and (B) Hoechst staining of the same. (C) Images of ALS31-001 patient fibroblasts stained with p62 and (D) Hoechst staining of the same. (E) Quantification of p62 puncta and (F) mean intensity of staining of p62 in patient and control fibroblasts. T-tests were used to determine statistical significance between patient and control cells. ** p<0.01 was deemed statistically significant. Experiment was repeated twice. Error bars represent mean +/- SEM. Scale bars represent 50 µm.
Figure 3.16. Patient fibroblasts demonstrate increased staining for LAMP-1, a lysosomal marker. To investigate levels of Lamp-1, western blot analyses
and immunostaining experiments were performed on patient (n=4) and control (n=3) fibroblasts. (A) Immunostaining of ALSC001 control fibroblasts with LAMP-1 and (B), Hoechst staining of the same. (C) Immunostaining of patient fibroblasts with LAMP-1 and (D), Hoechst staining of the same. (E) Western blot analysis of LAMP-1 in patient and control fibroblasts. (F) Quantification of LAMP-1 staining intensity and (G) western blot analysis. T-tests were performed to determine differences between patient and control fibroblasts. *p<0.05 was deemed statistically significant. Experiment was performed once. Error bars represent mean +/- SEM. Scale bars represent 50µm.

It is possible that western blot analysis detects total LAMP-1, whereas immunocytochemistry is only capable of detecting lamp one condensed into lysosomes. More thorough experimentation will be required to fully investigate this.

However, what appears to be extracellular staining is observed in LAMP-1 images. This is likely formed from precipitate within the antibody solution used. Several washes were unable to remove this background staining. A new batch or alternative antibody will be used in future.

Taken together these results suggest impaired autophagy due to impaired initiation and/or impaired autophagic flux. As such we aimed to determine whether pharmacological activation of the autophagy pathway could ameliorate deficits observed within the pathway.

3.4.9 Fibroblasts respond to autophagic induction via Rapamycin.

Recent studies have shown pharmacological activation of the autophagy pathway, such as Rapamycin, extend the lifespan of transgenic models of ALS (Staats et al. 2013; X. Zhang et al. 2014). As such, we investigated whether fibroblasts from patients and controls also respond to Rapamycin treatment. Treatment with Rapamycin reduced the phosphorylation of p70S6K in both patient and control cells, indicating that fibroblasts respond to Rapamycin via
inhibition of the mTORC1 complex as expected (Figure 3.17A). Subsequent analysis of protein levels indicated increased phosphorylation of ULK1 in a dose dependent manner, suggesting increased initiation of the autophagy pathway (Figure 3.17B). 200nM Rapamycin was shown to give the strongest induction of pULK1 (555), so this concentration was chosen for subsequent experiments. To further demonstrate activated autophagy we performed RT-PCR for ATG13 and WIPI1 expression. ATG13, as previously indicated plays a role in autophagy initiation. WIPI1 plays a role in autophagosome formation, and reliably indicates levels of autophagosome formation in several cell types (Tsuyuki et al. 2014). As expected, we observe significant upregulation of ATG13 and WIPI1 mRNAs, further indicating induced autophagy (Figure 3.17 C and D).

Rapamycin is known to induce phosphorylation of ULK1. However, the expression of total ULK1 was not performed in parallel with these experiments. This will be required to validate whether or not increased pULK1 is due to increased total protein, or specifically due phosphorylation of the protein.
Figure 3.17. Fibroblasts respond to autophagic induction via Rapamycin.
To determine whether Rapamycin could initiate autophagy in fibroblasts, cells were treated with a range of concentrations of Rapamycin from 0-300nM, after
which protein and RNA were harvested for western blot and RT-PCR analysis. (A) Western blot analysis of pULK1 and p-P70S6K. Red arrow indicates p-P70S6K, while the higher band observed is p85S6K, which is also detected by our antibody. (B) Densitometry of western blots analysis demonstrates a dose dependent increase in pULK1. Asterisks indicate significant differences relative to untreated cells. From this, 200nM was chosen as the optimum concentration of Rapamycin for further studies. (C) RT-PCR for ATG13 and (D) WIPI1, indicators of activated autophagy. Rapamycin increases expression of both ATG13 and WIPI1 mRNA, suggesting activated autophagy. Paired t-tests were used to determine significant differences in response to Rapamycin treatment. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ were deemed significantly different. Experiment was repeated for three patient and three control fibroblast samples. Error bars represent mean+/- SEM.

### 3.4.10 Rapamycin regulates ATG12

We subsequently aimed to identify molecular changes which Rapamycin induces to activate the autophagy pathway. No changes were observed in BECLIN-1, ATG3 or conjugated ATG12-ATG5 (Figure 3.18). Densitometry analysis demonstrated decreased levels of free ATG12 after treatment with 200nM Rapamycin in both patients and controls, suggesting that ATG12 becomes conjugated to other proteins for functional activation of the pathway. Additionally, analysis of basal ATG12 levels showed a significant decrease in levels of free, unconjugated ATG12. ATG12 conjugates with both ATG5 and ATG3 to regulate basal autophagy and promote autophagic flux (Murrow et al. 2015). As such, decreased levels of ATG12 may render cells less capable of regulating autophagic mechanisms.

No changes in p62 or LAMP-1 expressions were observed with Rapamycin treatment. 24 hour treatment with Rapamycin is sufficient to induce autophagic initiation and modulation of ATG12, but no discernible effect is observed regarding autophagic flux.
Figure 3.18. Patients demonstrate decreased availability of free ATG12. We further investigated the response of other autophagy related proteins to Rapamycin. Cells were treated with either 200nM Rapamycin, or a range from 0-300nM Rapamycin. (A) Western blot analysis of LAMP-1, ATG12 and ATG12 conjugated to ATG5 in patient (n=4) and control (n=3) fibroblasts. (B) Densitometry of ATG12 western blot analysis. No change in ATG5-ATG12 is observed, but significant reductions in free ATG12 are observed. (D) Western
blot analysis of p62 and BECLIN-1 in response to Rapamycin treatment. Rapamycin does not alter expression of p62 or BECLIN-1. Western blot analysis of ALS20 shown. *p<0.05, **p<0.01 were deemed statistically significant. Paired t-tests were used to determine significant differences observed in response to Rapamycin treatment. Unpaired t-tests were used to compare patient to control fibroblasts. Experiment was performed twice. Error bars represent mean+/- SEM.

3.4.11 Autophagy and Apoptosis

Altered autophagy has been implicated in the pathogenesis of ALS in various models of disease, and we have shown that patient fibroblasts demonstrate similar deficiencies. However, it is still unclear whether impaired autophagy is central to ALS, or a symptom of another underlying cause. It is well known that the autophagy and apoptosis pathways are intrinsically linked (Mariño et al. 2014). Animal models utilizing neural stem cells from mtSOD1 transgenic mice have demonstrated that cells expressing mtSOD1 are more sensitive to apoptosis induced via oxidative stress (Sui et al. 2014). Similar results were reported in myoblasts derived from ALS patients (Bradley et al. 2009).

At low levels reactive oxygen species (ROS) play a role in cell signaling, but at higher levels can result in oxidative damage (Lee et al. 2012). Autophagy is responsible for regulating cellular ROS, and impaired autophagy increases ROS (Rouschop et al. 2009).

As our patient cells demonstrate impairments of the autophagy pathway, we hypothesized they would be less capable of regulating ROS, thus leading to increased apoptosis in response to $H_2O_2$ treatment.
3.4.12 H$_2$O$_2$ induces apoptosis in fibroblasts.
To induce apoptosis, fibroblasts were treated with a range of H$_2$O$_2$ concentrations from 0-1mM for a period of 5 hours. After 5 hours cells were stained with Hoechst and analyzed by fluorescence microscopy. Condensation of nuclear material, a typical feature of apoptotic cells, was observed with 1mM H$_2$O$_2$. All other treatments yielded healthy appearing cells (Figure 3.19). We further treated patient and control cell lines with H$_2$O$_2$ for western blot analyses. We observe a significant increase in BAX, a pro-apoptotic protein, with a concomitant decrease in BECLIN-1, a pro-autophagic protein (Figure 3.20). This confirms a role for the interplay between the autophagy and apoptotic pathways.
Figure 3.19. *1mM H2O2 induces apoptosis in dermal fibroblasts.* To establish the optimum concentration of H$_2$O$_2$ required to induce apoptosis, fibroblasts were treated with a range of H$_2$O$_2$ concentrations from 0-1mM (A-D). Nuclear condensation can be readily observed at 5 hour treatment with 1mM H$_2$O$_2$ (F, enlarged insert of D) but not with lower concentrations of H$_2$O$_2$ (E, enlarged insert of A). 1mM H$_2$O$_2$ was used in subsequent experiments to induce apoptosis. Experiment was repeated three times. Scale bars represent 200µm.
Figure 3.20. 1mM H₂O₂ induces apoptosis in fibroblasts. To confirm that apoptosis directly affects the expression of autophagy related proteins, fibroblasts were treated with 1mM H₂O₂ for 5 hours, after which protein was harvested and analyzed by western blot. Increased expression of pro-apoptotic protein BAX is observed in response to H₂O₂ treatment, indicating induced apoptosis. Decreased expression of the pro-autophagic protein BECLIN-1 is observed upon treatment with H₂O₂ indicating impaired autophagy in response to induced apoptosis. Experiment was performed once.
3.4.13 ALS Patient fibroblasts are more sensitive to apoptotic induction

To quantitatively analyze the effect of H$_2$O$_2$ on cell viability, flow cytometry experiments were performed. Cells were treated with H$_2$O$_2$ for five hours as previously described, and analyzed via flow cytometry for the expression of Annexin V (a marker of early apoptosis) and 7-AAD (a marker of late apoptosis). Expression of Annexin V alone indicates early apoptosis, expression of both indicates late apoptotic cells, and subsequent loss of Annexin V indicates necrotic cells. In comparison to untreated cells we observe considerable increases in late apoptotic and necrotic cells in all samples. However, comparisons between patient and control cells show that patients are significantly more sensitive to induction of apoptosis, with higher proportions of both late apoptotic and necrotic cells (Figure 3.21).

3.4.14 Rapamycin does not ameliorate sensitivity to oxidative stress

Rapamycin has previously been shown to extend survival of ALS mouse models. As there is a balance between apoptosis and autophagy, we investigated whether pre-treatment with Rapamycin would increase the survival of patient and control fibroblasts in the presence of H$_2$O$_2$. Cells were pre-treated with 200nM Rapamycin for 24 hours, prior to H$_2$O$_2$ treatment and compared to untreated cells. We show that treatment with Rapamycin had no discernible effects on cell survival in either patient or control cells (Figure 3.22). Thus, induction of autophagy is insufficient to ameliorate increased sensitivity to oxidative stress induced apoptosis. Perhaps more prolonged treatment with Rapamycin or induction of autophagy with more potent activators may increase survival. Further studies will be needed.
Figure 3.21. **Patient fibroblasts are more sensitive to apoptotic induction via H$_2$O$_2$.** To determine whether patient fibroblasts are more sensitive to oxidative stress induced apoptosis patient (n=4) and control (n=4) fibroblasts were treated with 1mM H$_2$O$_2$ for 5 hours after which they were sorted for the expression of Annexin-V and 7AAD, markers of early and late apoptosis. (A) Flow cytometry gating strategy indicating populations of healthy, early apoptotic, late apoptotic and necrotic cells. (B) Quantitative analysis of different cell populations after 5 hours of 1mM H$_2$O$_2$ treatment, showing increased late apoptotic cells and decreased healthy live cells in patient fibroblasts. T-tests were used to determine statistical differences. * $p<0.05$ and ** $p<0.01$ were deemed statistically significant. Experiment was performed twice. Error bars represent mean±/SEM.
Figure 3.22. Pre-treatment with Rapamycin does not ameliorate increased sensitivity to apoptosis. To determine whether activation of autophagy could ameliorate increased sensitivity to apoptosis, patient (n=4) and control (n=4) fibroblasts were treated with 200nM Rapamycin for 24 hours prior to 1mM H\textsubscript{2}O\textsubscript{2} treatment for 5 hours. Cells were then sorted for expression of Annexin-V and 7-AAD, markers of early and late apoptosis. Rapamycin treatment has no effect on the percentage of live cells following H\textsubscript{2}O\textsubscript{2} treatment. Experiment was performed once. Error bars represent mean+/- SEM.

3.5 Discussion

The mechanisms underlying ALS disease pathogenesis remain elusive. Many studies have suggested that deregulated autophagy may be a critical component to the pathogenesis of ALS with perturbed autophagy reported in both cellular and animal models (Otomo et al. 2012). We aimed to determine
whether dermal fibroblasts from ALS patients would be a useful model to investigate impaired autophagy *in vitro*.

The expressions of ALS related proteins *SOD1*, *FUS*, *TDP-43* and *C9ORF72* were found to be expressed similarly to age matched control samples. However, we found a significant increase in nuclear staining of SOD1 in patient samples relative to controls. This may suggest deregulation of this protein as a common mechanism to all ALS patients, not just those with mutations within the SOD1 gene itself. Mutant SOD1 has been shown to destabilize the interaction between BECLIN-1 and Bcl-Xl, thus impeding the formation of autophagic vesicles (Nassif et al. 2014). However, this effect has not been confirmed in wild-type protein, so it is unclear as to whether over expression of SOD1 or a mutation within the gene is the cause of this phenomenon. Suppression of SOD1 has been shown to be protective in motor neurons, though the mechanisms by which SOD1 induce toxicity remain unclear (Haidet-Phillips et al. 2011).

It has recently been reported that levels of TDP-43 are significantly upregulated in ALS patient derived fibroblasts, in both sporadic and familial cases harboring C9ORF72 and TDP-43 mutations (Sabatelli et al. 2015). Exceptions were observed in patients with SOD1 mutations which conversely demonstrated a decrease in TDP-43 levels. While these data corroborate previously published data suggesting TDP-43 pathology affects all ALS cases, with the notable exception of SOD1 cases, our results did not confirm the same findings in our patients. No difference in TDP-43 expression was observed by western blot analysis, nor was there an increased cytoplasmic TDP-43 expression observed in patients. It may be possible that the use of different antibodies resulted in different results; perhaps our antibody is less potent and unable to detect the low levels of cytoplasmic staining. However, this would not explain differences observed obtained *via* western blot analyses. Further experimentation using
alternative antibodies and more patient and control samples will be required to investigate this.

The focus of this work was to investigate the role of the autophagy pathway in ALS. We found that both TDP-43 and FUS could be directly implicated in the autophagy pathway. As expected, inhibition of the pathway with Bafilomycin A1 increased the levels of TDP-43 due to decreased autophagic turnover. This has previously been demonstrated in neuronal models, and is recapitulated in our fibroblast samples (Barmada et al. 2014). Conversely, inhibition of autophagy led to a significant decrease in levels of FUS. This indicates not that autophagy regulates the turnover of FUS, but that FUS may play a crucial role in regulating the autophagy pathway. The majority of studies regarding FUS and autophagy, focus on the regulation of FUS positive stress granules. Activation of the autophagy pathway decreases the abundance of these stress granules, presumably via disassembly of the stress granule (Ling et al. 2013). The molecular mechanisms by which FUS may be directly regulated by autophagy remain unclear. It has been demonstrated that overexpression of mutant P525L-FUS and R522G-FUS is sufficient to impair autophagy in neuronal cells (Soo et al 2015). As such it is possible that the mutant protein results in a loss of function, thus impairing the autophagy pathway.

C9ORF72 has recently been identified as the most common cause of ALS. However, research regarding the role this protein plays in neurodegeneration has been hindered by the lack of antibodies which are known to be specific for C9ORF72. Our immunocytochemistry analysis demonstrated diffuse cytoplasmic staining with some microtubule-like staining. Additionally, strong perinuclear staining is also observed (Figure 3.4). A recent publication using novel antibodies detecting both short and long isoforms of the protein demonstrate that the short isoform specifically stains nuclear membrane
regions, while the longer isoform is present in a diffuse pattern in the cytoplasm (Xiao et al. 2015). Due to the similar patterns of staining observed, it is likely that specific staining is observed, though no study has reported microtubule-like staining which we show here. Future studies using knock-down or overexpression of the C9ORF72 protein will be useful in confirming specificity of antibodies used.

Little is known about the role of the C9ORF72 protein. It has been suggested that it may play a role in the autophagy process, but limited data is available (Farg et al. 2014). Our data suggest that C9ORF72 may co-localize with ULK1, a crucial component of the autophagy initiation complex, and to a lesser extent with p62. This suggests a likely role for the C9ORF72 in the autophagy process, possibly via endosomal trafficking as suggested by Farg et al. Additional localization was observed with cytoplasmic SOD1, suggesting a converging mechanism. Fibroblasts may prove useful for further elucidating the function of these ALS related proteins, each of which we have shown is highly expressed in both patient and control cells.

Investigating the levels of autophagy related proteins yielded two significant results. Firstly, ALS patients demonstrate decreased levels of autophagy initiation complex proteins (pULK1 and ATG13). And secondly, ALS patients demonstrate increased expression of p62, suggestive of impaired autophagic flux. Moreover, numbers of p62 puncta in patient fibroblasts are greatly increased. Investigating autophagic flux is a convoluted process; increased levels of the autophagy proteins p62 and LC3B can indicate both increased autophagy via increased formation of autophagosomes, or impaired autophagic flux, via decreased turnover of autophagosomes via fusion with the lysosome. Several animal models have identified increased levels of p62 and LC3B by immunohistochemistry analyses (Gal et al. 2007; Li et al. 2008). As such it
remains unclear as to whether autophagy is overactive or underactive in the setting of ALS. We identified increased LAMP-1 intensities in addition to increased p62 puncta. As such we hypothesize impaired autophagic flux is at play due to decreased turnover of autophagosomes and lysosomes.

Increased sensitivity to oxidative stress has been previously reported in neural progenitor cells from both mouse and rat mtSOD1 transgenic animals (Li et al. 2012; Sui et al. 2014). These authors suggest this effect is due to decreased activity of SOD1. We have shown that the same holds true for dermal fibroblasts, indicating this effect is not specific to neural lineages, though we did not investigate the activity of SOD1 in these cells. Further, we demonstrate that this effect occurs in both sALS and C9ORF72-ALS patient fibroblasts, suggesting a common pathological mechanism in different types of ALS, and may not be specific for SOD1 mutations. We hypothesize that increased sensitivity to apoptosis may be due to impaired autophagy observed in these cells.

Autophagic enhancers have been shown to extend the lifespan of transgenic mtSOD1 mice (Staats et al. 2013; Zhang et al. 2014). We show here that Rapamycin is sufficient to induce initiation of autophagy, but unable to modify the levels of total p62 expression. Further, Rapamycin was unable to ameliorate increased sensitivity to oxidative stress induced apoptosis. Perhaps more prolonged treatment, or treatment with superior autophagy enhancers, will be required to modulate p62 buildup and increased sensitivity to oxidative stress.

These data suggest that fibroblasts from patients may be a useful model for investigating the disease mechanisms underlying the development of ALS. They will be a useful tool for testing pharmacological activation of the autophagy
pathway and elucidating the mechanisms by which specific mutations disrupt the autophagy pathway.

**Limitations of Study**

Although demonstrating interesting results, there were several limiting factors in the analysis of results. Immunocytochemistry results often demonstrated Hoechst staining outside of the nucleus (Figures 3.3 -3.6). Hoechst specifically binds to DNA and should only identify the nucleus in cells. One possible explanation for the presence of staining within the cytoplasm may be mycoplasma infection. Mycoplasma are small bacteria (0.3-0.8µm in diameter), which are resistant to many antibiotics and capable of growing unnoticed in cell cultures (Olarrin-George & Hogenesch 2014). Notably, mycoplasma can induce changes in global gene expression. Prior to future studies PCR analysis should be performed for the detection of mycoplasma in these fibroblasts.

To determine whether or not autophagy regulates ALS related proteins we inhibited autophagic flux using Bafilomycin A1. While we observed differences in expressions of FUS and TDP-43, we also noted that the loading control, Beta Actin, also increased with treatment. These experiments will need to be repeated using alternative housekeeping controls which are not affected by autophagic inhibition.

Further, the efficacy and specificity of some antibodies were not ideal. pULK1 and ATG12 were difficult to detect and required long exposures. Although they are suggestive of differences, this makes interpreting the results difficult due to high background levels. Different antibodies should be tested in future studies.

The expression of pULK1 was demonstrated to be significantly decreased in patient samples relative to control samples, as assessed by a student's t-test. However, one control sample (ALSC001) demonstrated almost undetectable expression of pULK1. Why this sample demonstrated almost no expression is unclear and questions the validity of the result. Power analysis is a statistical method used in experimental design to identify the sample size required to
detect an effect of a given size, within a specific degree of confidence. In the case of pULK1 analysis here, online power analysis tools were used to determine the sample size required to confirm our findings (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). Analysis of pooled standard deviation with means determined for each group using densitometry analysis, a power of 0.58 is obtained. Although arbitrary, it is common practice to design experimental procedures with a power of 0.8. To reach a power of 0.8 in the comparison of pULK1 between ALS patient and control samples, a sample size of 9 would be required.

To measure autophagic flux we used western blot analysis of markers associated with the turnover of autophagosomes. During autophagy p62 is degraded, along with its bound cargo. Additionally, LC3-I is converted to LC3-II, which is also degraded. As such, increased levels of p62 and LC3-II may be used together to investigate autophagic flux (Zhang et al. 2013). However, more sophisticated methods for the analysis of autophagic flux may be used in future studies. These include the use of an LC3-construct fused with both acid-insensitive mCherry and acid-sensitive GFP. Expression of both mCherry and GFP yields yellow puncta with merged fluorescent images. Fusion with the lysosome results in degradation of GFP by lysosomal hydrolases, yielding mCherry-only puncta. As such, the balance between yellow and red puncta are an efficient method to monitor autophagic flux (Kimura et al. 2007).
Chapter 4
Investigating The Role Of Astrocytes In The Pathogenesis of Amyotrophic Lateral Sclerosis Using Patient Derived iPSCs
4.1 Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurological disease characterized by the progressive loss of motor neurons. Patients inevitably progress to full paralysis, with death typically occurring within 2-5 years of onset. There is currently no efficacious therapy available. As such, novel therapeutics are urgently required.

Despite decades of research, the molecular mechanisms underlying the development of ALS remain elusive. Cytoplasmic aggregations of misfolded ALS-related and RNA-binding proteins are hallmarks of disease progression, indicating impaired proteostasis may play a central role in disease progression. It has also been reported that astrocytes may induce motor neuron cell death in models of ALS via the secretion of toxic factors. To begin to model ALS in vitro we have established and characterized induced pluripotent stem cells (iPSCs) ALS patients and healthy controls. By generating both astrocyte and motor neurons from patients and controls we confirm that astrocyte conditioned medium induces cell death in both healthy and patient iPSC-derived motor neurons, as previously reported.

To investigate the mechanisms by which this occurs, we co-culture HEK293T cells with astrocyte conditioned medium from both patient and controls. We demonstrate that both patients and control conditioned mediums induce autophagy in HEK293T cells by increasing BECLIN-1 and ATG12 expressions. LC3B is additionally increased, but only in response to control conditioned medium suggesting defective signaling by patient astrocytes. We further show that p62 puncta accumulate more so in HEK293T cells treated with patient conditioned medium, suggesting impaired autophagic flux. These data may begin to elucidate a mechanism by which astrocytes, via impaired modulation of the autophagy pathway, induce the accumulation of cytoplasmic aggregations of autophagy and p62-regulated proteins, as often observed in animal models.
4.2 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive loss of both upper and lower motor neurons (Chiò et al. 2013). Early symptoms include muscle atrophy and weakness which eventually progress to full paralysis, ultimately leading to death by respiratory failure (Lechtzin et al. 2001). Up to 90% of all ALS cases have no known genetic cause. However, mutations in many genes have been identified as causative of ALS. Of these, mutations in C9ORF72, SOD1, TDP-43 and FUS may account for 50% of mutation-associated cases (Ling et al. 2013).

The majority of research surrounding ALS has focused on mutations within the SOD1 gene (Redler & Dokholyan 2012). However, the mechanisms by which SOD1 induces motor neuron cell death remain elusive. In vitro and in vivo studies have identified a wide variety of cellular processes perturbed in ALS. These include oxidative stress (Weiduschat et al. 2014), glutamate excitotoxicity (Aarts & Tymianski 2003), proteosome and autophagy dysfunction (Otomo et al. 2012; Scotter et al. 2014), secretion of toxic factors by glial cells (Haidet-Phillips et al. 2011), mitochondrial dysfunction (Allen et al. 2015), neuronal hyperexcitability (Brian J. Wainger et al. 2014) and deregulated RNA processing (Raman et al. 2014).

Recently an increasing focus has been directed towards non-neuronal cells in the pathogenesis of ALS. Notably, many studies have implicated astrocytes, and the secretion of toxic factors, as a key component of ALS pathogenesis (Nagai et al. 2007; Gallardo et al. 2014; Meyer et al. 2014; Marchetto et al. 2008). Some studies have identified molecular changes mediated by ALS astrocytes – increased PGD2 expression (Di Giorgio et al. 2008), activation of inflammatory responses and oxidative stress (Marchetto et al. 2008), as well as activation of NF-kB, MAPK, JNK and AKT signaling pathways (Haidet-Phillips et al. 2011) have all been reported. Further, a recent study has demonstrated increased levels of a α2-Na/K ATPase/α-adducin complex in SOD1 mice may be responsible for inducing motor neuron cell death via non cell-autonomous
mechanisms (Gallardo et al. 2014). Despite these findings, the identities of the factors secreted from astrocytes which mediate cell death are unknown. Identification of these secreted toxic factors will have immense implications for furthering our understanding of disease pathogenesis and for developing novel therapeutics.

Aggregations of proteins such as TDP-43, SOD1, FUS and p62 are hallmarks of ALS (Brady et al. 2011; Bruijn et al. 1998; Schwartz et al. 2014a). This highly suggests that impaired protein homeostasis plays a central role in ALS. Indeed, the majority of genes associated with the pathogenesis of ALS may be implicated in the autophagy process, a mechanism by which long lived and misfolded proteins are recycled (Scotter et al. 2014; Nassif et al. 2014; Ryu et al. 2014b; Farg et al. 2014). Several studies have demonstrated altered autophagy in animal and cellular models of ALS, but the mechanisms underlying this impairment remain to be elucidated (Li et al. 2008; Morimoto et al. 2007). Furthermore, it is still unclear as to whether altered autophagy is due to increased autophagic initiation, or impaired autophagic flux (Zhang et al. 2014).

We hypothesized that secretion of factors from ALS patient astrocytes may be the cause of impaired autophagy in neuronal cells. Herein we show that ALS patient astrocyte conditioned medium (ACM) reduces the viability of both control and patient motor neurons. We further investigate the effect ACM on HEK293T cells to examine whether ACM modulates the autophagy pathway via paracrine mechanisms. To confirm whether findings are specific to ALS patient samples, we additionally investigated the effect of conditioned medium derived from Fragile X patient iPSC-derived astrocytes. We show that both ALS patient and control ACMs induce autophagy in HEK293T cells via upregulation of pro-autophagic proteins BECLIN-1 and ATG12. Increases in LC3B-I and LC3B-II are also observed, but only in cells treated with control ACM. We also observed a significant increase in the formation of p62 puncta in cells treated with patient ACM, relative to control ACM. This may suggest that patient ACM may induce an imbalance in the autophagy pathway by increasing the expression of proteins
associated with autophagosome formation, but without a concomitant increase in LC3B expression required for the degradation of p62 tagged cargo (Maruyama et al. 2014). We further show that HEK293T cells treated with patient ACM show an increase in SOD1 expression. Whether this is due to upregulation of SOD1 and/or decreased turnover via autophagic mechanisms is unclear. These data may suggest a mechanism which promotes the aggregation of proteins in ALS patients and animal models, and demonstrates the validity of the autophagy pathway as a therapeutic target in ALS.

4.3 Methods

iPSCs from three patient and three control fibroblast lines were generated by Katya McDonagh. These were characterized by Alkaline Phosphatase staining, immunocytochemistry, RT-PCR and tri-germ layer differentiation. iPSCs were differentiated towards motor neurons and astrocytes, and characterized by immunocytochemistry and western blot analysis. The effect of astrocyte conditioned medium on viability was demonstrated on motor neurons derived from patients and controls by culturing the neurons in medium collected from either patient or control iPSCs for 5 days. The effect of astrocyte conditioned medium on the autophagy and ALS-related proteins was determined by culturing HEK293T cells with astrocyte conditioned medium for 5 days and analyzing protein expression by immunocytochemistry and western blot analyses. Standard microscopy, in addition to the Operetta high content imaging hardware coupled with Harmony image analysis software, were used to quantify motor neuron viability, puncta formations and identify protein localization. Autophagy was induced with Rapamycin and Trehalose. For full details on methodologies refer to Chapter 2.
4.4 Results

4.4.1 iPSC Characterization

iPSCs were generated by Katya McDonagh using either lentiviral transduction or episomal reprogramming methods. Details of patients and controls are provided in Table 4.1.

<table>
<thead>
<tr>
<th>iPSC Line</th>
<th>Age</th>
<th>Sex</th>
<th>Patient Status</th>
<th>Reprogramming Method</th>
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<tr>
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<td>ALS C9ORF72 R.E.</td>
<td>lentiviral</td>
</tr>
<tr>
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<td>lentiviral</td>
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Table 4.1. Details of iPSCs used in this study.

The first step in iPSC research is to characterize the cells as pluripotent. Cells which do not meet the necessary criteria may potentially lack the capability for differentiation towards specific cell types or a propensity for self renewal. As such we performed the standard array of molecular characterization techniques to confirm pluripotency in our cell lines. The first step of cell characterization performed was Alkaline Phosphatase (AP) staining. AP stains iPS cells highly with minimal staining typically observed in other cell types such as fibroblasts.
Figure 4.1 shows representative images of colonies from one patient and one control. The colonies are composed of very small cells with a high nuclear to cytoplasm ratio, typical of embryonic and induced pluripotent stem cells. Staining with AP demonstrated high staining at the edges of colonies, with moderate staining within. Cells which had clearly begun differentiation showed very low staining with AP as indicated by a white arrow (Figure 4.1 D).

While AP staining is a useful early indicator of pluripotency, AP is also known to highly express in some other cell types, such as osteoblasts (Beloti & Rosa 2005). As such, more robust assays are required for characterization. To this end we performed immunocytochemistry to show expression of OCT4, SOX2 and NANOG, as well as the cell surface markers SSEA4, TRA-1-60 and TRA-1-81 (Figure 4.2). All cell lines tested demonstrated high levels of expression of each of these markers, as expected by iPSCs.

Next, RT-PCR was performed to measure levels of endogenous OCT4 and SOX2 (Figure 4.3). Primers were designed to include the 3'UTR which were not present in the constructs of the reprogramming vectors. These results demonstrate that the iPSCs have begun to express endogenous levels of pluripotency markers and may no longer require expression of transgenes from the viral or plasmid vectors to maintain pluripotency.
Figure 4.1. iPSCs resemble ESCs and are positive for alkaline phosphatase staining. iPSCs were grown in pluristem culture medium and maintained on Geltrex coated dishes. Cells were imaged and shown to resemble ESC colonies. (A) Representative image of control-derived iPSCs from sample iPSC001. (B) Representative image of patient-derived iPSCs from sample iPS31c8. (C) Alkaline phosphatase staining of control-derived iPSCs. (D) Alkaline phosphatase staining of patient-derived iPSCs. Both patient and control cells demonstrate positive staining for alkaline phosphatase indicated by oink/purple staining. The white arrow in (D) indicates negative staining observed in differentiated cells as spontaneous differentiation occurs during iPSC maintenance, and black arrow in (D) indicates the colony with positive staining of alkaline phosphatase. Experiment was performed once for all iPS cell lines. Scale bars indicate 100μm and 200μm where indicated.
Figure 4.2. Immunocytochemistry analysis demonstrates expression of pluripotency markers in control and patient iPSCs. iPSCs were fixed and stained for the expression of OCT4, SOX2, NANOG, SSEA4, TRA-161 and TRA-180, all markers of pluripotent stem cells. iPSC1cx1 control cells stain positive for SOX2 (A), TRA-180 (B) with Hoechst staining in (C). iPS31c8 patient cells stain positive for SOX2 (D), TRA-180 (E) with Hoechst staining in (F). iPSC04c3 control cells stain positive for OCT4 (G), SSEA4 (H) with Hoechst staining in (I). iPS21c1 patient cells stain positive for OCT4 (J) and SSEA4 (K) with Hoechst staining shown in (L). iPSC04c3 control cells stain positive for NANOG (M) and TRA-161 (N) with Hoechst shown in (O). iPSC24cx1 patient cells stain positive for NANOG (P) and TRA-161 (Q) with Hoechst staining shown in (R). Experiment was performed once for all iPS cell lines. Scale bars indicate 100μm in length.

Figure 4.3. iPSCs express pluripotency markers as determined by RT-PCR. iPSCs were cultured and RNA extracted and cDNA generated. RT-PCR analysis for SOX2 and OCT4 were performed using primers to detect endogenous levels of gene expression, without detecting expression from viral or plasmid vectors. RT-PCR analysis of endogenous OCT4 (A) and SOX2 (B) expressions in iPSCs. All values were normalized to GAPDH expression, and relative to fibroblasts.
from control line ALSC001. Experiment was performed twice. Error bars represent mean +/-SEM.

4.4.2 iPSCs are capable of tri-lineage differentiation.
For iPSCs to be used for disease modeling they must be capable of differentiating to all three germ layers. To confirm tri-lineage differentiation potential, embryoid bodies (EBs) were generated from each cell line using the Aggrewell system as described in Chapter 2. EBs were allowed to mature in suspension for 4 days before being seeded into 24-well plates, and cells allowed to spontaneously differentiate for a further 7 days. Cells emerging from these EBs were stained with markers for each germ layer – smooth muscle actin (SMA, a mesodermal marker), beta -III tubulin (TUJ1, an ectodermal marker) and alpha fetal protein (AFP, an endodermal marker). We show that iPSCs from each line are capable of differentiating to cells of each germ layer (Figures 4.4, 4.5 & 4.6).

Taken together these results indicate that the iPSCs from our patients and controls are pluripotent, with the capabilities to differentiate to all three germ layers. As such they will be useful for the study of disease mechanisms in Amyotrophic Lateral Sclerosis.
Figure 4.4. Patient and control iPSCs are capable of differentiation towards ectoderm lineage. iPSCs were allowed to spontaneously differentiate in embryoid bodies for 8 days after which they were fixed and stained for expression of β-Tubulin. (A) β-Tubulin staining of iPSC1cx1 derived cells with Hoechst staining in (B). (C) β-Tubulin staining of iPS24c1 derived cells with Hoechst staining in (D). Experiment was performed once for all iPS cell lines. Scale bars 100μm in length.
Figure 4.5. Patient and control iPSCs are capable of differentiation towards endoderm lineage. iPSCs were allowed to spontaneously differentiate in embryoid bodies for 8 days after which they were fixed and stained for expression of Alpha Fetal Protein (AFP). (A) AFP staining of iPS04cx1 derived cells with Hoechst staining in (B). (C) AFP staining of iPS24c1 derived cells with Hoechst staining in (D). Experiment was performed once for all iPS cell lines.
Figure 4.6. Patient and control iPSCs are capable of differentiation towards mesoderm lineage. iPSCs were allowed to spontaneously differentiate in embryoid bodies for 8 days after which they were fixed and stained for expression of Smooth Muscle Actin (SMA). (A) SMA staining of iPSC3c2 derived cells with Hoechst staining in (B). (C) SMA staining of iPS31c8 derived cells with Hoechst staining in (D). Experiment was performed once for all iPS cell lines. Scale bars 100μm in length.
4.4.3 iPSCs can be used to generate neural progenitor cells (NPCs) with high efficiencies

To study ALS we need to generate disease specific cells; i.e. motor neurons and astrocytes. Many protocols are available for the differentiation towards specific cell types, each with advantages and disadvantages. For our studies we focused on the method published by Du et al., which utilizes small compounds to pattern differentiating cells to a motor neuron progenitor (MNP) cell intermediate (Du et al. 2015). During differentiation, the iPSCs condense to become more compacted small colonies of cells (Figure 4.7). Any cells which did not have this morphology were manually removed (Figure 4.7B, white arrow), though this was uncommon.

![Image of iPSCs](image)

Figure 4.7. iPSCs generate small rounded cells, typical of neural progenitors. Cells were differentiated in monolayer as per protocol and imaged on day 5 of differentiation to determine changes in morphology. Cells were shown to become smaller and more compact. (A) Representative image of iPSC3cx1 control iPSCs differentiated for 5 days. (B) Representative image of
iPS24c1 patient iPSCs differentiated for 5 days. The white arrow indicates cells of unexpected morphology. These were manually scraped away. Scale bars represent 100μm.

To confirm the generation of neural progenitor cells, we stained cells with NESTIN and PAX6, pan neural stem cell markers, at day 14 of monolayer differentiation (Figure 4.8). We showed that the majority of cells tested positive for both NESTIN and PAX6, indicating successful differentiation towards a neural stem cell fate. Some patches of cells did not express neural stem cell markers (arrow, Figure 4.8E), but this was uncommon in either patient or control lines. This suggests highly efficient differentiation towards a neural stem cell fate.

Neural stem cells are typically patterned to give rise to particular types of neurons upon further differentiation. During embryogenesis OLIG2 and NKX2.2 are co-expressed in a pool of progenitor cells which subsequently diverge to different paths; OLIG2 expression is maintained in motor neuron progenitor cells whereas NKX2.2 expression is maintained in interneuron progenitor cells (Du et al. 2015). Therefore we stained cells with OLIG2 to identify patterning of our stem cells. Our results indicate the vast majority of cells stained positive for OLIG2, suggesting a MNP fate (Figure 4.9).

Staining with OLIG2 and NESTIN demonstrated most cells maintained small compact morphology. However some cells had long axonal projections emerging from clustered cells. We hypothesized that these were stem cells that had spontaneously differentiated to a more advanced neuronal cell fate. To test this we stained cells with both Ki67, a marker of proliferating cells, and Beta-III-
Tubulin, a pan-neuronal marker (Figure 4.10). Cells which lack staining with Ki67 showed increased expression of Beta-III-Tubulin confirming the hypothesis (indicated by white arrows, Figure 4.10). The majority of cells maintained expression of Ki67 as proliferating cells.
Figure 4.8. Differentiating cells express NESTIN and PAX6, markers of neural stem cells. Cells were differentiated for a period of 14 days after which they were fixed and stained for NESTIN and PAX6 expression. Representative images from control and patient derived cells are shown. (A) iPSC1c1 derived MNPs express NESTIN, with Hoechst staining shown in (B). (C) iPSC2c1 derived MNPs express NESTIN, with Hoechst staining shown in (D). (E) iPSC3c2 derived MNPs express PAX6, with Hoechst staining shown in (F). (G) iPSC31c8 patient derived MNPs express PAX6, with Hoechst staining shown in (H). The white arrow in E and F indicates cells which are negative for PAX6 expression. These cells were extremely rare. Experiment was performed once for all iPS cell lines. Scale bars represent 100μm.

Figure 4.9. Differentiating cells express OLIG2, a marker of motor neuron progenitor cells. Cells were differentiated for 14 days after which they were
fixed and stained for OLIG2 expression. (A) OLIG2 expression in iPSC1c1 derived MNPs with Hoechst staining shown in (B). (C) OLIG2 expression in iPSC24c1 derived MNPs with Hoechst staining shown in (D). Scale bars represent 100μm.

Figure 4.10. iPSCs differentiated for 14 days express high levels of Ki67, a marker of proliferative cells. Cells were differentiated for 14 days after which they were fixed and stained for Ki67, a marker of proliferating cells, and β-Tubulin, a pan-neuronal marker. (A) β-Tubulin staining of iPSCs differentiated for 14 days. (B) Ki67 staining of iPSCs differentiated for 14 days. (C) Hoechst staining of cells. (D) Merged image of β-Tubulin, Ki67 and Hoechst. High levels of β-Tubulin indicate more terminally differentiated neuronal cells. These cells express lower levels of Ki67, indicating reduced proliferation, typical of neurons. White arrows indicate a region of increased β-Tubulin and decreased Ki67.
Images from iPSC1c1 derived MNPs shown. Experiment was performed once for all iPS cell lines. Scale bars represent 100µm.

We additionally performed western blot analyses to confirm the generation of motor neuron progenitor cells (Figure 4.11). Cells were shown to express high levels of NESTIN, but OLIG2 could not successfully be detected by western blot analysis. An alternative antibody will be tried to confirm Olig2 expression in future studies. Together with immunocytochemistry data, this demonstrates the successful generation of high proportion of neural progenitor cells (NESTIN+/PAX6+/Ki67+/OLIG2+), likely patterned toward a MNP fate.

(A)  
\[ \text{Control} \quad \text{Patient} \]

Nestin (220-240kDa)

Beta Actin (45kDa)

(B) MNPs derived from patient and control iPSCs express similar levels of NESTIN
Figure 4.11. MNPs derived from iPSCs express NESTIN, a marker of neural stem cells. In addition to analyzing proteins expression in differentiating cells via immunocytochemistry, protein was harvested at day 14 of differentiation and used for western blot analysis. (A) Western blot analysis of NESTIN expression in patient (n=4) and control (n=4) cell lines. (B) Densitometry analysis of western blot analysis. No difference in NESTIN expression is observed between patients and controls. Experiment was performed once for all iPS cell lines. Error bars represent mean +/-SEM.

4.4.4 Motor neuron progenitor cells successfully generate MNX1 positive neurons

To generate motor neurons, MNPs were cultured in suspension to form neurospheres for one week, and subsequently cultured on Geltrex coated plates to allow neuronal growth. The Aggrewell system was utilized to generate motor neuron spheres of a uniform size (Figure 4.12 A). Spheres were then either dissociated and cells cultured in monolayer (Figure 4.12 B), or the uniform sized spheres were seeded directly onto Geltrex-coated plates, and neurons allowed to emerge from the spheres (Figure 4.12 C). Cells cultured after enzymatic digestion of spheres showed poor viability and growth, possibly due to decreased levels of trophic factors produced. Neuronal cells which were allowed to emerge from spheres demonstrated rapid growth of neurites which grew long and relatively thick.

To confirm the identity of motor neurons, spheres were dissociated using Accutase and cultured as single cells prior to immunostaining (Figure 4.13 B). Cells were stained with MNX1 (Figure 4.13), a marker for motor neurons, and the positive cells were quantified (Figure 4.13C). Approximately 20% of cells were shown to stain positive for MNX1, suggesting successful generation of motor neurons, albeit to a relatively low level. Du et al reported the generation of
up to 90% pure populations. Further work will be needed to optimize the motor neuron differentiation protocols and improve the efficiency.

Western blot analysis was further performed in attempts to demonstrate MNX1 expression, but this was unsuccessful. Additionally, an alternative motor neuron marker antibody, ISLET1, was unsuccessful in both immunostaining and western blot analysis. As such, although MNX1 positive neurons were generated, future work will be needed to further confirm their identity as motor neurons using additional methodologies.
Figure 4.12. Generation of neurons from MNPs. iPSC-derived MNPs were enzymatically dissociated from monolayer culture and seeded into Aggrewell 400 plates to form uniform size spheres as shown in (A). Spheres were maintained in suspension culture for 7 days after which there were enzymatically
dissociated and seeded into monolayer culture, (B), or seeded directly onto dishes where neurons were allowed to grow from within (C). Representative images from iPSC1c1 control derived MNPs are shown. Scale bars represent 100μm.

Figure 4.13. **Differentiated neurons express MNX1, a marker of motor neurons.** Neurons derived by dissociation of MNP spheres were cultured for 7
days and stained with MNX1, a marker of motor neurons. Representative image for iPSC1c1 control derived neurons is shown in (A), with Hoechst staining in (B). Representative image for iPS21c1 patient derived neurons is shown in (C), with Hoechst staining in (D). Percentages of cells positive for MNX1 were quantified by calculating the number of MNX1 positive cells relative to total cells (E). Quantification was performed using Harmony software and Operetta imaging hardware. Scale bars represent 100μm. Experiment was performed once. Error bars represent mean +/- SEM.

4.4.5 Motor neuron progenitors are capable of generating astrocytes

OLIG2 is a master regulator of gliogenesis, and we next aimed to generate astrocytes from patient and control progenitor cells. To this end we cultured MNPs in medium containing 10% FBS. It has previously been suggested that bone morphogenic proteins (BMPs) in serum are sufficient to drive progenitor cells to differentiate towards a glial cell fate (Hu et al. 2010). Cells were seeded at a low density to prevent endogenous factors produced by stem cells inhibiting differentiation. Cells rapidly acquired a large fibroblastic morphology, typical of astrocytes (Figure 4.14). These cells were stained positive for Glial Fibrillary Acidic Protein (GFAP), a cytoplasmic intermediate filament protein characteristic of astrocytes, to confirm an astrocytic identity (Figure 4.14 A and C). Additionally, western blot analyses demonstrated expression of GFAP (Figure 4.15 A). All cell populations expressed similar levels of GFAP (Figure 4.15 B).
Figure 4.14. MNPs are capable of astrocyte differentiation. MNPs were cultured in medium containing 10% FBS to promote astrocyte differentiation. Differentiating cells acquired a fibroblastic morphology. To confirm astrocyte identity cells were fixed and stained for the expression of GFAP, a marker of astrocytes. (A) GFAP staining in iPSC3c2 control derived astrocytes with Hoechst staining in (B). (C) GFAP staining in iPS21c1 patient derived astrocytes with Hoechst staining in (D). Experiment was performed once for each iPS cell line. Scale bars represent 200μm.
Figure 4.15. Astrocytes express GFAP by western blot analysis. Protein was harvested from astrocytes at passage 2 and analyzed for the expression of GFAP, a marker of astrocytes. (A) Western blot analysis of GFAP expression in astrocytes. (B) Densitometry analysis of western blots indicates no significant difference in GFAP expression between patient and control derived astrocytes. Experiment was performed once for each iPS cell line. Error bars represent mean +/- SEM.
4.4.6 iPSC derived cells recapitulate disease mechanisms *in vitro*

It has recently been shown that ALS patient astrocytes induce cell death in motor neurons in a non-cell autonomous manner (Gallardo et al. 2014). We investigated whether conditioned medium from our astrocytes would recapitulate the same effect. Motor neuron culture medium was cultured on astrocytes for 7 days after which it was harvested and centrifuged to remove any cells and debris.

An equal number of motor neuron spheres were seeded into 24-well plates and allowed to proliferate. After 7 days of culture motor neurons from one control and one patient were treated with conditioned medium from patients and control astrocytes. After a further 5 days total numbers of MNX1 positive cells per well were quantified via automated imaging analysis (Figure 4.16). Significantly fewer MNX1 positive nuclei are observed in cells treated with patient conditioned medium, compared to control, confirming previously reported results (Figure 4.16 E and F).

The mechanisms by which astrocytes induce cytotoxicity in motor neurons remain unknown. It is interesting that the same effect is observed on both patient and control motor neurons, suggesting the possibility of a toxic factor secreted specifically by patient astrocytes, rather than intrinsic defects associated with patient motor neurons. Reasons as to why motor neurons are particularly susceptible to astrocyte induced toxicity remain unclear.

We have previously demonstrated that ALS patient fibroblasts display impaired autophagic mechanisms. As such we aimed to investigate the relationship between autophagy and astrocytes in both patients and controls derived cells.
Figure 4.16. Astrocyte conditioned medium from ALS patients decreases viability of both patient and control motor neurons. Conditioned medium was harvested from astrocytes from patients and controls. Each was used to culture motor neurons derived from a control cell line (iPSC1c1) and a patient cell line (iPS31c8). The effect on MNX1 positive cell numbers was then assessed via Operetta automated imaging analysis. (A) MNX1 positive motor neurons derived from iPSC1c1. (B) MNX1 positive nuclei quantified by Operetta imaging system outlined in white. (C) Hoechst staining of all nuclei. (D) Merged
image of MNX1, Hoechst and β-Tubulin (TUJ1). (E) Quantification of iPSC1c1 derived MNX1 positive nuclei with control and patient astrocyte conditioned medium after 5 days. (F) Quantification of iPS31c8 derived MNX1 positive nuclei with control and patient astrocyte conditioned medium after 5 days. Experiment was performed using ACM from all samples on motor neurons from one control cell line once and one patient cell line once. Error bars represent mean +/- SEM. Scale bars represent 100μm.

4.4.7 Patient astrocytes demonstrate significantly higher mTOR expression

mTOR plays a central role in many cellular mechanisms, including negatively regulating autophagy. We analyzed expression of total mTOR in patients and controls. We observed significantly higher expression in both ALS and Fragile X patients, with little observed in controls (Figure 4.17-4.18). However, we only examined levels of total mTOR. Further analysis of different phosphorylation sites will be needed to confirm the relevance of increased mTOR expression in the setting of ALS.

A significant decrease in ATG101 (an adaptor protein for the autophagy initiation complex) was observed in ALS iPSC-derived astrocytes, with a trend toward decreased pULK1 (a primary component of the autophagy initiation complex), suggesting a possible decrease in autophagy initiation (Figure 4.17-4.18). No differences in BECLIN-1 were observed. We further analyzed levels of proteins associated with autophagic flux. No differences in p62 or LAMP-1 were observed. These results demonstrate that patient astrocytes display relatively normal autophagy, with a higher level of mTOR inhibiting autophagy and a trend of decreased initiation of autophagy.
4.4.8 Patient astrocytes express normal levels of ALS-related proteins

We additionally examined the levels of ALS-related proteins in patient and control astrocytes. No changes in C9ORF72, SOD1 or TDP-43 expression were observed (Figure 4.19).

Figure 4.17. Expression of autophagy pathway components among the astrocytes derived from the control, ALS and FXS iPSCs. Protein was harvested from astrocytes and western blot analysis was performed to assess expression of autophagy components. Western blot analysis of autophagy related genes mTOR (A), pULK1 and ATG101 (B) and p62 and LAMP-1 (C). Beta Actin was used as a loading control. Experiment was performed once.
Figure 4.18. Densitometry of autophagy related proteins in ALS and Fragile X patient iPSC derived astrocytes. Blots from Figure 4.17 were quantified using densitometry analysis and expression was normalized to the expression of Beta Actin. ALS patients demonstrate increased levels of mTOR (A), but similar levels of other autophagy related proteins pULK1, p62, LAMP-1 and ATG101 (B-
E respectively). * p<0.05, *** p<0.001. For statistical analysis t-tests were used. Experiment was performed once. Error bars represent mean +/- SEM.
Figure 4.19. **Patient and control astrocytes express similar levels of ALS-related proteins.** Protein was harvested from astrocytes derived from control, ALS patient and FXS patient astrocytes and analyzed for the expression of ALS-related proteins by western blot analysis. No significant difference in expression of ALS related proteins among patient and control astrocytes. (A) Western blot analyses of C9ORF72, TDP-43 and SOD1. (B-E) Densitometry of western blot analyses of TDP43, SOD1, C9ORF72 (36kDa isoform) and C9ORF72 (55kDa isoform) respectively. Experiment was performed once. Error bars represent mean +/- SEM. T-tests were used for statistical analysis.

We next aimed to investigate how ACM may modify the autophagy pathway. Apoptosis and autophagy are intrinsically linked; increased apoptosis will decrease expression of pro-autophagic proteins such as BECLIN-1 (Figure 3.20). As such, patient ACM which may induce apoptosis in motor neurons would likely directly affect expression of autophagy related proteins. As the toxicity induced by astrocytes is specific to motor neuron cell types, we cultured another cell line (HEK293T cells, which should not demonstrate increased cell death) with ACM to investigate potential direct effects on the autophagy pathway. HEK293Ts were treated with ACM from 3 controls (iPS04c3, iPSC1cx1, and iPSc3c2), 3 ALS patients (iPS21c1, iPS21cx1, iPS24c1 and iPS31c8) and 2 Fragile X patients (Sc126 and Sc132). We first showed that
ACM from patients does not induce cell death in HEK293T cells (Figure 4.20). In fact ACM from patients and controls was shown to support HEK293T cell growth, relative to HEK293T cells maintained in fresh medium. Without ACM the cells grew less compact and showed a more spiky morphology. Medium was not changed for five days, which would explain the spiky, unhealthy morphology. This indicates that astrocytes produce factors which are supportive of cell growth. After 5 days of culture, HEK293T cells were fixed for immunocytochemistry and protein was harvested for western blot analysis.

For comparative analysis the following treatments were assessed: (1) HEK293Ts with medium changed every day, (2) HEK293Ts maintained in culture for 5 days without changing medium, (3) HEK293T cells treated with control ACM, (4) HEK293T cells treated with ALS patient ACM, (5) HEK293T cells treated with Fragile X ACM and (6) HEK293T cells treated with 1mM H₂O₂ to induce apoptosis.
Figure 4.20. Astrocyte conditioned medium supports the growth of HEK293T cells. To determine whether conditioned medium from astrocytes would negatively affect HEK293T cells, cells were cultured in DMEM + 10% FBS for 5 days (A), iPSC1c1 astrocyte conditioned medium (B), iPSC21c1 astrocyte conditioned medium (C) and iPSC-Sc132 astrocyte conditioned medium (D). Cells cultured with ACM, from patients and controls grow better with less spiky morphology observed in cells grown in DMEM + 10% FBS. No obvious differences were observed using different sources of ACM. No obvious signs of cell death were observed. Scale bars represent 100μm.

4.4.9 ACM induces autophagy in HEK293T cells
We performed western blot analysis for a range of autophagy related proteins. No changes in total mTOR expression or pULK1 were observed (Figure 4.21). However, increased expression of BECLIN-1 (Figure 4.22) and free ATG12
(Figure 4.23) were observed, suggesting increased autophagy via an mTOR independent pathway. Additionally, we observed a significant increase in the expression of LC3B-I and LC3B-II, but this effect was limited to control ACM (Figure 4.23). No changes in LC3B-I or LC3B-II expression were observed with ACM from either ALS or Fragile X patients.

4.4.10 Patient ACM increases accumulation of p62 puncta in HEK293T cells

We next investigated expression of p62 by western blot analysis and immunocytochemistry (Figure 4.23). No changes in total p62 were observed by western blot analysis. To further investigate the effect of ACM on p62, immunocytochemistry was performed and p62 positive puncta were quantified. This demonstrated a significant increase in the number of p62 puncta in HEK293T cells treated with patient ACM, suggesting decreased autophagic flux.
Figure 4.21. Astrocyte conditioned medium induces autophagy in HEK293T cells. HEK293T cells were cultured for 5 days in DMEM + 10% FBS (One group medium was changed daily, another group the medium was not changed for the 5 day period), iPSC-derived astrocyte-conditioned medium from
controls, ALS and FXS iPSCs. We additionally treated one sample with H2O2 for 5 hours to induce apoptosis, to observe the effect on autophagy related proteins. HEK293T protein lysates were then harvested and blotted with antibodies against autophagy components. (A) Western blot analyses of autophagy related proteins pULK1 and BECLIN1. (B) Western blot analyses of autophagy related proteins mTOR, ATG3 and ATG12. (C) Western blot analyses of autophagy related proteins p62 and LC3BI/II. Beta Actin was used as a loading control. Experiment was performed once.
Figure 4.22. Astrocyte conditioned medium induces autophagy in HEK293T cells. Densitometry of western blots from Figure 4.21. Expression of proteins was normalized to Beta Actin. (A/B) No changes in mTOR or pULK1 were observed. (C) Both patient and control ACM increases levels of BECLIN-1, a pro-autophagic protein. (D) A significant decrease in ATG3 expression was observed in response to FXS ACM treatment. T-tests and Anova were used for statistical analysis. * p<0.05 was deemed statistically significant. Experiment was performed once. Error bars represent mean +/- SEM.
Figure 4.23. Astrocyte conditioned medium induces autophagy in HEK293T cells. Densitometry of western blots from Figure 4.21. Expression of proteins was normalized to Beta Actin. (A) Both patient and control ACM increase the expression of ATG12, a pro-autophagic protein, indicating increased autophagy. (B) No change in p62 is observed by either control or patient ACM. (C/D) LC3B-I and LC3B-II are each increased in response to control ACM, but not in response to patient ACM. One-way Anova were used for statistical analysis. * p<0.05 was deemed statistically significant. Experiment was performed once. Error bars represent mean +/-SEM.
Figure 4.24. Patient ACM results in increased accumulation of p62 positive puncta. HEK293T cells were treated with ACM from patient and control astrocytes for 5 days, after which cells were fixed and stained for p62 expression. (A) Immunocytochemistry of p62 in HEK293T cells treated with control ACM with Hoechst staining in (B). (C) Immunocytochemistry of p62 in
HEK293T cells treated with patient ACM with Hoechst staining in (D). (E) Quantification of p62 puncta/cell. (F) Mean intensity staining of p62 in HEK293T cells. n.s. indicates no significant difference. T-tests were used for statistical analysis. Experiment was performed three times. Error bars represent mean +/- SEM. Scale bars represent 50μm.

### 4.4.11 Patient ACM increases levels of SOD1 in HEK293T cells

From these data we hypothesized that increased induction of autophagy (via increased BECLIN-1 and ATG12) results in the increased formation of autophagosomes. However, without a concomitant increase in LC3B expression (as observed in cells treated with control ACM) p62 accumulates in the cytoplasm. Prolonged impairment such as this may result in the buildup of p62 and other proteins regulated by autophagy and p62-dependent degradation. As such we next aimed to determine whether patient ACM would have any effect on ALS related proteins in HEK293T cells. We found that cells treated with patient ACM show an increase in SOD1 expression (Figure 4.25). SOD1 may play a direct role in autophagy via interaction with the pro-autophagic protein BECLIN-1, and BCL2L1, a suppressor of BECLIN-1 activity (Nassif et al. 2014b). No changes in TDP-43 expression were observed.
Figure 4.25. Patient astrocyte conditioned medium increases levels of SOD1 expression. (A) Western Blot analysis of ALS related proteins SOD1 and TDP-43 in HEK293T cells treated with ACM. The higher band observed upon detection with Beta Actin is residual identification of TDP-43. Densitometry analysis was performed on the lower band for Beta Actin expression. (B) Densitometry of western blot for SOD1. Although SOD1 appears to be increased in response to control ACM, this is due to increased protein loaded onto the western blot. Normalization to Beta Actin verifies no difference between cells treated with control ACM and regular medium. (C) Densitometry of western blot
for TDP-43. T-tests were used for statistical analysis. Experiment was performed once. Error bars represent mean +/-SEM.

4.4.12 Rapamycin and Trehalose modify numbers of p62 puncta in HEK293T cells

Induction of autophagy has been reported to extend the lifespan of mtSOD1 transgenic mice (Staats et al. 2013; Zhang et al. 2014). We investigated how both Rapamycin, an mTOR dependent regulator of autophagy, and Trehalose, an mTOR independent regulator of autophagy, would affect the number of p62 puncta in HEK293T cells treated with ACM (Figure 4.26). Conflicting results were obtained between ALS patients and controls. We found that Trehalose greatly increased the number of p62 puncta in cells treated with control ACM. This is presumably by increasing proteins associated with autophagosome formation and recruitment of p62 within. However, this effect was not observed with patient ACM. Conversely, Rapamycin was shown to decrease the levels of puncta in HEK293T cells treated with patient ACM. We hypothesize that this is due to increased activation of LC3B to regulate the turnover of p62. However, Rapamycin had no effect on HEK293T cells treated with control ACM. Further investigation into autophagy mechanisms, with larger patient cohorts, will be needed to elucidate the mechanisms underpinning differential responses to pharmacological inducers of autophagy.
Figure 4.26. Autophagy inducers modulate p62 puncta in HEK293T cells treated with ACM. Trehalose increases the number of p62 positive puncta in HEK293T cells treated with control ACM, but not patient ACM. Rapamycin decreases the number of p62 positive puncta in HEK293T cells treated with patient ACM, but not control ACM. * p<0.05. ** p<0.01. Experiment was performed once. Error bars represent mean +/-SEM. Previous experiments demonstrated that HEK293T cells treated with regular medium generate large amounts of p62 positive puncta (Figure 4.24). This may be due to starvation induced autophagy, in combination with detrimental affects due to not changing the medium for five days. As such, this experiment only compared cells treated control ACM to those treated with patient ACM for direct comparisons.
Figure 4.27. Hypothesized mechanism of action. Astrocytes release factors which positively regulate autophagy by increasing pro-autophagic proteins including BECLIN-1, ATG12 and LC3B. Patient astrocyte conditioned medium fails to induce LC3B expression, thus leading to an imbalance in autophagic mechanisms. Increased p62 puncta may form due to decreased turnover mediated by LC3B. This may explain a possible mechanism by which autophagy-regulated proteins accumulate and form cytoplasmic aggregations in ALS.
4.5 Discussion

In the present study we established and validated a model of non-autonomous cell death in ALS using iPSCs. iPSCs were successfully characterized and shown to display gene expression patterns as expected in pluripotent stem cells. We successfully used these patient and control derived cells to generate disease specific cells, i.e. motor neurons and astrocytes. Although highly pure populations of astrocytes were obtained, we only obtained neuronal populations that contained approximately 20-30% motor neurons (Figure 4.13), and these numbers are likely inaccurate as discussed below. The most recently established methodologies for generating motor neurons from iPSCs report generation efficiencies of up to 90% (Du et al. 2015). Further work will be needed to optimize these methods to achieve more pure neuronal populations for reliable investigations into disease mechanisms. Despite this, conditioned medium from ALS patient astrocytes was shown to decrease motor neuron viability after 5 days of co-culture, as previously reported (Meyer et al. 2014; Gallardo et al. 2014). This confirms that astrocytes induce cytotoxicity in motor neurons via paracrine mechanisms.

Impaired autophagy may play a role in the pathogenesis of ALS (Otomo et al. 2012). However, the majority of research regarding autophagy in the setting of ALS focuses on neuronal cells (Di Malta et al. 2012). This work focused on elucidating the relationship between autophagy and astrocytes in both ALS patient and control derived cells. We demonstrate that both ALS and Fragile X patient astrocytes express significantly higher levels of mTOR. mTOR is responsible for the regulation of many cellular processes, including autophagy, which has been implicated in the pathogenesis of each of these neurological diseases (Lee et al. 2013; Morimoto et al. 2007). Indeed, increased mTOR activity is a well known feature of Fragile X, but little has been reported regarding ALS (Sharma et al. 2010). Recently it has been shown that knockdown of p62 increases mTOR levels in zebrafish (Lattante et al. 2014). As
such, deregulation of autophagy may contribute to increased levels of mTOR observed in our cells, but more experiments will be required to confirm this. Further, we only investigated total mTOR expression. Future work will investigate whether increased phosphorylation of specific mTOR sites is observed, and the effect this may have on autophagy and other cellular processes.

We hypothesized that astrocytes may modulate the autophagy pathway via secreted factors. ACM from both patient and control astrocytes improved cell growth and morphology relative to cells maintained in normal medium. This indicates that astrocytes from patients do not induce cell death in HEK293T cells. Investigating the autophagy pathway we show that astrocyte conditioned medium induces autophagy in HEK293T cells as indicated by increased levels of BECLIN-1 and ATG12. No change in pULK (Ser555) was observed suggesting that modification of the autophagy pathway was not mediated through mTOR (Kim et al. 2011).

Increased expression of LC3B was also observed, but only in HEK293T cells treated with control ACM. LC3B is indispensable for the regulation of p62 (Maruyama et al. 2014). Analysis of total p62 by western blot analysis showed no difference between HEK293T cells treated with patient or control astrocyte conditioned medium. However, a significant increase in the formation of p62 positive puncta was observed, suggesting decreased turnover of p62-containing autophagosomes. Indeed, p62 is known to form cytoplasmic inclusions in many cases of ALS (Teyssou et al. 2013; Mizuno et al. 2006). It is possible that long term exposure to factors secreted by astrocytes results in the continuous build up of p62, due to insufficient levels of LC3B required to regulate autophagy. Although no increase in total p62 was observed via western blot analysis, cells were only cultured for 5 days in the presence of astrocyte conditioned medium. More prolonged exposure may more readily recapitulate disease mechanisms associated with ALS.
Further to this, we observed a significant increase in SOD1 in response to patient astrocyte conditioned medium. SOD1 is known to aggregate and accumulate in ALS patient cells (Bruijn et al. 1998). Whether increased SOD1 here is due to impaired autophagy, or increased translation is presently unknown. Interestingly, this was only observed in cells treated with ALS patient ACM, and not with those treated with Fragile X ACM. Both Fragile X and ALS ACMs had similar effects on p62 and LC3B differences observed. This indicates that modulation of SOD1 via paracrine mechanisms may be specific to ALS pathogenesis. Although no increase in TDP-43 was observed, it will be interesting to see whether prolonged exposure to patient conditioned medium will have any effect. Figure 4.27 illustrates our proposed mechanism of astrocyte induced impairment of autophagy in ALS.

The accumulation of p62 aggregates in ALS is well documented (Gal et al. 2007) and here we suggest that astrocytes may be the cause of this phenomenon. Activators of autophagy such as Rapamycin and Trehalose have been shown to be beneficial to the survival of motor neurons (Staats et al. 2013; Zhang et al. 2014; Cheng et al. 2015). As such we investigated the effects of both Rapamycin and Trehalose on the levels of p62 puncta in HEK293T cells treated with ACM. Conflicting results were obtained. Trehalose was shown to greatly increase the numbers of p62 positive puncta in cells treated with control ACM, while no significant difference was observed in cells treated with ALS patient ACM. Conversely, Rapamycin was shown to decrease the levels of p62 puncta in cells treated with patient ACM, with no effect observed in cells treated with control ACM. Rapamycin acts to induce autophagy via inhibition of mTOR, whereas Trehalose operates through an mTOR independent mechanism. The differences in response to autophagic inducers may be due to impairments in the autophagy process, induced by patient ACM, which we have not investigated here, resulting in differential responses to autophagy induction.

Taken together these results suggest that astrocytes may be the source of impaired autophagy often described in the literature. Future work will focus on
further elucidating the mechanisms by which astrocytes modulate the autophagy pathway, and determine whether chronic exposure to patient ACM will result in the development of cytoplasmic inclusions containing ALS related proteins.

**Limitations of Study**

Although differences between ALS patient and control cells were identified this study was limited in some ways. Firstly, quantification of MNX1 positive motor neurons (Figure 4.13) yielded questionable results. The percentages of positive numbers identified by automated image analysis are clearly higher than the actual numbers observed. This is likely due to under-counting of Hoechst stained nuclei which clustered together. As such it will be imperative to optimize quantification methods for accurate calculation of MNX1 positive motor neurons in future studies. New protocols should be established and verified using manual counting techniques to develop optimized methodologies. Furthermore, the efficiencies of MNX1 positive motor neuron generated here are considerably lower than those previously reported by Du et al (Du et al. 2015).

This problem further extends to quantification of MNX1 positive neurons after culture in the presence of ACM (Figure 4.16). Many positive cells are identified within neurospheres, but due to high density of cells these are not readily quantifiable. Rather, analyses focused on regions outside of dense neurospheres. As such, further optimization of differentiation protocols may be required. Despite this, we have demonstrated the successful generation of MNX1 positive motor neurons, albeit at low efficiencies.

Secondly, although we demonstrate that ACM derived from patients yields significantly fewer MNX1 positive motor neurons, we cannot confirm that this is due to apoptosis of motor neuron cells. Although induced cell death is likely as this has been reported previously (Meyer et al. 2014a), our results could also be due to impaired generation of new MNX1 neurons, rather than cell death mechanisms. To confirm this future studies will be required to examine apoptotic
markers such as Caspase-3. Additionally, although we examined the effects of ACM from three patients and three controls, we only utilized MNX1 positive neurons from one patient and one control. Further optimization of these experimental procedures will be highly valuable in furthering our understanding of glial-neuronal interactions in the setting of ALS.

Additionally, we only used MNX1 as an indicator of motor neurons. Other markers which may be specific for motor neurons include choline acetyl transferase (ChAT) and Islet-1/2. The use of these additional markers may improve methods to quantify and validate the generation of motor neuron cells in vitro.

Thirdly, the quality of several western blot experiments is debatable (Figure 4.17, 4.18). Highly dense or uneven beta-actin levels make quantification of differences between samples unreliable. These will need to be repeated to ensure reliability of any findings. The use of an alternative housekeeping protein (such as GAPDH) may be warranted due to the vast amount of protein detected by the Beta Actin antibody, which yields highly dense bands, complicating quantification by densitometry methods.

Chapter 5
Investigating the potential of IGF2 and RARβΔ384 to aid in the generation of induced neural stem cells (iNSCs)
5.1 Abstract

The development of induced pluripotent stem cell technology has been a powerful tool for investigating molecular mechanisms of disease using patient specific cells. However, this technology is time consuming and costly. As such, more rapid methods for the direct conversion of somatic cells to cells of interest have been developed.

Several studies have demonstrated the direct conversion of fibroblasts to induced neural stem cells (iNSCs). These methods are not without their own disadvantages; many studies fail to indicate the regional identity of reprogrammed cells, and do not show the generation of specific neuronal subtypes. Other studies which report the generation of specific neuronal subtypes do not report the efficiency at which they are generated.

Herein we investigated the potential of IGF2 and RARΔ384 to improve reprogramming efficiency towards a neural stem cell fate, directly from dermal fibroblasts. We additionally investigate the regional identities of reprogrammed cells – the use of SOX2, BRN2 and FOXG1 in direct reprogramming results in the generation of iNSCs with a ventral hindbrain identity. This is not modified by either IGF2 or RARΔ384.

We further demonstrate the differentiation potential of reprogrammed cells – iNSCs generated using SOX2+BRN2+FOXG1 with or without IGF2 or RARΔ384 are capable of differentiating to astrocytes, oligodendrocyte precursor cells and neurons, indicating tri-lineage differentiation potential. We further show that these iNSCs have a propensity for differentiating towards GABAergic neurons, while also generating glutamatergic neurons.
To overcome several of the issues associated with induced pluripotent stem cells (iPSCs), recent research has focused on the direct conversion of somatic cells to other cell fates. These include conversion of cells to blood cells (Heyworth et al., 2004; Xie et al., 2006), brown fat cells (Kajimura et al., 2009), hepatocytes (Sekiya and Suzuki, 2010; Huang et al., 2011), sertoli cells (Buganim et al., 2012a), as well as neurons and neural stem cells (Veirbuchen et al., 2010; Kim et al., 2011; Son et al., 2011; Han et al., 2012; Lujan et al., 2012; Najm et al., 2013; Yang et al., 2013). The generation of neurons from fibroblasts is a fascinating discovery, which may avoid the potential tumorigenicity associated with the iPSCs, and rapidly accelerate the study of neurons associated with specific diseases. However, these methodologies yield heterogeneous cell cultures with limited proliferative capacity, thus limiting their use (Wapinski et al. 2013).

Many efforts have been made towards improving methodologies to directly induce neural stem cells (iNSCs) from somatic cells, whether by increasing efficiencies, reducing the numbers of factors required for reprogramming or avoiding the use of viral vectors altogether (Table 1.3). iNSCs have been generated with the potential to differentiate towards astrocytes, oligodendrocytes and various neuronal subtypes as indicated by immunocytochemistry. However, most publications only show the expression of GFAP as an astrocytic marker which is also expressed in adult NSC in the subventricular zone, and do not report the efficiencies at which different neuronal cell types may be generated.

Furthermore, the regional identity of iNSCs in most cases remains undetermined. During development, and indeed neuronal differentiation from pluripotent cell sources, NSCs or neural progenitor cells (NPCs) are patterned by morphogens to give rise to cell populations with the propensities to differentiate towards specific types of neurons. As such it will be highly valuable to establish the regional identity of directly induced neural stem cells if they are to be used for disease modelling – for example, iNSCs capable of generating
motor neuron cell types will be highly advantageous for the investigation of disease mechanisms of Amyotrophic Lateral Sclerosis and other motor neuron diseases.

Insulin-like growth factor 2 (IGF2) is a growth factor which has previously been identified as highly expressed in Sox2 positive cells from the hippocampus (Bracko et al. 2012). IGF2 is a key regulator of proliferation in these stem cells, and is significantly downregulated during differentiation. The mechanism by which IGF2 modulates proliferation is predominantly via an autocrine manner, acting through Akt signalling. We hypothesized that over-expression of IGF2 may increase the generation and/or proliferation of iNSCs.

Retinoic acid signalling plays a role in many cellular processes including proliferation, differentiation, apoptosis, cell type specification, survival and immune-regulation (Niederreither & Dollé 2008). Importantly, retinoic acid receptor beta (RARβ) plays a role in neuronal differentiation (Bi et al. 2010). As such, we hypothesized that inhibition of RARβ, via a dominant negative mechanism by truncation Δ384, may aid cells in maintaining a stem cell fate by reducing their sensitivity to retinoic acid mediated differentiation (Shen et al. 1993).

Herein we investigate whether IGF2 or RARβΔ384 could improve reprogramming efficiencies or replace other previously reported reprogramming factors. We further investigate the regional identities of established iNSCs and determine their differentiation potential to various neuronal and glial lineages.

5.3 Methods

Lentiviral vectors encoding a range of transcription and potential reprogramming factors were cloned by PCR and restriction cloning methodologies. Lentiviruses were generated in HEK293T cells by transfecting lentiviral vectors encoding transgenes with lentiviral packaging vectors. Various combinations of lentiviruses were used to transduce human dermal fibroblasts and mouse embryonic fibroblasts in attempts to reprogram somatic cells directly to induced
neural stem cells. Reprogramming cells were cultured in neural stem cell medium, in both suspension and monolayer cultures. The formation of potential neurospheres was quantified by manual counting of solid spheres at thirty days in culture. Neurospheres were grown on Geltrex for the proliferation of neural stem cells. RT-PCR analysis was used to identify neural stem cell identity, as well as regional identity of neural stem cells. Induced neural stem cells were differentiated to astrocytes, oligodendrocytes and neuronal subtypes including GABAergic and Glutamatergic neurons. Confirmation of differentiated cell subtype was done by immunocytochemistry for the expression of lineage specific factors. For full details on methodologies see Chapter 2.

5.4 Results

5.4.1 Generation of lentiviral vectors

Over-expression of transcription factors is the most common method used for cellular reprogramming. This can be achieved using either viral or non-viral methods, though the latter demonstrates significantly lower efficiency. This may be due to decreased transfection efficiency resulting in decreased transgene expression per cell. For this reason we focused on overexpressing factors using lentiviral systems. Lentiviral vectors may be superior to retroviral vectors in that they can infect and integrate into slow and non-dividing cells (Cooray et al. 2012).

Using the pWPT-GFP vector (obtained from Addgene) we excised the GFP sequence using BamHI and Sall/Notl restriction digests and replaced it with sequences for transgenes of interest. OCT4 (O), SOX2 (S), KLF4 (K) and CMYC (M) were subcloned from pMXs retroviral vectors developed by Yamanaka for cellular reprogramming. BRN2 (B), FOXG1 (F) and LIN28 (L) were amplified by PCR methods using primers designed to contain BamHI (at the 5’ end) and SalI (at the 3’ end) for subsequent digestion, to subclone into the pWPT vector. IGF2 (I) was PCR amplified from cDNA clone MGC:8683 IMAGE:2964584, again with primers designed to contain BamHI and Sall sites flanking the coding sequence. A HIF1α (H) plasmid was also obtained from Addgene for the
generation of HIF1α retrovirus. The sequence coding for RARβΔ384 (R) was PCR amplified from a construct previously generated by Professor Shen (Shen et al. 1993). All sequences cloned via PCR methods were subjected to sequencing analysis to confirm no mutations were present.

Lentiviral and retroviral vectors were generated using viral packaging vectors (obtained from Addgene) in HEK293T cells. Transfection of HEK293T cells was determined using the pWPT-GFP construct (Figure 5.1). We observed approximately 50% transfection efficiency. After 48 hours, virus containing culture medium was harvested and stored at -80°C until use. To confirm the generation of functional virus in the harvested culture medium, dermal fibroblasts were transduced with 200μl of culture medium containing pWPT-GFP lentivirus. After 48 hours, we observe greater than 90% transduction efficiency, indicated by GFP expression, confirming the generation of functional viruses (Figure 5.1).
Figure 5.1. Generation of high titer retroviruses and lentiviruses in HEK293T cells. (A) HEK293T cells transfected with pWPT-GFP and lentiviral
packaging vectors demonstrate >50% transfection efficiency at 24 hours. (B) Brightfield view of the image in A. (C) HEK293T cells transfected with pMXs-GFP and retroviral packaging vectors demonstrate <50% transfection efficiency at 24 hours. (D) Brightfield view of the cell image in C. (E) Transduction of human dermal fibroblasts with HEK293T culture medium containing pWPT-GFP lentivirus demonstrates >90% transduction efficiency at 48 hours. (F) Brightfield image of lentiviral transduced fibroblasts. (G) Transduction of dermal fibroblasts with pMXs-GFP retrovirus demonstrates >90% transduction efficiency at 48 hours. (H) Brightfield image of retroviral transduced fibroblasts.

5.4.2 OKSM are sufficient to induce neurosphere formation

Initial experiments utilized combinations of retroviruses to attempt to generate iNSCs from human dermal fibroblasts. Cells from a 3-year old healthy control (PWC3Y) were transduced with two different combinations of retroviruses: (i) OCT4, SOX2, KLF4 and C-MYC. (ii) SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARβΔ384, LIN28. Transductions were performed on day 1, and repeated on day 2 to improve transduction efficiencies and increase transgene expression. Cells were then cultured in neural stem cell medium and to promote reprogramming towards a neural stem cell fate. At day 7 cells were enzymatically dissociated and cultured in suspension to induce sphere formation. In cells transduced with OCT4, SOX2, KLF4 and C-MYC (OKSM), preliminary cell clusters resembling spheres were observed at day 13 (Figure 5.2). These became more compact and rounded by day 15, and by day 21 they formed large spheres which resembled typical neurospheres. This was consistent with previous report that OKSM can convert fibroblasts into NSCs in neural stem cell medium (Janghwan Kim et al. 2011; Thier et al. 2012).

Transductions with 8 factors (KLF4 + SOX2 + C-MYC + BRN2 + FOXG1 + IGF2 + RARβΔ384 + LIN28) yielded no such spheres. It is likely that decreased expression of individual transcription factors occurred due to increased
competition between retroviruses to enter the cell, and/or dilution of each virus presented in the culture medium.

![Figure 5.2. Induction of neurosphere morphology from human dermal fibroblasts.](image)

5.4.3 OKSM derived neurospheres display characteristics typical of neural stem cells

Spheres formed by overexpression of OKSM were seeded onto plates coated with laminin and poly-L ornithine, and cells allowed to proliferate from attached spheres. Cells which proliferated had morphology distinctly different from dermal fibroblasts; they comprised a smaller cell body and several neurite like projections. To assess their identity we stained cells with the neural stem cell marker NESTIN. This showed that the majority of cells expressed NESTIN, confirming stem cell identity (Figure 5.3). We further validated NESTIN expression by western blot analysis. This showed that NESTIN expression was expressed in our iNSCs and neural stem cells derived from iPSCs, but not in dermal fibroblasts.
For spontaneous differentiation iNSCs were cultured in 10% FBS. After 7 days, cells were stained with Beta Tubulin and GFAP, markers of neuronal and astrocytic cells respectively (Figure 5.4). We show cells positive for both of these markers could be generated, demonstrating both neuronal and glial differentiation propensities. Together these results confirm that iNSCs may be directly generated from dermal fibroblasts, without the need for generating iPSCs, as previously reported (Janghwan Kim et al. 2011; Thier et al. 2012).

**Figure 5.3.** Cells proliferating from induced neurospheres express NESTIN, a marker of neural stem cells. (A) Cells proliferating from within a neurosphere. (B) Immunocytochemistry of cells grown from neurospheres and passaged in monolayer culture. Cells are stained with NESTIN, a marker of neural stem cells. (C) Brightfield image of cells shown in (B). Approximately 50% of cells stain strongly for NESTIN. (D) Western blot analysis confirms NESTIN expression in iNSCs, similar to iPSC derived neural stem cells. Experiment was performed once.
Figure 5.4. iNSCs can generate both neurons and astrocytes. Passage 3 iNSCs were cultured in DMEM + 10% FBS for 7 days and stained with anti-TUJ1 and anti-GFAP, markers of neurons and astrocytes respectively. (A) TUJ1 staining indicates neuronal cells. (B) High GFAP expression is indicative of astrocytes. (C) Merged image with DAPI to show nuclei. A mix of neuronal and glial cells was obtained from OKSM-iNSCs. Experiment was performed once.

5.4.4 Reprogramming using novel combinations of factors

One of the primary aims of this work was to determine whether we could generate iNSCs using novel reprogramming factors. It has previously been shown that mouse cells are more amenable to cellular reprogramming (Eminli et al. 2009). As such, to investigate the potential of our novel factors we switched to using mouse embryonic fibroblasts (MEFs).

As a starting point to investigate novel factors, we based our study on a previously published method which demonstrated a combination of SOX2, BRN2 and FOXG1 were sufficient to induce a neural stem cell fate in MEFs (Lujan et al. 2012). In addition to this combination, we added either IGF2 or RARβΔ384 to determine whether or not they could improve reprogramming efficiency. We further removed FOXG1 from combinations to see if our novel factors could replace FOXG1. Additionally, we determined whether just SOX2 with either of our novel factors could reprogram cells. An alternative study also used SOX2 and HMGA2 to reprogram cells (K.-R. Yu et al. 2015). We also attempted this combination, but without the use of a feeder layer described in their protocol to determine if that was essential. SOX2 has been suggested as
essential for the generation of iNSCs. We used several combinations of factors in the absence of SOX2 to see if we could replace this factor. A full list of combinations is provided in Table 5.1. Cells were transduced twice with each reprogramming factor. We also cultured transduced in monolayer or in suspension. Changes in morphology were monitored and documented every two days.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Reprogramming Factors</th>
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<tbody>
<tr>
<td>1</td>
<td>GFP</td>
</tr>
<tr>
<td>2</td>
<td>OCT4, SOX2, KLF4, C-MYC</td>
</tr>
<tr>
<td>3</td>
<td>SOX2, HMGA2</td>
</tr>
<tr>
<td>4</td>
<td>SOX2, BRN2, FOXG1</td>
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<tr>
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<td>SOX2, BRN2, FOXG1, IGF2</td>
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<td>7</td>
<td>SOX2, IGF2</td>
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<td>8</td>
<td>IGF2</td>
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<tr>
<td>9</td>
<td>SOX2, IGF2, HIF1A</td>
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<tr>
<td>10</td>
<td>SOX2, BRN2, FOXG1, RARBΔ384</td>
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<td>11</td>
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<td>13</td>
<td>RARBΔ384</td>
</tr>
<tr>
<td>14</td>
<td>SOX2, IGF2, RARBΔ384</td>
</tr>
<tr>
<td>15</td>
<td>SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARBΔ384, LIN28</td>
</tr>
<tr>
<td>16</td>
<td>RARBΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A</td>
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<tr>
<td>17</td>
<td>BRN2, FOXG1, IGF2</td>
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<tr>
<td>18</td>
<td>RARBΔ384, IGF2, HIF1A</td>
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Table 5.1. Combinations of reprogramming factors used for the generation of iNSCs.
5.4.5 Changes in morphology

MEFs were transduced with various combinations of reprogramming factors as indicated in Table 5.1. After transduction, cells were allowed to recover in fibroblast medium for 2 days after which medium was switched to neural stem cell medium to promote reprogramming.

After 8 days of monolayer culture in neural stem cell medium, the majority of conditions yielded cells which remained similar to fibroblasts; heterogeneous populations with many cells having elongated shape. However, in two conditions, iPSC-like colonies appeared (Figure 5.5 B and O). It was not surprising that the canonical reprogramming factors OCT4, SOX2, KLF4 and C-MYC yielded iPSCs (Figure 5.5 B). However, a combination without OCT4 also generated iPSC like colonies (KSMBFIRL, Figure 5.5 O). It is likely that the additional reprogramming factors were able to compensate for the lack of OCT4 expression. Additionally, the number of colonies which emerged in this condition was notably lower, presumably due to the lack of OCT4 expression. Recent reports have confirmed that a combination of OKSM does in fact require transient activation of a pluripotent stage for the generation of iNSCs (Bar-Nur et al. 2015).
Figure 5.5. Morphology of MEF-derived cells at day 8 of monolayer reprogramming with different sets of factors. Images in A-R represent
combinations 1-18 respectively. (A) negative control with GFP; (B) Positive control with OCT4, SOX2, KLF4, C-MYC; (C) SOX2, HMGA2; (D) SOX2, BRN2, FOXG1; (E) SOX2, BRN2, FOXG1, IGF2; (F) SOX2, BRN2, IGF2; (G) SOX2, IGF2; (H) IGF2; (I) SOX2, IGF2, HIF1A; (J) SOX2, BRN2, FOXG1, RARβΔ384; (K) SOX2, BRN2, RARβΔ384; (L) SOX2, RARβΔ384; (M) RARβΔ384; (N) SOX2, IGF2, RARβΔ384; (O) SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARβΔ384, LIN28; (P) RARβΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A; (Q) BRN2, FOXG1, IGF2; (R) RARβΔ384, IGF2, HIF1A. Significant alteration of the cell morphology were seen in (B) and (O) at day 8 of reprogramming. Red arrows highlight iPSC-like colonies, with enlarged inserts to demonstrate morphology more clearly. Cells are condensed and round with low a ratio of cytoplasm to nucleus. Each condition was replicated in triplicate wells.

5.4.6 Neurosphere Generation – Monolayer Vs. Suspension Culture

Neural stem cells have the intrinsic capability to form neurospheres, 3D structures which can be cultured in suspension. Further, culturing cells in suspension has been shown to increase stem cell gene expression (Shim et al. 2013). As such we compared the effect of culturing reprogramming cells either in monolayer on Geltrex to form colonies as previously described, or in suspension in 6-well plates with no coating to induce suspension culture.

By day 18 of reprogramming iPSC colonies were still present in monolayer culture. Further, colonies began to emerge in three other conditions; #4, #5 and #10, comprising the factors SBF, SBFI and SBFR respectively (Figure 5.6 D, E and J, Blue arrows). These colonies were morphologically distinct to iPSC colonies (Figure 5.5 B and O, Red arrows), with less compact cells. They resembled colonies more akin to iNSCs previously reported (Lujan et al. 2012). No colony formation was yet observed in other conditions. In conditions SBI (Figure 4 F) and SR (Figure 4 L) clustering of cells was observed showing sign of early reprogramming, but not distinct enough to infer any implications. Other conditions showed mild changes in morphology with a decrease in elongated
fibroblastic morphology, and more round flat cells apparent (Figure 5.6 G and H).

*Figure 5.6. Morphology of MEF-derived cells at day 18 of reprogramming in monolayer culture.* Images A-R represents virus combinations 1-18.
respectively. (A) negative control with GFP; (B) Positive control with OCT4, SOX2, KLF4, C-MYC; (C) SOX2, HMGA2; (D) SOX2, BRN2, FOXG1; (E) SOX2, BRN2, FOXG1, IGF2; (F) SOX2, BRN2, IGF2; (G) SOX2, IGF2; (H) IGF2; (I) SOX2, IGF2, HIF1A; (J) SOX2, BRN2, FOXG1, RARBΔ384; (K) SOX2, BRN2, RARBΔ384; (L) SOX2, RARBΔ384; (M) RARBΔ384; (N) SOX2, IGF2, RARBΔ384; (O) SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARBΔ384, LIN28; (P) RARBΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A; (Q) BRN2, FOXG1, IGF2; (R) RARBΔ384, IGF2, HIF1A. Significant morphological changes are seen in B, D, E, F, J, L, and O. Each condition was replicated in triplicate wells.

Regarding cells cultured in suspension at day 18 of reprogramming, many conditions yielded no viable spheres and only small clumps of cells were observed (Figure 5.7 E, N, P and Q). Conditions #4 (Figure 5.7 B, OCT4, KLF4, SOX2, C-MYC) and #15 (Figure 5.7 O, KLF4, SOX2, C-MYC, BRN2, FOXG1, IGF2, RARBΔ384, LIN28) which contained iPSC-like colonies previously yielded large clumps of cells. However, these did not have typical neurosphere morphology – they did not form defined borders with dark centres as expected. Some conditions including GFP, SOX2+HMGA2, SOX2+BRN2+IGF2, SOX2+IGF2, SOX2+BRN2+RARBΔ384 and SOX2+RARBΔ384, (Figure 5.7 A, C, F, G, K, L) yielded spheres more akin to neurospheres. However, these spheres in many conditions had a propensity to attach to the dish, despite no coating. Further, the addition of GFP alone yielded some spheres, indicating that suspension culture alone may allow cells to aggregate into sphere like structures. Conditions #11 (Figure 5.7 K, SOX2, BRN2, RARBΔ384) and SR (Figure 5.7 L, SOX2, RARBΔ384) yielded the most likely candidates for neurospheres as they have a defined border and the beginnings of a dark centre, hallmark characteristics of NSCs (Figure 5.7 K and L, Green arrows). This may suggest that inhibition of retinoic acid signalling by a dominant negative RARBΔ384 may increase stemness to support sphere formation.
Figure 5.7. Morphology MEF-derived cells at day 18 of reprogramming in suspension culture. Images A-R represents virus combinations 1-18 respectively. (A) negative control with GFP; (B) Positive control with OCT4,
SOX2, KLF4, C-MYC; (C) SOX2, HMGA2; (D) SOX2, BRN2, FOXG1; (E) SOX2, BRN2, FOXG1, IGF2; (F) SOX2, BRN2, IGF2; (G) SOX2, IGF2; (H) IGF2; (I) SOX2, IGF2, HIF1A; (J) SOX2, BRN2, FOXG1, RARBΔ384; (K) SOX2, BRN2, RARBΔ384; (L) SOX2, RARBΔ384; (M) RARBΔ384; (N) SOX2, IGF2, RARBΔ384, LIN28; (O) RARBΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A; (Q) BRN2, FOXG1, IGF2; (R) RARBΔ384, IGF2, HIF1A. Notable morphological changes are seen in B, K, L, and O. Each condition was replicated in triplicate wells.

By day 27 in monolayer culture, many colonies had emerged in conditions #4 (Figure 5.8 D, SOX2+BRN2+FOXG1), #5 (Figure 5.8 E, SOX2+BRN2+FOXG1+IGF2), and #10 (Figure 5.8 J, SOX2+BRN2+FOXG1+RARBΔ384) confirming that SOX2, BRN2 and FOXG1 were potent reprogramming factors. Other conditions demonstrated some changes in morphology, such as #6 (Figure 5.8 F, SOX2+BRN2+IGF2;) and #17 (Figure 5.8 Q, BRN2+FOXG1+IGF2), which showed formation of some cobble stone like cells which grew in a monolayer (Figure 5.8 F and Q). Interestingly, combinations #7 (Figure 5.8 G, SOX2+IGF2), #14 (Figure 5.8 N, SOX2+IGF2+RARBΔ384) and #18 (Figure 5.8 R, IGF2+RARBΔ384+HIF1A) demonstrated the formation of what appeared to be lipid droplets (Figure 5.8 G, N and R, Green Arrows.). Previous reports have indicated the potential of fibroblasts to differentiate towards adipocytes (Tontonoz et al. 1994). It may be possible that over-expression of IGF2 and RARBΔ384 mediated signalling pathways may induce this transdifferentiation. This was particularly evident in cells transduced with SOX2+IGF2+RARBΔ384 (Figure 5.8 N). No such cells were observed in cells transduced with GFP only.
Figure 5.8. Morphology of MEF-derived at day 27 of reprogramming in monolayer culture. Images A-R represents virus combinations 1-18 respectively. (A) negative control with GFP; (B) Positive control with OCT4,
SOX2, KLF4, C-MYC; (C) SOX2, HMGA2; (D) SOX2, BRN2, FOXG1; (E) SOX2, BRN2, FOXG1, IGF2; (F) SOX2, BRN2, IGF2; (G) SOX2, IGF2; (H) IGF2; (I) SOX2, IGF2, HIF1A; (J) SOX2, BRN2, FOXG1, RARΔ384; (K) SOX2, BRN2, RARΔ384; (L) SOX2, RARΔ384; (M) RARΔ384; (N) SOX2, IGF2, RARΔ384, LIN28; (O) RARΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A; (Q) BRN2, FOXG1, IGF2; (R) RARΔ384, IGF2, HIF1A. Notable morphological changes are seen in D, E, F, J Q. Red arrows indicate typical neural stem cell colony formation as previously reported (Lujan et al. 2012). Green arrows indicate the formation of what appear to be lipid droplets in some conditions. Each condition was replicated in triplicate wells. Note: images for (B) and (O) are not present as these cells generated iPSC-like colonies. As this was not the aim of this work these cells were discarded. Blank images are shown to maintain consistency with other images.

By day 27, reprogrammed cells maintained in suspension showed increases in size of spheres over time. Notably, combination #6 (Figure 5.9 F, SOX2+BRN2+IGF2) generated numerous spheres where previously only few were detected (Figure 5.7 F). Proliferation of cells may have occurred, which may then have proceeded to detach and form neurospheres due to the lack of coating. However, many spheres still attached to the dish, despite not having any coating. Additionally, cells which emerged from these spheres were often fibroblastic.
Figure 5.9. Morphology MEF-derived cells at day 27 of reprogramming in suspension culture. Images A-R represent virus combinations 1-18 respectively. (A) negative control with GFP; (B) Positive control with OCT4, SOX2, KLF4, C-MYC; (C) SOX2, HMGA2; (D) SOX2, BRN2, FOXG1; (E) SOX2,
BRN2, FOXG1, IGF2; (F) SOX2, BRN2, IGF2; (G) SOX2, IGF2; (H) IGF2; (I) SOX2, IGF2, HIF1A; (J) SOX2, BRN2, FOXG1, RARβΔ384; (K) SOX2, BRN2, RARβΔ384; (L) SOX2, RARβΔ384; (M) RARβΔ384; (N) SOX2, IGF2, RARβΔ384; (O) SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARβΔ384, LIN28; (P) RARβΔ384, IGF2, BRN2, HMGA2, FOXG1, HIF1A; (Q) BRN2, FOXG1, IGF2; (R) RARβΔ384, IGF2, HIF1A. Each condition was replicated in triplicate wells. Note: images for (B) and (O) are not present as these cells generated iPSC-like colonies. As this was not the aim of this work these cells were discarded. Blank images are shown to maintain consistency with other images.

5.4.7 Culturing of reprogrammed spheres
On day 28, cells from all conditions were subjected to suspension culture as they should by now be capable of neurosphere formation if they have successfully generated iNSCs. Spheres formed from monolayer culture and suspension cultures were pooled together. Efficiency of cellular reprogramming is often difficult to ascertain with iNSCs. However, typically efficiency is measured by number of colonies or neurospheres obtained. We chose to determine efficiency by counting the number of neurospheres obtained in each condition at day 30 (Figure 5.10). The highest sphere formation was observed using the canonical reprogramming factors OCT4+ SOX2+KLF4+C-MYC, which has previously been reported to generate iNSCs, albeit through a pluripotent state (Bar-Nur et al. 2015). High sphere formation was also observed with SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARβΔ384, LIN28. Again, these likely formed iPSCs, based on morphology of the early colonies obtained. Further, these did not resemble typical neurospheres, as they had a less defined border than would be expected of neurospheres, such as those observed in Figure 5.2. Combinations SOX2+BRN2+FOXG1 and SOX2+BRN2+FOXG1+RARβΔ384 generated similar numbers of spheres, suggesting that RARβΔ384 had no additive effect on reprogramming efficiency.
Conversely, a combination of SOX2+BRN2+FOXG1+IGF2 had significantly fewer neurospheres at day 30, suggesting IGF2 may have inhibited iNSC generation.

**Figure 5.10. Mean number of neurospheres obtained using different combinations of reprogramming factors.** Numbers are the mean values of three wells. (1) GFP; (2) OCT4, SOX2, KLF4, C-MYC; (3) SOX2, HMG A2; (4) SOX2, BRN2, FOXG1; (5) SOX2, BRN2, FOXG1, IGF2; (6) SOX2, BRN2, IGF2; (7) SOX2, IGF2; (8) IGF2; (9) SOX2, IGF2, HIF1A; (10) SOX2, BRN2, FOXG1, RARΒΔ384; (11) SOX2, BRN2, RARΒΔ384; (12) SOX2, RARΒΔ384; (13) RARΒΔ384; (14) SOX2, IGF2, RARΒΔ384; (15) SOX2, KLF4, C-MYC, BRN2, FOXG1, IGF2, RARΒΔ384, LIN28; (16) RARΒΔ384, IGF2, BRN2, HMG A2, FOXG1, HIF1A; (17) BRN2, FOXG1, IGF2; (18) RARΒΔ384, IGF2, HIF1A. Error bars represent mean +/-SEM.

Conditions #2 (OCT4, SOX2, KLF4, C-MYC), #4 (SOX2, BRN2, FOXG1), #10 (SOX2, BRN2, FOXG1, RARΒΔ384), and #15 (SOX2, KLF4, C-MYC, BRN2,
FOXG1, IGF2, RARβΔ384, LIN28) had significantly higher number of sphere formation.

These spheres were maintained in suspension for one week, after which they were cultured on Geltrex and cells allowed to migrate from the spheres. Cells were passaged when they reached approximately 80% confluency.

Figure 5.11. Morphology of cells in monolayer culture at passage 3. (A) MEFs transduced with GFP (Brightfield) and (B) GFP. The majority of cells are still expressing lentiviral GFP. (C-I) Representative images of combinations #4 (C, SOX2+BRN2+ FOXG1), #5 (D, SOX2+BRN2+FOXG1+IGF2), #6 (E, SOX2+BRN2+IGF2), #7 (F, SOX2+IGF2), #10 (G, SOX2+BRN2+FOXG1+RARβΔ384), #11 (H, SOX2+BRN2+ RARβΔ384); and #12 (I, SOX2+RARβΔ384), respectively.
Only a limited number of conditions yielded cells which were capable of proliferating (Figure 5.11). These were combinations #4, #5, #6, #7, #10, #11, #12. Further, combinations #11 and #12 only proliferated for 2-3 passages, after which they failed to proliferate. The remaining conditions yielded cells which did not proliferate or spheres which would not attach to the Geltrex coated dishes.

The remaining work focused on analyzing combinations #4, #5, #6, #7 and #10. They were transduced with SOX2+BRN2+FOXG1 in #4 (Figure 5-11 C), SOX2+BRN2+FOXG1+IGF2 in #5 (Figure 5-11 D), SOX2+BRN2+IGF2 in #6 (Figure 5-11 E), SOX2+IGF2 in #7 (Figure 5-11 F) and SOX2+BRN2+FOXG1+RARΒΔ384 in #10 (Figure 5-11 G).

5.4.8 Reprogrammed cells express markers of neural stem cells

To confirm stem cell identity RT-PCR analysis was performed for neural stem cell markers. We investigated levels of Nestin, Pax6, endogenous Sox2 and Sox9 (Figure 5.12). Sox2 was induced in all conditions of #4, #5, #6, #7 and #10. Nestin was upregulated in #4, #5, #6 and #10 but not #7. Pax6 was highly elevated in #4, #6 and #10 and marginally increased in #5, but not in #7. These data demonstrate various expressions of Sox2, Nestin and Pax6 in different combinations. The condition #7 transduced with SOX2+IGF2 was the worst in inducing Nestin or Pax6 or Sox9, suggesting that IGF2 alone with SOX2 may not be sufficient to induce a neural identity. Interestingly, Sox9 was specifically upregulated in samples #4, #5 and #10. These three samples also showed the greatest reduction of Col1a1, a fibroblast marker. Condition #6 (SOX2+BRN2+IGF2) does not show decreased Col1a1, but were highly expressing Nestin, Pax6, and Sox2; therefore it might have mixed populations of neural stem cells and fibroblasts. From these PCR data, the most likely candidates for iNSCs are #4 (SOX2+BRN2+FOXG1), #5 (SOX2+BRN2+FOXG1+IGF2) and #10 (SOX2+ BRN2+FOXG1+RARΒΔ384). The common factors shared by #4, #5 and #10 are SOX2, BRN2 and FOXG1. Therefore SOX2, BRN2 and FOXG1 are the core factors required for neural conversion.
Figure 5.12. Expression of stem cell markers in reprogrammed cells. The neural stem cell markers, Sox2, Nestin, Pax6 and Sox9, are increased in some
populations, with a decrease in Col1a1 observed. Combinations #1 (GFP), #4 (C, SOX2+BRN2+FOXG1), #5 (D, SOX2+BRN2+FOXG1+IGF2), #6 (E, SOX2+BRN2+IGF2), #7 (F, SOX2+IGF2), #10 (G, SOX2+BRN2+FOXG1+RARΒΔ384), respectively. Experiments were performed once. Error bars represent mean +/-SEM.

5.4.9 Investigating the regional identity of iNSCs
During development, early stage neural stem cells maintain the ability to differentiate to region specific cell fates (Elkabetz et al. 2008). They gradually acquire a specific regional identity, thus limiting their differentiation potential to specific neuronal and glial subtypes.

We aimed to determine whether iNSCs generated herein already possess a regional identity. To this end we analyzed expression of various regional identity markers by RT-PCR. The differentiation of iPSCs to neural stem cells generates a default telencephalic identity. As such we first investigated the expressions of Foxg1, Emx1 and Nkx2.1, markers typically expressed in telencephalon neural stem cells. We found that the expressions of these genes were not highly altered, suggesting they did not possess this identity (Figure 5.13).

We subsequently assessed the expressions of hindbrain markers Nkx6.1 and Hoxb4. Each of these was shown to be increased in several cell populations, suggesting a hindbrain identity (Figure 5.14).

We next investigated dorso-ventral patterning. Analysis of ventral markers Olig2 and Hes5 suggest our cells possess a ventral identity (Figure 5.15). We also observed the expression of Lmx1a, a midbrain marker. However, we were unable to detect levels of Foxa2, another well established midbrain marker. Although typically a midbrain marker, Lmx1a may also be expressed in the hindbrain (Mishima et al. 2009). These data suggest reprogrammed cells using Sox2, Brn2 and Foxg1 develop a ventral hindbrain identity. However, no positive controls were used for PCR analyses. Future experiments will use RNA/cDNA
derived from cells known to express transcription factors of interest (such as mouse-derived neural stem cells) for positive controls.

Figure 5.13. Expression of rostral NSC patterning markers Foxg1, Emx2 and Nkx2.1 in reprogrammed cells. Endogenous Foxg1 was increased in #1 (GFP) and #6 (SOX2+BRN2+IGF2), but not in other samples, despite overexpression of Foxg1. Further, expressions of either Nkx2.1 or Emx2 were not highly increased. This suggests reprogrammed cells do not possess a forebrain regional identity. Experiments were performed once. Error bars represent mean +/-SEM.
Figure 5.14. Expression of hindbrain markers Nkx6.1 and Hoxb4 in reprogrammed cells. Increased expression of Nkx6.1 and Hoxb4, hindbrain markers in conditions #4, #5, #6, and #10, and to a lesser extent #7, suggestive of caudal neural identity. Experiments were performed once. Error bars represent mean +/- SEM.
Figure 5.15. Expression of ventral stem cell markers Olig2 and Hes5, and midbrain marker Lmx1a. Some reprogrammed cells (in condition #4, #5 and #10) express high levels of Olig2; Hes5 was moderately increased in all five conditions. These data suggest that reprogrammed cells possess a ventral brain identity. However we also observe an increase in Lmx1a, suggesting a midbrain identity. Experiments were performed once. Error bars represent mean +/-SEM.

We further assessed the expression of Olig2 by immunocytochemistry (Figure 5.16), and found that Olig2 was expressed in #4, #5 and #10, with #10 demonstrating the highest numbers of positive cells. #6 and #7 did not yield any positive cells.
Figure 5.16. Analysis of Olig2 positive cells by immunocytochemistry. A-E, staining of Olig2 in iNSCs #4, #5, #6, #7, #10. (F) Quantification of Olig2 positive cells. Experiment was performed once for each line. Error bars represent mean +/- SEM.
5.4.10 iNSCs are capable astroglial differentiation

Previously, it was shown that iNSCs were capable of differentiation into tri-lineages of neuron, astrocyte and oligodendrocyte. Here we examined the differentiation potential of iNSCs to generate specific neuronal subtypes, as well glial cells including astrocytes and oligodendrocytes. Reprogrammed cells were cultured in DMEM with 10%FBS and 0.3% N2 to begin differentiation. Previous studies have shown that this may induce astrocytic differentiation, likely via BMP signalling (Hu et al. 2010). We demonstrate that iNSC populations #4, #5 and #10 readily give rise to astrocytic populations as imaged at 21 days post differentiation (Figure 5.17). iNSC populations #6 and #7 only demonstrate minimal staining, which is presumably non-specific. S100B was not detected at 21 days of differentiation. This may be due to cells not being fully matured yet. To further test this we differentiated more iNSCs for up to 5 weeks. At this time point a large portion of cells demonstrated high S100B staining surrounding the nucleus (Figure 5.18). Furthermore, these cells demonstrated a more mature astrocyte morphology. This confirms the generation of astrocytes from iNSC populations #4, #5 and #10, but not from #6 or #7.
Figure 5.17. Astrocyte differentiation of reprogrammed cells at three weeks. Images of cells stained for GFAP, Hoechst and merged images from iNSCs #4 (A-C), #5 (D-F), #6 (G-I), #7 (J-L) and #10 (M-O) are shown. Combinations #4, #5 and #10 yielded GFAP positive cells by
immunocytochemistry (A, D, M), but no expression of S100B was observed. The GFAP positive cell in #6 did not have typical astrocyte morphology (G). Experiment was performed once. Scale bars represent 50µm.

Figure 5.18. Differentiation of reprogrammed cells towards astrocytes for 5 weeks. Images of cells stained for GFAP, S100b and merged image (with Hoechst) from iNSCs #4 (A-C), #5 (D-F) and #10 (G-I) are shown. Cell morphology in #4, #5 and #10 showed typical mature astrocytes (A,D,G), compared to cells at three weeks of differentiation. Further, expression of S100b was observed in many cells (B, E, and H). Combinations #6 and #7 did not generate significant GFAP or S100b positive astrocytes. Scale bars represent 50µm.
5.4.11 iNSCs can generate oligodendrocyte precursor cells

We next aimed to determine whether iNSCs could be differentiated towards an oligodendrocyte lineage. Cells were cultured in neural stem cell medium without the addition of FGF or EGF, and supplemented with T3 supplement (30ng/ml) for three weeks and stained with NG2, a marker specific for oligodendrocyte precursors. Similar to samples astrocytic differentiation we found that iNSC populations #4, #5 and #10 were capable of generating NG2 positive cells, with typical bipolar or multipolar morphology (Figure 5.19). iNSC population #6 generated some cells which stained positive for NG2, but high back ground staining was observed and morphology was not quite as expected. iNSC population #7 again demonstrated no positive cells. We attempted to differentiate cells for five weeks to assess morphology at a later stage but high levels of cell death were observed. This may be due to the basic medium used. Many protocols use a variety of growth factors for the differentiation towards oligodendrocytes, which were not used here. Regardless, we have shown that some populations may generate oligodendrocyte precursor cells.
Figure 5.19. Differentiation of reprogrammed cells towards oligodendrocytes for 3 weeks. Images of cells stained for NG2, Hoechst and the merged image are shown for iNSCs #4 (A-C), #5 (D-F), #6 (G-I), #7 (J-L) and #10 (M-O) are shown in A-E respectively. Combinations #4, #5 and #10
yielded NG2 positive cells by immunocytochemistry (A,D,M), indicated by white arrows. Although it appeared some cells in #6 were positive (G), they did not display a typical oligodendrocyte morphology. No positive cells were obtained from iNSC#7. Experiment was performed once. Scale bars represent 50µm.

5.4.12 iNSCs can generate neurons

A previous study has demonstrated the establishment of neural stem cell lines established from the human embryonic hindbrain (Tailor et al. 2013). These cells maintain the capabilities to differentiate towards GABAergic, glutamatergic and serotoninergic neurons. As we also showed a hindbrain identity, we hypothesized that we would observe similar differentiation potential. To determine neuronal differentiation capabilities, our iNSCs were differentiated in the presence of retinoic acid (0.5µM) and purmorphamine (0.1µM). Alternatively, in attempts to generate dopaminergic neurons, cells were treated with TGFβ (10ng/ml) and FGF8a (100ng/ml). Cells were differentiated for three weeks and stained for neuronal markers.

We first investigate whether differentiated cells would express DCX, a marker of immature post-mitotic neurons. iNSC populations #4 and #5 generated some Dcx positive cells, albeit quite few (Figure 5.20). Surprisingly, iNSC population #10 did not yield any Dcx positive cells. It is possible that these cells have matured more rapidly, as such matured past the point of Dcx expression. No positive cells were observed in iNSC#6 or iNSC#7, nor were cells with neuronal morphology observed.

We further investigated the expression of MAP2, a marker of mature neurons. iNSC#4, iNSC#5 and iNSC#10 generated neurons positive for MAP2, indicating the successful generation of mature neurons after 3 weeks of differentiation (Figure 5.21). Again, no neurons were obtained in iNSC#6 or iNSC#7.

These results demonstrate that iNSC populations #4, #5 and #10 have each generated induced neural stem cells with tri-lineage potential. Despite
expression of some neural stem cell markers, populations #6 (SOX2+BRN2+IGF2) and #7 (SOX2+IGF2) displayed incorrect morphology and were incapable of generating astrocytes, oligodendrocytes or neuronal cells. As such these cells were excluded from subsequent analysis. These data also suggest that IGF2 was not able to replace FOXG1 or BRN2 for iNSC generation.
Figure 5.20. Differentiation of reprogrammed cells towards Dcx positive neurons for 3 weeks. Images of cells stained for DCX, DAPI and a merged image for iNSCs #4 (A-C), #5 (D-F), #6 (G-I), #7 (J-L) and #10 (M-O) are shown. Combinations #4 and #5 yielded Dcx positive cells by immunocytochemistry.
No DCX positive neurons were obtained in other conditions. Experiment was performed once. Scale bars represent 50µm.

Figure 5.21. Differentiation of reprogrammed cells towards Map2 positive neurons for 3 weeks. Images of cells stained for MAP2, DAPI and merged
image for iNSCs #4 (A-C), #5 (D-F), #6 (G-I), #7 (J-L) and #10 (M-O) are shown. Combinations #4 and #5 and #10 yielded Map2 positive cells by immunocytochemistry (A, D, G). No Map2 positive neurons were obtained in other conditions. Experiment was performed once. Scale bars represent 50µm.

5.4.13 iNSCs can generate GABAergic neurons

We next aimed to generate specific subtypes of neurons from different iNSC populations. For the generation of glutamatergic and GABAergic neurons iNSCs were exposed to retinoic acid and purmorphamine. Alternatively, to induce a dopaminergic fate, cells were treated with purmorphamine, TGFβ and FGF8a.

We found that iNSC#4, #5 and #10 were each capable of generating GABAergic neurons as indicated by the expression of GABA (Figure 5.22). Despite relatively low numbers of positive cells obtained, we quantified the numbers of GABA positive neurons (Figure 5.26). We found that #10 yielded significantly higher numbers of GABA positive neurons, relative to #4 and #5. iNSC#10 was generated using SOX2, BRN2, FOXG1 and RARβΔ384. This was unexpected, because RA was previously shown to define anterior–posterior neuronal identity in a concentration-dependent manner (Okada et al. 2004), and high concentration of RA was shown to differentiate ES cells into homogenous GABAergic neurons (Chatzi et al. 2009). Therefore we would expect reduced GABAergic neurons in cells with dominant negative RARBΔ384. However there is only an increase from ~1% to ~2% of total cells, a very small number of cells. Further, the expression level of the RARBΔ384 is yet to be determined was not confirmed by PCR analyses.

We next aimed to determine whether longer culture would increase the numbers of GABAergic neurons. However, we found decreased neuronal viability at five weeks of differentiation (Figure 5.23). No GABA positive neurons were observed in iNSC#4 differentiated cells. Conversely, several GABA positive cells were found in iNSC#5 and iNSC#10. Each of these populations were generated using SOX2, BRN2 and FOXG1 plus either IGF2 or RARβΔ384 overexpression. As such it is possible that each of these factors may extend the survival of
GABAergic neurons *in vitro*. However, PCR analyses will need to be performed to determine whether these genes are still being expressed.
**Figure 5.22. Differentiation of reprogrammed cells towards GABAergic neurons for 3 weeks.** Images of cells stained for GABA, Hoechst and merged image from iNSCs #4 (SOX2+BRN2+FOXG1) (A-C), #5 (SOX2+BRN2+FOXG1+IGF2) (D-F) and #10 (SOX2+BRN2+FOXG1+RARβΔ384) (G-I) are shown. (D) Quantification of GABAergic neurons in each condition. Condition #10 (SOX2+BRN2+FOXG1+RARβΔ384) showed significantly higher proportion of GABAergic neurons, suggesting that RARβΔ384 may be involved in GABAergic neuronal differentiation. * p<0.05, ** p<0.01. Experiment was performed once. Error bars represent mean +/-SEM. Scale bars represent 50µm.

**Figure 5.23. Differentiation of reprogrammed cells towards GABAergic neurons for 5 weeks.** Images of cells stained for GABA, Hoechst and a merged image from iNSCs #5 (A-C) and #10 (D-F) are shown. No viable GABAergic neurons were observed in other populations of reprogrammed cells. Experiment was performed once. Scale bars represent 50µm.
5.4.14 iNSCs can generate Glutamatergic neurons, but not TH positive neurons

We also investigated whether iNSCs could generate glutamatergic neurons. For this cells were stained with the glutamatergic neuron marker VGlut1. Each iNSC population #4, #5 and #10 successfully generated some positive cells (Figure 5.24). However, these cells were rare, even less common than GABAergic neurons. Further, attempts to identify glutamatergic neurons at five weeks differentiation was unsuccessful in any cell population. This is likely due to high levels of cell death observed. Alternative or improved methodologies for differentiation will be needed for improving the generation of different neuronal populations.

We further investigated whether we could generate neurons which express tyrosine hydroxylase, a marker of dopaminergic neurons, but no positive cells could be identified at three weeks differentiation. We cannot yet rule out the possibility of dopaminergic differentiation. Due to lack of a positive control we could not confirm the functionality of our antibody. Additionally, it may take more than three weeks to generate different types of neurons. But preliminary evidence suggests that iNSCs using our factors are capable of generating both excitatory (glutamatergic) and inhibitory (GABAergic) neurons.
Figure 5.24. Differentiation of reprogrammed cells towards Glutamatergic neurons for 3 weeks. Images of cells stained for VGlut, Beta Tubulin (TUJ1) and a merged imaged (with Hoechst staining) from iNSCs #4 (A-C), #5 (D-F) and #10 (G-I) are shown, showing VGlut positive Glutamatergic neurons. Too few VGlut positive neurons were generated for reliable quantification. Experiment was performed once. Scale bars represent 50µm.

5.5 Discussion

Herein we investigated the potential of IGF2 and RAR βΔ384 overexpression to aid in cellular reprogramming towards a neural stem cell fate and investigate stem cell regional identities. To establish the methodology we first demonstrated that overexpression of the canonical reprogramming factors OCT4, SOX2, KLF4 and C-MYC could be used to generate iNSCs with at least bi-lineage potential. However, differentiation towards oligodendrocytes was not examined.
Different cell sources may be more easily reprogrammed and more readily reprogrammed. Typically, more immature cells will differentiate more efficiently than terminally differentiated cells (Eminli et al. 2009). As such, more immature cells may be a more useful tool for investigating the factors for cellular reprogramming. For this reason we switched to using mouse embryonic fibroblasts to investigate the effects of potentially novel reprogramming factors.

Of 18 combinations of viruses two yielded colonies typical of iPSCs. These were OCT4, SOX2, KLF4 and C-MYC, the canonical reprogramming factors, and SOX2, KLF4, C-MYC, LIN28, BRN2, FOXG1, IGF2 and RARβΔ384. This indicates that OCT4 is dispensable for the induction of pluripotency, though the specific factor(s) which facilitates this is unclear with the addition of five extra factors with SOX2, KLF4 and C-MYC. Further, these cells were not confirmed as iPSCs using methods such as immunostaining and RT-PCR, as we were primarily interested in the induction of neural stem cells, not iPSCs.

Two recent studies have demonstrated the generation of iNSCs using just SOX2, or SOX2 with HMGA2 alone (Yu et al. 2015; Ring et al. 2012). However these studies utilized a mouse feeder layer for successful reprogramming. We investigated whether this feeder layer was essential by attempting to reprogram cells without it, but transduced with SOX2 and HMGA2. We found that this combination was insufficient to generate neurospheres or induced neural stem cells.

We were able to confirm the successful generation of iNSCs using the previously reported factors SOX2, BRN2 and FOXG1 (Lujan et al. 2012). Our factors were unable to increase the efficiency of reprogramming. Conversely, IGF2 significantly decreased the number of neurospheres formed. We hypothesized that this is due to activation of alternative pathways or mechanisms which may promote the differentiation of reprogramming cells or reprogramming towards alternative cell fates. By replacing FOXG1 with IGF2 we were able to generate a proliferating cell population which expressed some neural stem cell markers (such as Pax6). Nestin, Sox2 and Pax6 were all shown
to be upregulated in various cell populations. These alone would not be sufficient for the identification of iNSC populations. However, Sox9 was specifically expressed in #4, #5 and #10, samples we later confirmed as bonafide iNSCs. As such, this may be a more useful factor for early identification of iNSC populations.

Despite lower efficiency of reprogramming, a combination of SOX2, BRN2, FOXG1 and IGF2 were capable of generating neural stem cell populations with high expression of Nestin, Sox2, Pax6 and Sox9. Additionally, GABAergic neurons derived from these iNSCs demonstrated increased survival over a period of five weeks. This may suggest that recombinant IGF2 may be useful to increase survival of GABAergic neurons in vitro in future studies.

Overexpression of RAR βΔ384 with SOX2, BRN2 and FOXG1 had no effect on reprogramming efficiency, demonstrating similar generation of neurospheres relative to cells transduced with SOX2, BRN2 and FOXG1 only. Further, these cells demonstrated increased numbers of GABAergic neurons upon differentiation with retinoic acid and purmorphamide. This is counterintuitive to our hypothesis, as we expected inhibition of retinoic acid signalling, via our dominant negative mechanism, would decrease neuronal differentiation, if anything. GABAergic neurons are still present at five weeks post differentiation, whereas none remained viable from SOX2, BRN2, and FOXG1 iNSCs. Further investigation will be required to understand the molecular mechanisms at play.

Neither RARβΔ384 nor IGF2 differentially modulated the regional identity of iNSCs generated using SOX2, BRN2 and FOXG1. We have shown that these stem cells display a ventral hindbrain regional identity based on high expressions of Olig2. Despite overexpression of FOXG1, only limited expression of endogenous Foxg1 was observed in iNSCs.

The generation of GABAergic and glutamatergic neurons from these cells may be useful for investigating different neurological disorders such as autism, but methodologies will need to be greatly improved to increase the populations of
specific neuronal subtypes. We were unable to generate either dopaminergic or motor neurons from these cells. Future studies may focus on utilizing alternative factors for the generation of stem cells which may give rise to motor neurons. But for now, iNSCs (in lieu of iPSCs) may offer a more rapid and cost effective method for the generation of astrocytes from ALS patients to study glial-neuronal interactions in a “disease in a dish” model.
Chapter 6 - Discussion

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease with limited therapeutic options. The drug discovery in the field of mental and neurological disorders, which represent 14% of the global burden of disease generally, has been particularly affected due to the lack of human disease models. Consequently, in the last three decades, the new drug output has been unproportional to the exponential increase of the pharmaceutical investment. The lack of suitable human disease models during the early phases of drug screening, test and validation was considered to significantly contribute to the failure of many clinical trials. The species difference was partially highlighted by a recent research report, that only one VPAC2 antagonist was identified from the screening of 1.67 million of small molecules. Yet, this compound was specific for one species (human VPAC2 protein), but completely inactive for another (mouse Vpac2 receptor) (Chu et al. 2010). Therefore, human disease cell models of ALS are urgently needed for drug development. The focus of this thesis has been to develop and validate novel cellular models and methodologies which may be used for the identification of disease mechanisms in vitro, and for developing subsequent screening assays.

6.1 Autophagy in neurodegeneration

Autophagy is a cellular mechanism required for the degradation of long lived and misfolded proteins by incorporating targeted proteins to the developing autophagosome and subsequent degradation via fusion to the lysosome. This process, regulated by many proteins and physiological stressors, is essential in maintaining cellular homeostasis. Disruptions in the autophagy pathway have been observed in many neurological diseases, including Alzheimer’s disease, Parkinson’s disease and ALS (Wong & Holzbaur 2014; Nixon et al. 2005; Ahmed et al. 2012). Notably, these diseases are often characterized by the accumulation of misfolded proteins, such as SOD1, TDP-43 and FUS in the case of ALS, which has been hypothesized to be due to failure of the autophagy
system to regulate the turnover of these proteins (Cheng et al. 2015). It has been demonstrated that inhibition of the autophagy pathway results in neurodegeneration (Hara et al. 2006; Komatsu et al. 2006). Further, disruption of the balance between autophagosome formation and degradation (i.e. autophagic flux), also induces neurodegeneration (Zhang et al. 2013). The accumulation of autophagosomes has been observed in Parkinson’s disease and Alzheimer’s disease (Nixon et al. 2005; Stefanis et al. 2001). While impaired autophagy may induce neurodegeneration, excessive autophagy may have the same effect (Lee & Gao 2009). Additionally, disruption of autophagosome transport may also disrupt autophagy by impairing fusion to the lysosome (Webb et al. 2004). Furthermore, decreased activity of lysosomal hydrolases or decreased lysosomal acidity may also be causative of impaired autophagy (Shen & Mizushima 2014). Although many of these defects have been observed in models of neurodegenerative diseases, the cause of these molecular defects remains unclear. Additionally, it remains to be seen whether impaired autophagy is causative of neurodegeneration in these diseases, or merely symptomatic to other underlying pathological mechanisms.

6.2 Modeling ALS using dermal fibroblasts
Our investigations demonstrated that the expressions of key autophagy initiation proteins, phosphorylated ULK1 (Serine 555) and ATG13, were decreased in ALS patient fibroblasts. This was shown for patients harboring the repeat expansion in the C9ORF72 gene, and patients with no known genetic mutation. Other studies have also suggested that autophagic initiation may be compromised in ALS – a mutation in the CHMP2B gene may negatively affect ULK1 mRNA expression (Cox et al. 2010). However, this study did not examine ULK1 on the protein level. Another study used a G85R SOD1 transgenic model of ALS demonstrates decreased levels of p-ULK1 (Serine 757). mTOR inhibits autophagy by actively phosphorylating ULK1 at serine 757 (Kim et al. 2011). Thus, decreased p-ULK1 at serine 757 may suggest increased initiation of
autophagy. It is possible that mutant SOD1 animals differentially regulate autophagy from humans due to species differences, and/or mutations in SOD1 may differentially modulate autophagy, relative to other mutations. It has been reported that SOD1 mutations result in the development of pathology distinct from other mutations – TDP-43 aggregations are hallmark of ALS cases attributed to mutations and those with no known genetic defects. The only exception to this is in patients presenting with SOD1 mutations, who are characteristically devoid of TDP-43 pathology (Mackenzie et al. 2007). Further work additionally utilizing fibroblasts from patients with mutations in the SOD1 gene will allow us to elucidate whether this holds true.

Following autophagic initiation, activation of BECLIN-1 is required for phagophore formation. Our results indicated no differences in levels of protein associated with autophagosome formation – BECLIN-1, ATG3 and ATG5-ATG12 were all similarly expressed. Despite this, a trend towards decreased BECLIN-1 was observed (Figure 3.13). The use of a larger patient cohort would allow more precise identification of differences in protein expression. It has recently been shown that mutant SOD1 may interact with BECLIN-1, disrupting its interaction with Bcl-xl, thus impairing autophagosome formation, though this was not determined with wild type SOD1 (Nassif et al. 2014). Our results showed that patient cells express significantly higher levels of nuclear SOD1, as determined by immunocytochemistry staining intensity (Figure 3.3). This was observed in all patient samples, with and without known genetic mutations. It will be interesting to observe whether increased wild type SOD1 is a common pathological mechanism to ALS, and whether increased SOD1 may negatively regulate the function of BECLIN-1, and thus the formation of autophagosomes.

Although no difference in conjugated ATG5-ATG12 was observed, we demonstrated that patient fibroblasts express significantly lower levels of free ATG12 (Figure 3.18). ATG12 conjugates to ATG5, and subsequently ATG16L to expand the developing autophagosome (Mizushima et al. 2011). Despite no difference in conjugated ATG5-ATG12 observed, it is possible that decreased
availability of free ATG12 to form this complex may hinder the biogenesis of autophagosomes. We have further shown that free ATG12 is dramatically decreased in response to activation of autophagy by Rapamycin. Several studies have investigated the potential of activating autophagy to alleviate ALS (Cheng et al. 2015; Li et al. 2015). Whether decreased availability of ATG12 may hinder the activation of autophagy and the biogenesis of autophagosomes remain to be seen.

Recent identification of mutations in autophagy receptor proteins has helped to solidify a role of impaired autophagy in the pathogenesis of ALS. These include mutations in p62, Optineurin and Ubiquilin-2 (Majcher et al. 2015). Of these, p62 is the most widely studied in relation to ALS, with little known about the Optineurin and Ubiquilin-2. p62, a ubiquitin binding protein, contains an LC3 interacting region for the inclusion of ubiquitinated cargo into the developing autophagosome (Lippai & Low 2014). Upon fusion with the lysosome, p62 and its targeted cargo are degraded and recycled in the cell. Mutations in p62 result in cytoplasmic accumulation of the protein (Teyssou et al. 2013). Further, accumulation of p62 has been observed in patients without mutations in the gene itself (Troakes et al. 2012). From this it has been hypothesized that impaired autophagic flux be a key component of ALS. Analysis of p62 in patient and control fibroblasts demonstrated increased levels in patient cells by western blot analysis (Figure 3.14). To further investigate this we performed immunocytochemistry analysis and observed significantly increased numbers of p62 positive puncta in patient fibroblasts (Figure 3.15). These p62 puncta may represent ubiquitinated cargo targeted for degradation by autophagy, and increased numbers may suggest impaired turnover of this ubiquitinated cargo, thus impaired autophagic flux. This indicates that patient fibroblasts recapitulate the accumulation of p62 as observed in other models of ALS (Gal et al. 2007; Sasaki 2011). The underlying cause of increased p62 is unclear. It may be due to decreased autophagosome formation, thus limiting the levels of p62 which may be degraded. Conversely, decreased degradation of the autophagosomes may be the cause. This could potentially be due to impaired transport of
autophagosomes to lysosomes, or impaired fusion to the lysosomes. Mutations in proteins associated with endosomal trafficking have been reported, including C9ORF72 and Dynein (Ravikumar et al. 2005; Farg et al. 2014). Further, mutations in the CHMP2B gene may directly impair fusion of the autophagosome to the lysosome (Parkinson et al. 2006). No difference in total LAMP-1, a key lysosome protein, was observed by western blot analysis. However, we show an increased accumulation of LAMP-1 vesicles in patient cells (Figure 3.16). This may be due to decreased fusion of the autophagosomes to the lysosomes for degradation, thus allowing the accumulation of LAMP-1 positive vesicles. This would also explain the increased accumulation of p62.

We have identified impaired autophagic initiation (decreased pULK1, ATG13), possibly impaired autophagosome formation (decreased free ATG12), impaired autophagosome degradation (increased p62) and impaired fusion to lysosomes (increased LAMP-1) in ALS patient fibroblasts, suggesting gross alterations to autophagy in these cells. Autophagy plays a vital role in clearing cells of irreversibly oxidized biomolecules which may damage cellular systems via oxidative stress (Filomeni et al. 2014). As such, due to impaired autophagy, we hypothesized that this may render cells more susceptible to oxidative stress induced apoptosis. Indeed, increased oxidative stress has been widely implicated in the pathogenesis of ALS (Shaw et al. 1995; Li et al. 2012). We demonstrated that $H_2O_2$ treatment decreased expression of pro-autophagic protein BECLIN-1, while increasing expression of pro-apoptotic protein BAX (Figure 3.20). For a quantitative analysis of cell death we performed flow cytometry experiments for the expression of Annexin V and 7-AAD (Figure 3.21). These results demonstrated that ALS patient fibroblasts were significantly more sensitive to oxidative stress induced apoptosis. It has previously been demonstrated that embryonic stem cell derived neural stem cells from SOD1G93A mice are more susceptible to oxidative stress (Sui et al. 2014). A similar study demonstrated the same in male SOD1G93A rat pup derived neurospheres (Li et al. 2012). Our studies further corroborate these findings,
demonstrating fibroblasts again recapitulate findings previously reported in neural cells and tissues. Further, we show that this phenomenon may be applicable to more than SOD1-related ALS cases as our patients do not contain mutations within the SOD1 gene.

Activation of autophagy has been demonstrated to be beneficial for survival of ALS animal models (Cheng et al. 2015; Zhang et al. 2014). We further aimed to identify whether activation of the autophagy pathway may promote survival of fibroblasts under conditions of oxidative stress. Activation of autophagy with 200nM Rapamycin for 24 hours prior to H₂O₂ treatment has no discernible effect on survival. This may be due to several reasons. Perhaps 24 hours is insufficient time to completely activate autophagy. Moreover, we demonstrated that Rapamycin was insufficient to alter levels of p62, thus limiting its effect on autophagic flux (Figure 3.18). Sui et al demonstrated in their model that inhibition of AMPK signaling by treatment with compound C rescued the survival of neural stem cells in their model (Sui et al. 2014). Compound C is also known to activate autophagy by blocking mTOR signaling (Vucicevic et al. 2011), though whether increased survival in this case is due in any part to activated autophagy was not determined. Further studies utilizing other autophagy inducers may further shed light on the interplay between autophagy, oxidative stress and ALS.

Other studies have also used patient fibroblasts for rapid identification of potential biomarkers of ALS. Increased accumulation of TDP-43 in the cytoplasm of patient cells has been reported in patients with mutations in TDP-43 and C9ORF72 genes (Sabatelli et al. 2015). Our results did not demonstrate the same findings in our samples, though we only had two patients harboring a mutation in the C9ORF72 gene. Higher numbers of patients with specific mutations may be required to replicate these findings. Another study has demonstrated altered localization of SMN in patients harboring mutations in FUS and TDP-43 genes (Yamazaki et al. 2012). However, these findings do not extend to patients with no known mutations (Kariya et al. 2014). This indicates
that patient fibroblasts may be useful for the identification of pathogenic mechanisms associated with specific mutations. However, our sample size was too limited to identify differences specifically associated with the repeat expansion in the C9ORF72 gene (n=2), and our remaining samples have unknown mutations.

It has been demonstrated that the UPS, another key protein degradation mechanism, may be impaired in ALS patient fibroblasts (Yang et al. 2015). However, only one study to date has suggested ALS patient fibroblasts may demonstrate altered autophagy – immunohistochemical analysis of LC3 in sectioned dermal tissue indicates significantly increased LC3 expression (Wang et al 2015). The authors suggest this may be a useful biomarker for use ALS, though further experimentation on the autophagy pathway or underlying dysfunction was not examined.

Together these results have validated dermal fibroblasts as a useful tool for studying the pathogenic mechanisms associated with ALS and the autophagy pathway. These cells allow rapid identification of pathogenic mechanisms, relative to the use of animal models or other cellular models such as iPSCs. As such, patient fibroblasts may be a useful tool for further investigating disease mechanisms in vitro.

6.3 Modeling ALS using iPSCs
To study ALS in vitro using iPSCs, it will be crucial to generate pure populations of disease specific cells, the most widely studied being motor neurons and astrocytes. Various methods for the generation of astrocytes and motor neurons have been reported (Juopperi et al. 2012; Shaltouki et al. 2013; Du et al. 2015; Hu & Zhang 2009). For the generation of astrocytes we utilized the protocol by Du et al to first generate neural progenitor cells, followed by inducing glial differentiation with FBS. While alternative protocols yield astrocytes with typical star-like morphology, this protocol yields cells with more fibroblastic morphology.
Regardless, they still express GFAP, as determined by western blot and immunocytochemistry (Figures 4.14, 4.15). It is hypothesized that BMP proteins in serum are responsible for inducing glial cell differentiation (Hu et al. 2010). Moreover, the use of FBS to generate astrocytes from neural stem cells has been used to demonstrate the non-cell autonomous toxicity induced by patient ACM on motor neurons (Meyer et al. 2014). This protocol is also simpler, requiring only basic medium supplemented with FBS, and no other growth factors.

An increasing focus is now directed toward non-neuronal cells in the pathogenesis of ALS. Several studies have demonstrated a non-cell autonomous toxic effect of astrocytes, and astrocyte conditioned medium on the motor neurons (Ilieva et al. 2009; Marchetto et al. 2008; Meyer et al. 2014; Di Giorgio et al. 2007; Gallardo et al. 2014; Di Giorgio et al. 2008; Rojas et al. 2014). Despite this, the factors secreted by astrocytes which cause these toxic effects remain unidentified. Prostaglandin D2 was initially identified to be increased by patient astrocytes (Di Giorgio et al. 2007). However, inhibition of this offered only mild protection from cell death. More recently, a complex comprising α2- Na+ K+ ATPase/α-adducin was shown to be upregulated in mtSOD1 transgenic mice (Gallardo et al. 2014). Both pharmacological and genetic disruption of Na+ K+ ATPase were protective in response to mtSOD1 astrocyte conditioned medium. Na+ K+ ATPase has been shown to regulate autosis, a mechanism of cell death triggered by increased BECLIN-1 (a key autophagy protein), starvation and hypoxia-ischemia (Liu et al. 2013). It is possible that increased autosis plays a part in the pathogenesis of ALS, though current considerably more data is required to confirm this.

To corroborate previous findings we demonstrated that ALS patient derived astrocyte conditioned medium decreased the viability of motor neurons, relative to conditioned medium from control derived iPSCs (Figure 4.16). Our study did not determine whether this was due to increased cell death or decreased generation of motor neurons, we simply assessed numbers of viable motor
neurons. A similar methodology was employed by Meyer et al where motor neurons were cultured directly on patient and control astrocytes, and numbers of motor neurons used to determine a toxic effect (Meyer et al. 2014). Similarly, Rojas et al use the percentage of surviving SMI-32 (in the spinal cord expressed specifically in motor neurons) positive cells in random fields of spinal motor neurons treated with astrocyte conditioned medium (Rojas et al. 2014). While it is clear that ALS astrocytes induce toxicity on motor neurons, a more robust and reliable assay to measure this induced toxicity will help further advances in ALS research. Additionally, confirmation of induced apoptosis or other form of cell death will be essential, whether by measuring caspase activity or other proteins.

Our fibroblast model demonstrated gross impairments in the autophagy pathway. As such, we hypothesized that astrocyte conditioned medium may directly modulate the autophagy pathway, and this may be compromised in ALS patients. Autophagy and apoptosis are intrinsically linked pathways; autophagy will typically inhibit apoptosis while the activation of apoptosis associated caspases will inhibit the autophagy process (Mariño et al. 2014). This makes investigating autophagy in response to ALS ACM a convoluted process. Apoptosis induced by astrocyte conditioned medium will modulate the expression of autophagy related proteins, thus making unclear which changes are primarily due to ACM, and which are due to the induction of cell death. As this cell death mechanism is only observed in motor neurons, we cultured ACM on cells which do not undergo apoptosis in response to patient conditioned medium. For this we used HEK293T cells.

Little has been reported regarding astrocytes and autophagy. One study published by Perucho et al demonstrated that glial cell conditioned medium induced the autophagy pathway in a mouse model of Huntington’s disease (Perucho et al. 2013). We could not find any other studies suggesting similar results. Further, investigations into autophagy in iPSC and astrocyte models of ALS has not been reported.
Our initial data demonstrated that astrocytes from patients demonstrated greatly increased levels of mTOR. mTOR regulates a wide variety of cellular pathways including apoptosis, autophagy, proliferation, senescence, differentiation, survival and cell fate (Maiese 2015). However, the phosphorylation status of mTOR was not determined making it difficult to ascertain the significance of this in relation to ALS. Interestingly, increased mTOR activation, indicated by increased pS6 expression, has been observed in response to misfolded SOD1 in motor neurons (Saxena et al. 2013). This was not investigated further as the primary focus was to identify the effect of ACM from ALS patients and controls on the autophagy pathway.

We investigated the expressions of key autophagy proteins in HEK293T cells in response to ACM from patient and controls, by western blot analysis. We found that mTOR and phosphorylated ULK1 (Serine 555) were not modified by ACM, suggesting activation of the autophagy initiation complex is not affected. However, we found that BECLIN-1, associated with autophagosome formation was increased in response to both patient and control ACM (Figure 4.22). The same was observed for free ATG12, a protein required for elongation of the autophagosome. These data suggest that astrocytes may modulate the autophagy process by increasing the expression of pro-autophagy proteins. However, this is likely not mediated through mTOR, as no change in phosphorylated ULK1 (Serine 555) was observed.

We also identified changes in the expression of LC3B-I and LC3B-II. Each of these proteins is required for the selective degradation of cargo by binding p62, which selectively binds ubiquitinated proteins. However, this increase is only observed in response to control conditioned medium (Figure 4.23). Increased LC3B-II is also observed in cells which remained unfed for 5 days. As such, it is unclear whether this increase is due to starvation induced autophagy. If this is the case it would suggest that patient ACM directly prevents the conversion of LC3B-I to LC3B-II.
LC3B is required for the selective degradation of p62. Immunocytochemistry analysis further show increased accumulation of p62 puncta in response to patient ACM, relative to control ACM (Figure 4.24). This may be due to a combination of increased autophagosome formation (increased BECLIN-1/ATG12) and a lack of concomitant increase in LC3B-I/II expression required to degrade p62 and its cargo. Indeed, p62 is known to accumulate in both patient neurons and animal models (Al-Sarraj et al. 2011; Gal et al. 2007), and we have demonstrated the same in our patient fibroblast model. p62 enhances the formation of aggregates in animals expressing mtSOD1 (Gal et al. 2007), likely by linking mutated SOD1 to other cellular proteins. Whether increased p62 may affect aggregation formation in our cells, which do not contain mutations in the SOD1 gene is unclear.

p62 is required for the selective degradation of many proteins, including ALS related proteins SOD1 and TDP-43 (Brady et al. 2011; Li et al. 2015). We show that in addition to increased accumulation of p62 an increase in SOD1 expression is also observed in response to patient ACM. Though no change in TDP-43 is observed we only investigated expression of these proteins at one time point. It is possible that extended incubation with patient astrocyte conditioned medium would modify TDP-43. Accumulation of TDP-43 aggregates in response to astrocyte conditioned medium would be highly interesting as TDP-43 aggregates are observed in all ALS cases, except patients harboring mutations in the SOD1 gene (Mackenzie et al. 2007).

The accumulation of aggregated ALS-related proteins is hallmark of ALS, and is likely due to impaired autophagy/autophagic flux (Blokhuis et al. 2013). However, the exact underlying mechanism resulting in impaired autophagic flux is yet to be elucidated. It has been demonstrated that imbalances in autophagosome formation and degradation result in neurodegeneration. Our results indicate both patient and control ACM induces expression of BECLIN-1, a key protein involved in autophagosome formation. However, due to impaired LC3B in response to patient ACM, it is possible that an imbalance occurs
between formation and degradation of autophagosomes in cells treated with patient conditioned medium. This would also explain the increased accumulation of p62, and thus impaired autophagic flux. To corroborate this hypothesis it has been demonstrated that mutant SOD1 mice haploinsufficient for BECLIN-1 demonstrate increased lifespan (Nassif et al. 2014). This may indicate that these mice demonstrate lower basal autophagosome formation, and thus the effect of astrocyte mediated induction of autophagosome formation does not result in as extreme an imbalance in autophagosome formation/degradation. Indeed, impaired autophagic flux has been reported in animal models of ALS (Zhang et al. 2014). As such, autophagic flux may be a key therapeutic target in the development of new therapeutics for ALS.

Activation of autophagy, by Rapamycin and Trehalose treatment, have been demonstrated to be beneficial to the survival of ALS animal models (Cheng et al. 2015; Staats et al. 2013; Lattante et al. 2014; Li et al. 2015; Zhang et al. 2014). Both of these compounds are known to increase the expression of LC3B, a protein we hypothesize is inhibited by patient ACM (Li et al. 2015; Cherra et al. 2010). For this reason we aimed to determine whether Rapamycin or Trehelose may decrease the accumulation of p62 puncta in cells treated with ACM from patients and controls. Rapamycin had no effect on cells treated with control ACM, with similar levels of puncta observed in cells treated with ACM only. Conversely, Rapamycin decreased the accumulation of p62 puncta in cells treated with patient ACM, returning to levels similarly observed in cells treated with control ACM. This may be due to increased expression of autophagy components (including LC3B), thereby increasing autophagic flux and the turnover of p62 puncta. On the other hand, Trehalose was shown to significantly increase the accumulation of p62 puncta in cells treated with control ACM, with no change observed in cells treated with patient ACM. The reason for this is unclear. However, the mechanism by which Trehalose acts to induce autophagy remains unclear, making this finding more difficult to explain. Further, it will be crucial to investigate whether this decrease in p62 puncta, observed in response
to Rapamycin, may prevent the accumulation of SOD1 observed by treatment with patient ACM.

Together these data suggest that impairments in the autophagy pathway, notably regarding autophagic flux, may be induced by patient ACM in a non-cell autonomous manner. To date is unclear why motor neurons are susceptible to astrocyte mediated toxicity where other neuronal subtypes are spared.

While the generation of astrocytes from iPSCs readily yielded pure populations of GFAP positive astrocytes, the generation of motor neurons was not as easily achieved. Following the protocol by Du et al. we generated motor neuron populations comprising very low numbers of MNX1 positive cells. The protocol suggests that greater than 90% of cells should be MNX1 positive (Du et al. 2015). As such further optimization of this protocol in our laboratory will be required to reliably identify motor neuron associated phenotypes in ALS patient iPSC-derived cells.

Several studies have reported the generation of motor neurons from ALS patients and identification of pathological mechanisms therein (Alves et al. 2015; Zhang et al. 2013; Kiskinis et al. 2014; Wainger et al. 2014). However, these studies utilize older protocols which result in lower purity of motor neurons, relative to the recent protocol established by Du et al. As such, the generation of glial cells mixed with motor neuron cells is almost certain. For this reason it is difficult to determine whether patient derived motor neurons contain intrinsic defects, or whether perturbations are induced by glial cells producing toxic effects. The generation of iPSCs which express mutant proteins (such as SOD1, TDP-43, FUS and C9ORF72) specifically in motor neuron lineages would circumvent this issue.
6.4 Induced Neural Stem Cells

In efforts to improve cellular reprogramming methodologies many recent reports have focused on the direct conversion of somatic cells (such as fibroblasts) to cells of interest. These include the direct induction of neural stem cells, neurons, astrocytes and oligodendrocytes (Kalani & Martirosyan 2012; Lee et al. 2015; Najm et al. 2013). Our aim was to generate neural stem cells with the potential to generate neurons, astrocytes and oligodendrocytes for disease modeling purposes.

The various methods used to generate iNSCs are shown in Table 1.2. Several protocols rely on the transduction of cells with the canonical reprogramming factors (OCT4, SOX2, KLF4 and C-MYC) to initiate reprogramming towards a pluripotent state, and prematurely induce differentiation towards a neural stem cell fate. These methods however require the passage of cells through a transient pluripotent state (Maza et al. 2015). As such, the use of these cells may still retain the risk of teratoma formation by residual pluripotent cells in the mixed population.

Alternative strategies use lineage specific transcription factors for the direct induction of neural stem cells. Of these methods, a combination of Sox2, Brn2 and FoxG1 was one of the first methods to successfully demonstrate direct conversion to neural stem cells with tri-lineage differentiation potential (Lujan et al. 2012). As such, we based our experimental protocols on this method. We aimed to investigate whether overexpression of IGF2 or RARβΔ384 would improve reprogramming efficiency, be capable of replacing transcription factors in the reprogramming protocol, alter the regional identity of iNSCs or modulate their differentiation potential. IGF2 is a growth factor identified to be highly expressed in neural stem cells derived from the hippocampus (Bracko et al. 2012). It is known to play a role in memory enhancement and consolidation (Alberini & Chen 2012; Chen et al. 2011). Further, low oxygen tension has been shown to mediate the expression of IGF2 to maintain stemness of mesenchymal stem cells (Youssef & Han 2016). RARβ is known to mediate neuronal
differentiation (S et al. 2015). We hypothesized that inhibition of RARB by overexpression of a truncated dominant negative isoform would hinder neuronal differentiation, thus aiding to maintain stemness of reprogrammed cells.

Our results demonstrated that a combination of eight transcription factors (SOX2, BRN2, KLF4, FOXG1, C-MYC, IGF2, RARB384 and LIN28) may generate iPSCs, based on the formation of ESC-like colonies (Figure 5.6). OCT4 is often considered the master regulator of pluripotency, with most reprogramming strategies utilizing it during reprogramming methods (Jerabek et al. 2014). It was initially believed that Oct4 was indispensable for reprogramming (JC et al. 2010). Our study indicates that OCT4 may be replaced by other transcription factors. However, the formation of ESC-like colonies with eight transcription factors was dramatically lower relative to transduction with OCT4, SOX2, KLF4 and C-MYC. Improvements in reprogramming technology will avoid the use of transcription factors which may act as oncogenes, and be replaced by other factors such as growth factors. As such, it will be interesting to see if this replacement of OCT4 was mediated by IGF2 and/or RARBΔ384. However, this was not studied further as the primary aim of this project was to establish iNSCs.

The generation of iNSCs typically involvessubjecting reprogramming cells to suspension culture in which they should form neurospheres. Many of our reprogramming groups formed spheres in suspension. However, it was difficult to ascertain differences between bona fide neurospheres and clusters of cells which had aggregated together (Figure 5.9). By day 30 spheres in culture for all groups were quantified. We found that cells transduced with SOX2, BRN2 and FOXG1 (+/-RARBΔ384) readily yielded high numbers of spheres. RARBΔ384 had no effect on the number of spheres formed. Interestingly, IGF2 decreased the number of spheres formed when added in addition to SOX2, BRN2 and FOXG1. The mechanism by which reprogramming efficiency was reduced is
unclear. All other populations yielded very few spheres or sphere-like cell clusters.

As it was difficult to determine whether cells had formed bona fide neurospheres, or simply clusters of aggregating cells, we subjected spheres at day 30 to monolayer culture to proliferate and expand populations of NSCs (Figure 5.11). Of the eighteen combinations tested, seven yielded proliferative populations. This included cells transduced with GFP alone. It was quite unlikely that overexpression of GFP was sufficient to reprogram cells to a stem cell fate. Further, these cells maintained an elongated morphology, typical of fibroblasts (Figure 5.11 A), whereas other cell populations were smaller and rounder (Figure 5.11 C).

To determine whether these populations contained bona fide NSCs they were subjected to tri-lineage differentiation. Only cells transduced with SOX2, BRN2 and FOXG1 (+/- IGF2 or RARBD384) generated populations capable of generating neurons, astrocytes and oligodendrocytes as indicated by immunocytochemistry (Figures 5.17, 5.19 and 5.21). This demonstrates that neither IGF2 nor RARBD384 are sufficient to replace FOXG1 in the reprogramming process.

Lujan et al demonstrated that SOX2, BRN2 and FOXG1 were sufficient to generate iNSCs with tri-lineage differentiation potential. However, the generation of specific neuronal subtypes was not investigated (Lujan et al. 2012). We demonstrate that cells reprogrammed with these factors, with or without IGF2 or RARBD384 may generate both GABAergic and Glutamatergic neurons, indicated by the expression of GABA and Vglut (Figures 5.22 and 5.24). Further, we attempted to differentiate these cells towards dopaminergic neurons. No cells expressing Tyrosine Hydroxylase (a marker of dopaminergic neurons) were observed. However, a suitable positive control was not available. As such, these experiments will need to be repeated to verify this finding. Other reports have demonstrated the generation of Tyrosine Hydroxylase positive neurons from iNSCs (Tian et al. 2013; Kim et al. 2011; Sheng et al. 2012). It is possible that
the use of alternative reprogramming factors yields the generation of regionally distinct neural stem cell populations with unique differentiation potentials.

Interestingly, we found that cells overexpressing RARBΔ384 were capable of generating significantly more GABA positive neurons, relative to cells expressing SOX2, BRN2 and FOXG1 (+/-IGF2) (Figure 5.22). This was surprising as we hypothesized that inhibiting RAR signaling by overexpression of RARBΔ384 would hinder neuronal differentiation. It is unclear why this increase was observed. Future experiments to investigate expression of RARBΔ384 by RT-PCR will be required to begin to elucidate this finding. Additionally, it should be noted that despite generating more GABA positive neurons, the yields of these neurons was very low, reaching only ~2%. As such, more optimum differentiation protocols will be required if these cells are to be used in disease modeling. Notably, many of the published methods to date do not quantify the percentages of cell types obtained. For this reason we cannot compare our results to other publications in this respect.

We also examined the levels of neurons surviving at five weeks in culture. We found that cells overexpressing SOX2, BRN2 and FOXG1 only did not survive this long. However, cells additionally expressing either IGF2 or RARBΔ384 did show some survival at this time point, suggesting each of these factors may promote the survival of neurons. However, at this time point there were significantly fewer viable neurons, relative to three weeks in culture.

Some reports demonstrate the generation of many neuronal subtypes from iNSC populations. For example, Lee et al demonstrate the generation of neurons positive for TUJ1 (pan neuronal), Tyrosine Hydroxylase (dopaminergic neurons), Isl-1 (motor neurons), BRN3A (sensory neurons), Glutamate (Glutamatergic neurons), GABA (GABAergic neurons) and Nurr1 (dopaminergic neurons) (Lee et al. 2015). These cells may be less specified, representing an early stage NSC population. However, the regional identity/specification of these cells was not reported. Indeed, most reports to not determine the regional identity of reprogrammed neural stem cells. We aimed to identify NSC
populations by RT-PCR analysis. We first showed that reprogrammed cells express markers typical of NSCs including Sox2, Pax6 and Nestin (Figure 5.12 A-C). However, the expression of these markers were variable and slight increases in expression were observed in cells transduced with GFP alone. Of the cells later identified as bona fide NSCs with tri-lineage potential, the decreased expression of Col1a1 and the increased expression of Sox9 were the most reliable indicators of iNSC phenotype (Figure 5.12 D/E). These may be used in future studies for the more rapid elimination of non-iNSC groups.

We further demonstrated the expression of region-specific markers and found that our bona fide iNSCs express high levels of hindbrain markers Nkx6.1 and Hoxb4, as well as midbrain marker Lmx1a. However, we could not detect the expression of Foxa2, a typical midbrain marker. A previous study has demonstrated that Lmx1a may also be expressed in the hindbrain, as well as the midbrain (Mishima et al. 2009). As such, we suggest that cells reprogrammed with SOX2, BRN2 and FOXG1 possess a hindbrain identity. These cells may be useful for disease modeling, but improvements in methodologies to generate pure populations of specific neuronal subtypes will be required. However, these cells readily generate astrocytes. As astrocytes are highly implicated in the pathogenesis of ALS, direct conversion methods may more rapidly be used to study these mechanisms in vitro, relative to the use of iPSCs. The generation of iNSCs which can readily generate relatively pure populations of motor neurons will greatly advance neurodegenerative research in ALS.

6.5 Cellular Models of ALS.
In this thesis we have developed and validated cell models with the potential to study disease mechanisms in vitro. Dermal fibroblasts are a readily obtainable cell source, which are easily cultured, with low cost. Using this model we have demonstrated clear differences in the autophagy pathway, in a relatively short space of time. These cells may be ideal for the rapid identification of drug targets and impaired molecular mechanisms in a variety of diseases. However,
these cells have limited capability for self renewal. Further, they are unable to recapitulate disease mechanisms which may be associated specifically in neural cells. For example, while conditioned medium from patient astrocytes demonstrates toxicity towards motor neuron cells, this is not observed with conditioned medium from fibroblasts. As such, it will not be possible to model all aspects of disease pathology in this cell type.

The development of iPSC technology has allowed the emergence of an entirely new field of research. These cells, capable of unlimited self renewal, are capable of generating any cell type, holding promise in the fields of disease modeling, drug screening, and possibly therapeutic applications. They are highly advantageous for the study of neuronal diseases, where access to primary patient tissue is extremely difficult, often only obtainable post mortem. However, the culture of iPSCs is significantly more laborious than that of fibroblasts. Further, the generation, characterization, maintenance and differentiation of iPSCs is lengthy and highly costly. It has been estimated that to generate iPSCs from 3 patients and 3 controls, a cost of approximately $10,000 is incurred, prior to any characterization or differentiation experiments (Beers et al. 2015). Furthermore, variability between clones may affect differentiation potential. Additionally, the differentiation protocols required are often time consuming, with efficiencies of cell generation highly variable.

More recent advances in cellular reprogramming have developed methodologies to generate cells of interest from somatic cell sources, bypassing the need to generate iPSCs. These include the generation of induced neural stem cells (iNSCs) and induced neurons (iNs). While iNs may be a rapid means to generate patient specific neuronal cells, the efficiencies reported are low and these cells are incapable of proliferation, thus limiting their use (Kalani & Martirosyan 2012). Conversely, the generation of iNSCs yields a stem cell population which may be expanded for many passages while retaining the potential to generate oligodendrocytes, astrocytes and neurons. Using this technology, the successful generation of ALS patient specific astrocytes has
been reported (Meyer et al. 2014). These astrocytes demonstrate non-cell autonomous toxicity towards motor neurons (Mitchell et al. 2014). However, to date, these cells have not been used to generate motor neuron cells. More advances will be required to fully apply these methodologies to disease modeling in the setting of ALS.

6.6 Conclusions and Future Perspectives

Autophagy may be deregulated in various models of ALS. Herein we have confirmed that patient fibroblasts as well as patient derived iPSCs may be useful for investigating disease mechanisms in the pathogenesis of ALS. We focused on analyzing the autophagy pathway and demonstrated that autophagic flux may be a prime therapeutic target for future studies. Future work may focus on establishing these same methodologies using larger cohorts of patients and age-matched controls to further clarify disease mechanisms. Ideally, patients with various genetic mutations associated with the development of ALS could be recruited, as well as sporadic ALS.

Furthermore it will be interesting to develop these methodologies in neuronal cell lines, including SH-SY5Y cells and the motor neuron-like cell line NSC-34. Establishing cell lines harbouring reporter constructs for autophagy proteins such as LC3B and p62 may also be advantageous. These may offer more relevant disease models instead of HEK293T cells and will be highly useful prior to the generation of motor neurons from iPSCs.
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Appendices

Appendix A: Details of plasmids obtained from Addgene.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Addgene Plasmid #</th>
<th>Encodes</th>
<th>Type of Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMXs-hOCT 3/4</td>
<td>17217</td>
<td>OCT4</td>
<td>Retroviral</td>
</tr>
<tr>
<td>pMXs-hSOX2</td>
<td>17218</td>
<td>SOX2</td>
<td>Retroviral</td>
</tr>
<tr>
<td>pMXs-hKLF4</td>
<td>17219</td>
<td>KLF4</td>
<td>Retroviral</td>
</tr>
<tr>
<td>pMXs-hc-ymc</td>
<td>17220</td>
<td>C-MYC</td>
<td>Retroviral</td>
</tr>
<tr>
<td>pUMVC</td>
<td>8449</td>
<td>Packaging plasmid</td>
<td>Retroviral Packaging</td>
</tr>
<tr>
<td>pCMV-VSVG</td>
<td>8454</td>
<td>Envelope protein</td>
<td>Retroviral Packaging</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>12259</td>
<td>Envelope protein</td>
<td>Lentiviral Packaging</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>12253</td>
<td>REV</td>
<td>Lentiviral</td>
</tr>
<tr>
<td>psPAX2</td>
<td>12260</td>
<td>Packaging plasmid</td>
<td>Lentiviral Packaging</td>
</tr>
<tr>
<td>pWPT-GFP</td>
<td>12255</td>
<td>Green Fluorescent Protein (GFP)</td>
<td>Lentiviral</td>
</tr>
<tr>
<td>pEB-C5</td>
<td>28213</td>
<td>LIN28, OCT4, SOX2, KLF4, C-MYC</td>
<td>Non-Viral</td>
</tr>
<tr>
<td>IRES-GFP-FOXG1</td>
<td>14935</td>
<td>FOXG1</td>
<td>Non-viral</td>
</tr>
</tbody>
</table>
Table 2.1. Plasmids obtained from Addgene.

Appendix B: Western blot buffer recipes and gel preparations.

12% Acrylamide gels were made in 1.5mm thick plates. Recipes for resolving (lower) and stacking (upper) gels as follows:

**Resolving Gel Recipe (Per gel)**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H2O</td>
<td>2.94 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>3.6 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>2.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>90 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>90 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3.6 µl</td>
</tr>
</tbody>
</table>

**Stacking Gel Recipe (Per gel)**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H2O</td>
<td>2.55 ml</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>622.5 µl</td>
</tr>
<tr>
<td>1.0 M Tris-HCl (pH 6.8)</td>
<td>472.5 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>37.5 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>37.5 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3.75 µl</td>
</tr>
</tbody>
</table>

30% Acrylamide (Sigma, Cat# A3699). Tris-HCl (Sigma, Cat# T5941). SDS (Sigma, Cat# L3771). Ammonium persulphate (APS), (Sigma, Cat# A3678).
Protein Loading Buffer: (250 mM TrisHCl pH6.8, 10% SDS, 30% Glycerol (Sigma, Cat# G5516), 5% β-mercaptoethanol (Sigma, Cat# M3148), 0.02% bromophenol blue (Sigma, Cat# B0126).

10X Running buffer (For 1L): 30.28g Tris Base (Sigma, Cat# T1503), 144.2g Glycine (Sigma, Cat# G8898), 10g SDS.

1X Running Buffer: 100mls 10X Running buffer, 900mls distilled H₂O.

10X Transfer Buffer: (For 1L): 30.28g Tris Base, 144.2g Glycine.

1X Transfer Buffer: 100mls 10X Transfer buffer, 200mls Methanol (Sigma, Cat# 651133), 700mls distilled H₂O.

10X TBS (For 1L): 1.94g Tris Base, 13.22 Tris-HCl, 87.66g NaCl (Sigma, Cat# S9888).

1X TBS-T: 100mls 10X TBS, 1ml Tween-20 (Sigma, Cat# P1379), 899mls distilled H₂O.