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INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Michelle Antoinette Ricketts
PhD – The University of Edinburgh – 2015
2.2.3 DNA Sequencing ................................................................. 53
2.2.4 Statistical Analysis ............................................................... 54
2.2.5 Bioinformatic Analysis ........................................................... 54
2.3 Results ...................................................................................... 56
2.4 Discussion ............................................................................... 61

Chapter 3: Expression of GNL3 in Joint Tissue ........................................ 68
3.1 Introduction ............................................................................. 68
3.2 Materials and Methods .............................................................. 70
  3.2.1 Human Joint Samples ............................................................ 70
  3.2.2 Non-Human Joint Samples ..................................................... 72
  3.2.3 Cell Line Culture ................................................................. 72
  3.2.4 Passaging Cells .................................................................. 73
  3.2.5 Freezing Cells .................................................................. 74
  3.2.6 Histology .......................................................................... 74
  3.2.7 DNA Extraction ................................................................. 75
  3.2.8 Total RNA Extraction .......................................................... 77
  3.2.9 Reverse Transcription .......................................................... 79
  3.2.10 cDNA Amplification ............................................................. 79
  3.2.11 Quantitative PCR (qPCR) ..................................................... 81
  3.2.12 Total Protein Extraction ....................................................... 83
  3.2.13 Western Blot ..................................................................... 84
  3.2.14 Cytokine Treatment of Chondrocyte Cultures ....................... 86
3.3 Results ...................................................................................... 89
  3.3.1 GNL3 Immunohistochemistry ................................................. 89
  3.3.2 DNA Genotyping at rs11177 and rs2289247 in Primary Human Articular Chondrocytes ......................... 89
  3.3.3 mRNA Expression of GNL3 in Human Joint Tissues and Cell Lines ... 89
  3.3.4 Expression of GNL3 in Primary Human Articular Chondrocytes ........ 90
  3.3.5 Protein Expression of GNL3 in Primary Cultured Human Articular Chondrocytes .................................. 95
  3.3.6 Expression of GNL3 in Cytokine- and Growth Factor-Treated Chondrocyte Cultures ........................................ 101
3.4 Discussion ............................................................................... 108

Chapter 4: Functional Analysis of GNL3 ................................................... 118
4.1 Introduction .......................................................................................................................... 118
4.2 Materials and Methods ......................................................................................................... 125
  4.2.1 Knockdown of GNL3 using siRNA in Cultured Human Articular Chondrocytes and JJ012 Chondrosarcoma Cells ............................................................. 125
  4.2.2 Investigating the Effect of rs11177 Variants on the Chondrogenic Potential of Human Articular Chondrocytes and Mesenchymal Stem Cells ...................................... 127
  4.2.3 Gateway® Cloning .......................................................................................................... 130
4.3 Results .................................................................................................................................. 150
  4.3.1 siRNA GNL3 Knockdown ................................................................................................. 150
  4.3.2 Genotyping Cells Cultured in Chondrogenic Media ......................................................... 153
  4.3.3 Association between rs11177 variants and mRNA Expression of Chondrogenic Markers in Human Articular Chondrocyte and Mesenchymal Stem Cell Cultures .................. 154
  4.3.4 Confirming Site Directed Mutagenesis in Expression Clones ........................................... 159
  4.3.5 Confirming mRNA Expression of GNL3 in Transfected A375 Cells ............................... 159
  4.3.6 Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to Cisplatin Treatment ............................................................................................................. 160
  4.3.7 Influence of Hypoxia on A375 Cell Survival ..................................................................... 164
  4.3.8 Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to Hypoxia . 164
4.4 Discussion ............................................................................................................................ 175

Chapter 5: Role of gnl3 in Zebrafish Cartilage Development ................................................. 188
5.1 Introduction .......................................................................................................................... 188
5.2 Materials and Methods ......................................................................................................... 194
  5.2.1 Zebrafish ........................................................................................................................ 194
  5.2.2 Histology ........................................................................................................................ 195
5.3 Results .................................................................................................................................. 197
  5.3.1 The Effect of Nonsense Mutation ns$^{hu3259}_3$ on Cartilage in Zebrafish ....................... 197
5.4 Discussion ............................................................................................................................ 201

Chapter 6: Discussion ............................................................................................................... 204

References .................................................................................................................................. 210

Chapter 7: Appendix .................................................................................................................. 252
7.1 Supplementary Tables .......................................................................................................... 252
7.2 Solutions ............................................................................................................................... 262
  7.2.1 Orange-G Loading Dye .................................................................................................... 262
<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2.2</td>
<td>DNA Wash Solution</td>
<td>262</td>
</tr>
<tr>
<td>7.2.3</td>
<td>NaOH</td>
<td>262</td>
</tr>
<tr>
<td>7.2.4</td>
<td>Hepes</td>
<td>262</td>
</tr>
<tr>
<td>7.2.5</td>
<td>EDTA</td>
<td>262</td>
</tr>
<tr>
<td>7.2.6</td>
<td>Loading Buffer</td>
<td>262</td>
</tr>
<tr>
<td>7.2.7</td>
<td>Transfer Buffer</td>
<td>262</td>
</tr>
<tr>
<td>7.2.8</td>
<td>TBS</td>
<td>263</td>
</tr>
<tr>
<td>7.2.9</td>
<td>Stripping Buffer</td>
<td>263</td>
</tr>
<tr>
<td>7.2.10</td>
<td>StemPro® Chondrogenic Media</td>
<td>263</td>
</tr>
<tr>
<td>7.2.11</td>
<td>RIPA Lysis Buffer</td>
<td>263</td>
</tr>
</tbody>
</table>
Table of Figures

Figure 1.1: Diagrammatic representation of a diarthrodial synovial joint, the human knee ................................................................. 3

Figure 1.2: The zonal architecture of articular cartilage (adapted from Jackson et al. 2001 (review); Poole 2011 (review)) Grey box inset showing the chondrocyte microenvironment, namely the pericellular (dark grey), territorial (medium grey), inter-territorial (light grey) matrices. Please refer to text for details (Section 1.2.2.3). ........................................................................................................ 8

Figure 1.3: OARSI cartilage histopathology grading and staging Sections are stained with safranin-O and a light green counterstain. Staging % refers to the percentage of the length, volume or area of cartilage involved. ECM, Extracellular matrix; HAC, Human articular chondrocyte (Pritzker et al. 2006). ............................................................................................................................ 10

Figure 1.4: Disease variant distribution (adapted from Koeleman et al. 2013 (review)). ...................................................................................................................... 33

Figure 1.5: Regional association plot for locus 3p21.1 showing the statistically significant rs6976 associated with TJR Chromosome 3 positions (mega base pairs (Mb) (NCBI build 36) are plotted against discovery set case-control association values (-log_{10}(p value)) for each SNP. $r^2$ values are the estimated correlation coefficients for each SNP in relation to rs6976 in the CEU population (International HapMap Project 2005). Red line, recombination rates (centimorgan (cM)/Mb); Purple diamond, p value for the discovery set; Purple square, p value for combined discovery and replication meta-analysis (Zeggini et al. 2012). .................................................................................................................. 40

Figure 1.6: Schematic representation of nucleostemin domains and protein binding sites N, N-terminus; G4 and G1, GTP-binding sites (G5, G2 and G3 not shown); C, C-terminus. Red bar, rs11177. The intermediate domain lies between the GTP-binding sites and the acidic (adapted from Ma & Pederson 2008b (review)). ...................................................................................................................... 41

Figure 2.1: Mutations identified in GNL3 mutation screen (Table 2.2) Black bar indicates amino acid codon. All DNA sequences are 5’ to 3’ orientation. Chromatograms from Chromas LITE v2.1.1 (Technelysium 2012); Green, Adenine; Blue, Cytosine; Black, Guanine; Red, Thymine. ................................................................. 56

Figure 2.2: Transcription factor binding sites upstream of GNL3 at c.-134G>A (bold) Chromosome location refers to H. sapiens (hg19) reference sequence. DNA sequence is in 5’ to 3’ orientation. Image adapted from SwissRegulon (Swiss Institute of Bioinformatics 2013). ...................................................................................................................... 58

Figure 2.3: Consensus binding motifs for AHR/ARNT/ARNT2 and ZFP161 transcription factors (Swiss Institute of Bioinformatics 2013). Black arrows
indicate the novel polymorphic site (c.-134G>A) identified in the mutation screen. ................................................................. 58

Figure 2.4: Linkage disequilibrium plot ($r^2$) of GNL3 variants (Table 2.2 and Figure 2.1) and rs6976 (GLT8D1) at 3p21.1 (Barrett et al. 2005). .......................... 60

Figure 3.1: Dot-positive nuclear staining for GNL3 IHC in HACs (a) Primary cultured HAC cytospin from OA cartilage (bar = 10µ, inset bar = 5µ); (b) HAC in a frozen cartilage section (bar = 2.5µ); (c) Primary non-cultured HAC cytospin from OA cartilage (bar = 10µ); (d) Negative control for primary cultured HAC cytospin from OA cartilage (bar = 20µ). Brown, Nucleostemin; Blue, Nucleus. ........................................ 89

Figure 3.2: mRNA expression of GNL3 (1587bp) and GAPDH (451bp) in human joint tissues and cell lines cDNA PCR products were run on a 1.5% agarose gel at 75V for 45min in TBE buffer (1X). No bands were seen in the no template control (not shown). L1, 1kb DNA ladder (New England BioLabs®); L2, Low molecular weight DNA Ladder (New England BioLabs®); 1-2, Osteoblast-like cells from OA bone; 3-6, Primary cultured OA HACs; 7, Primary cultured normal HACs; 8-9, Synovial cells; 10, C20A4 chondrocytes; 11, U2OS osteosarcoma cells; 12, JJ012 chondrosarcoma cells................................. 90

Figure 3.3: mRNA expression of GNL3 (208bp) and β-Actin (250bp) in primary non-cultured HACs taken from normal femoral head and OA knee cartilage cDNA PCR products were run on a 2% agarose gel at 75V for 45min - 1hr in TBE buffer (1X). No bands were seen in the no template control. L, Low molecular weight DNA Ladder (New England BioLabs®); 1, No template control for GNL3; 2, No template control for β-Actin. ................................................. 91

Figure 3.4: qPCR results for mRNA expression of GNL3 in primary cultured HACs taken from normal and OA femoral head cartilage Values are average GNL3/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM........ 92

Figure 3.5: qPCR results for mRNA expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs11177 GG, GA and AA refer to rs11177 genotypes. Values are average GNL3/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM. .................................................. 93

Figure 3.6: qPCR results for mRNA expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs2289247 GG, GA and AA refer to rs2289247 genotypes. Values are average GNL3/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM................................. 94
Figure 3.7: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression in primary cultured HACs taken from normal and OA femoral head cartilage L, Magic Mark™ XP (Life Technologies™). ................................................................. 96

Figure 3.8: Western blot results for protein expression of GNL3 in primary HACs taken from normal and OA femoral head cartilage Values are average GNL3/β-Actin. All samples were run in triplicate. Error bars are ± SEM ......................... 97

Figure 3.9: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression in primary HACs taken from femoral head cartilage of different genotypes at rs11177 GG, GA and AA refer to rs11177 genotypes. All samples were run on the same western blot. Samples of unknown genotype are omitted from the picture. L, Magic Mark™ XP (Life Technologies™). .................................................... 98

Figure 3.10: Western blot results for protein expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs11177 GG, GA and AA refer to rs11177 genotypes. Values are average GNL3/β-Actin. All samples were run in triplicate. Error bars are ± SEM. .................................................. 99

Figure 3.11: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression in primary HACs taken from femoral head cartilage of different genotypes at rs2289247 GG, GA and AA refer to rs2289247 genotypes. The first three samples were run on the same western blot and the last five samples on different blot. Samples of unknown genotype are omitted from the picture. L, Magic Mark™ XP (Life Technologies™). ........................................ 100

Figure 3.12: Western blot results for protein expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs2289247 GG, GA and AA refer to rs2289247 genotypes. Values are average GNL3/β-Actin. All samples were run in triplicate. Error bars are ± SEM. ...................................... 101

Figure 3.13: mRNA expression of GNL3 and GAPDH in murine (A), canine (B) and bovine (C) articular cartilage cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X). L, 100bp DNA ladder (New England BioLabs®). (A) Murine: 1-2, CBA 26 weeks; 3-4, CBA 40 weeks; 5-6, Str/ort 26 weeks; 7-8, Str/ort 40 weeks; 9, Cb2+/− untreated; 10, Cb2+/− TNFα-treated; 11, Cb2+/− IL1β- treated; 12, Cb2−/− untreated; 13, Cb2−/− TNFα-treated; 14, Cb2−/− IL1β-treated; 15, No template control. (B) Canine: 1-6, Cartilage treated with IL1β; 7-12, Untreated controls; 13, No template control. (C) Bovine: 1, Untreated control; 2, IL13-treated; 3, IL1β-treated, 4, No template control. .. 102

Figure 3.14: qPCR results for mRNA expression of GNL3 in control, FGF2- and IL1β-treated primary cultured HACs Values are average GNL3/18S percentage calculated using molecules/µl values. Each experiment was carried out in duplicate. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM. ................................................................. 104
Figure 3.15: qPCR results for mRNA expression of GNL3 in control, FGF2- and IL1β-treated chondrocyte cells lines, C20A4 (A) and C28I2 (B) Values are average GNL3/18S percentage calculated using molecules/µl values. Each experiment was carried out in duplicate. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM. ........................................... 104

Figure 3.16: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression GNL3 in control, FGF2- and IL1β-treated chondrocyte cells lines, C20A4 and C28I2 L, Magic Mark™ XP (Life Technologies™). .................. 106

Figure 3.17: Western blot results for protein expression of GNL3 in control FGF2- and IL1β-treated chondrocyte cells lines, C20A4 (A) and C28I2 (B) Values are average GNL3/β-Actin. Each experiment was carried out in duplicate. .......... 107

Figure 4.1: Cisplatin structure (Barabas et al. 2008 (review)).................................. 123

Figure 4.3: BP reaction results in recombination between the attB-GNL3 PCR product and the attP sites of the donor vector to create an entry clone containing attL sites and a by-product containing attR sites (Invitrogen™ 2010a)......... 131

Figure 4.4: LR reaction results in recombination between attL sites of the entry clone and attR sites of the destination vector to create an expression clone containing attB sites and a by-product containing attP sites (Invitrogen™ 2010a)......... 131

Figure 4.5: Primer design for the attachment of attB sites Shine-Dalgarno and Kozak sequences allow the native protein to be expressed in both mammalian and E.coli cells. The GNL3 stop site was excluded from the reverse primer sequences. ATG, is the GNL3 start site; ORF, Open Reading Frame (adapted from (Invitrogen™ 2010a; GibcoBRL® 2012). ..................................................... 134

Figure 4.6: pDONR™221 donor vector (Invitrogen™) map (4761bp) T2, rrmB T2 transcription termination sequence (c) (268-295bp) and T1, rrmB T1 transcription termination sequence (c) (427-470bp), prevent the expression of the cloned gene by vector-encoded promoters; M13, sites for the attachment of forward (537-552bp) and reverse (3026-3042bp) sequencing primers; attP1 (570-801bp) and attP2 (c) (2753-2984bp), allow for recombination with attB-GNL3; ccdB gene (c) (1197-1502bp), allows for negative selection of the plasmid; CmR, chloramphenicol resistance gene (c) (1825-2505bp); Kanamycin, kanamycin resistance gene (3155-3964bp); pUC origin (4065-4758bp), allows for replication and maintenance in E.coli; (c), complementary strand (Invitrogen™ 2012). .............................................................. 136

Figure 4.8: Methylated Dpn1 recognition site (adapted from (New England BioLabs® 2014)). .......................................................... 141

Figure 4.9: Overview of the Flp-In™ system (Invitrogen™) Recombination takes places between the FRT sites of the A375 cells and the GNL3 expression vector (Figure 4.7) in the presence of Flp-recombinase which is expressed from
pOG44. This results in GNL3 protein expression in hygromycin-resistant A375 cells (Invitrogen™ 2002).

Figure 4.10: FRT site The minimal FRT site consists of two imperfect inverted repeats, both 13bp in length (black arrows), situated either side of an 8bp spacer region which also contains a Xba I restriction site. An additional 13bp (grey arrow) is often found but is not essential for cleavage. Blue triangle, FRT cleavage site (adapted from (Invitrogen™ 2002)).

Figure 4.11: cDNA end-point PCR results for mRNA expression of GNL3 (208bp) and β-Actin (250bp) in cultured HACs and JJ012 chondrosarcoma cell line cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X). All amplicons from the same cell type were run on the same gel. No bands were seen in the no template PCR control.

Figure 4.12: qPCR results for mRNA expression of GNL3 in control and GNL3 knockdown HACs and JJ012 chondrocyte cell lines HAC, Cultured HACs. Transfection carried out once (n=1). All samples were run in duplicate. JJ012, JJ012 chondrosarcoma cell line. Transfection carried out in triplicate (n=3). Samples from the first transfection were run in duplicate. Samples from all other transfections were run in triplicate. Values are average GNL3/18S percentage calculated using molecules/µl values. Blank subtracted from all values. Values are normalised to the untreated control. Error bars are ± SEM.

Figure 4.13: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in control and GNL3 knockdown cultured HACs cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X) alongside a low molecular weight marker (New England BioLabs®). All genes are represented by a single sample. All samples for the same gene were run on the same gel. No bands were seen in the no template PCR controls.

Figure 4.14: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in control and GNL3 knockdown JJ012 chondrocyte cell lines cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X) alongside a low molecular weight marker (New England BioLabs®). All genes are represented by a single sample (Results may vary in alternate samples). No bands were seen in the no template PCR control.

Figure 4.15: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in HACs cultured in StemPro® chondrogenic media for 0, 7, 14 or 21 days cDNA PCR products were run on a 2% agarose gel at 75V for 1hr in TBE buffer (1X). All genes are represented by a single sample for each genotype (Results may vary in alternate samples). No bands were seen in the no template PCR control. B, No template PCR control; L, Low molecular weight DNA ladder (New England BioLabs®).

Figure 4.16: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in MSCs cultured in StemPro® chondrogenic media for 0, 7,
14 or 21 days cDNA PCR products were run on a 2% agarose gel at 75V for 1hr in TBE buffer (1X). All genes are represented by a single sample for each genotype (Results may vary in alternate samples). No bands were seen in the no template PCR control (Figure 4.15). L, Low molecular weight DNA ladder (New England BioLabs®).

Figure 4.17: Site directed mutagenesis of expression clones Chromatograms show expression clones sequences for primers ‘DEST_FRT’ and ‘Insert_2’ (Appendix Table 7.22). Primer sequences for ‘R39Q’ and ‘V367M’ are listed in Appendix Table 7.23. Black bar indicates amino acid codon. All DNA sequences are 5’ to 3’ orientation. Chromatograms from Chromas LITE v2.1.1 (Technelysium 2012); Green, Adenine; Blue, Cytosine; Black, Guanine; Red, Thymine.

Figure 4.18: mRNA expression of GNL3 confirmed in A375 cells PCR products for GNL3 and β-Actin run on a 2% agarose gel at 75V for 30min in TBE buffer (1X). Results shown here are from the first transfection; FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247). L, 100bp DNA ladder (New England BioLabs®); B, No template PCR control.

Figure 4.19: Western blot for GNL3, MDM2, p53 and β-Actin expression in control and cisplatin-treated (5µM) A375 cells Both bands were taken into account when quantifying MDM2. Only the lower darker band was taken into account when quantifying GNL3 and β-Actin. (-) Control cells not treated with cisplatin; (+) Cisplatin-treated cells. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247). L, Magic Mark™ XP (Life Technologies™).

Figure 4.20: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in control and cisplatin-treated (5µM) A375 cells (A) GNL3 expression in first transfection cells; (B) MDM2 expression in first transfection cells; (C) p53 expression in first transfection cells; (D) GNL3 expression in second transfection cells; (E) MDM2 expression in second transfection cells; (F) p53 expression in second transfection cells. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in duplicate for each transfection (n=2). Dark grey, Protein/β-Actin expressed in control cells; Light grey, Protein/β-Actin expressed in cisplatin-treated cells. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).
Figure 4.21: A375 cell survival after 48hrs under hypoxic stress. Values are average fluorescence readouts for alamarBlue® redox reaction. All samples were run in triplicate. All values corrected for background. Error bars are ± SEM (n=3 (except QV, n=2)). Dark grey, Control samples under 48hrs normoxic conditions; Light grey, Cells under 48hrs hypoxic conditions. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).

Figure 4.22: Western blot for GNL3, MDM2, p53 and β-Actin expression in A375 cells placed under normoxic (N) or hypoxic (H) conditions for 24hrs. Both bands were taken into account when quantifying MDM2. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247). L, Magic Mark™ XP (Life Technologies™).

Figure 4.23: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in A375 cells placed under normoxic or hypoxic conditions for 24hrs. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in triplicate. Error bars are ± SEM (n=3). Dark grey, Protein/β-Actin expressed in cells under normoxic conditions for 24hrs; Light grey, Protein/β-Actin expressed in cells under hypoxic conditions for 24hrs.

Figure 4.24: Western blot for GNL3, MDM2, p53 and β-Actin expression in A375 cells placed under normoxic (N) or hypoxic (H) conditions for 48hrs or ‘re-oxidated’ (R) from 24hrs hypoxic to 24hrs normoxic. Both bands were taken into account when quantifying MDM2. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247). L, Magic Mark™ XP (Life Technologies™).

Figure 4.25: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in A375 cells placed under normoxic or hypoxic conditions for 48hrs or ‘re-oxidated’ from 24hrs hypoxic to 24hrs normoxic. (A) GNL3 expression in first transfection cells; (B) MDM2 expression in first transfection cells; (C) p53 expression in first transfection cells; (D) GNL3 expression in second transfection cells; (E) MDM2 expression in second transfection cells; (F) p53 expression in second transfection cells.
expression in second transfection cells. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in triplicate for each transfection. Error bars are ± SEM (n=3). Dark grey, Protein/β-Actin expressed in cells under normoxic conditions for 48hrs; Light grey, Protein/β-Actin expressed in cells under ‘re-oxidative’ conditions; Medium grey, Protein/β-Actin expressed in cells under hypoxic conditions. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).

Figure 5.1: Wildtype zebrafish cartilaginous pharyngeal skeleton (A-C) Whole mount alcian blue staining and (D-F) schematic representations of pharyngeal skeleton. (A, D) Lateral view, (B, E) Ventral view, (C, F) Dorsal view. (D, E) Pharyngeal arches: 1, blue; 2, yellow; 3, pink; 4, orange; 5, green; 6, purple; 7, black. (F) Neurocranium, grey. abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; c, cleithrum; cb, ceratobranchial; ch, ceratohyal; e, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; m, Meckel’s cartilage; n, notochord; ot, otic capsule; pc, parachordal; pq, palatoquadrate; t, trabeculae cranii. (Adapted from Schilling et al. 1996).

Figure 5.2: The ns^{hu3259} allele is a nonsense mutation in exon 5 of gnl3 in the zebrafish genome This results in the alteration of a glutamic acid at position 117 (GAA) being converted to a premature stop codon (TAA) (Paridaen et al. 2011).

Figure 5.3: Graticule used to quantify cartilage, other tissue, chondrocytes and ECM in zebrafish

Figure 5.4: Whole mount alcian blue staining of wildtype and ns^{hu3259} mutant gnl3 zebrafish at both 3 and 5dpf Mutant fish show a general dysmorphia with shortened anterior cartilage and no ceratobranchials. These images were kindly provided by Alyson MacInnes and Paul Essers (Hubrecht Institute, Utrecht). m, Meckel’s cartilage; pq, palatoquadrate, cb, ceratobranchial arches (1-5); ch, ceratohyal; pf, pectoral fin.

Figure 5.5: Histological staining of wildtype and ns^{hu3259} mutant gnl3 zebrafish at both 3 and 5dpf Cartilage was detected using alcian blue staining (blue) and counterstained with safranin-O (red). Sections were visualised at 40X magnification.

Figure 5.6: Percentage cartilage in wildtype and ns^{hu3259} mutant gnl3 zebrafish at both 3 and 5dpf Values are average percentage cartilage/other tissue. Error bars are ± SEM (n=10 (except ns^{hu3259} mutant zebrafish at 5dpf, n=6)). Dark grey, wildtype; Light grey, ns^{hu3259} zebrafish.
Figure 5.7: Percentage chondrocytes in wildtype and ns hu3259 mutant gnl3 zebrafish at both 3 and 5dpf. Values are average percentage chondrocytes/ECM in cartilage tissue. Error bars are ± SEM (n=10 (except ns hu3259 mutant zebrafish at 5dpf, n=6)). Dark grey, wildtype; Light grey, ns hu3259 zebrafish.
Declaration

I hereby declare that this thesis has been composed by myself and the work described within, except where specifically acknowledged, is my own and that it has not been accepted in any previous application for a degree. The information obtained from sources other than this study is acknowledged in the text or included in the references.

Michelle A. Ricketts
Abstract

Osteoarthritis (OA) is a common disease with a strong genetic component. Despite this, previous attempts to identify genetic variants that predispose to OA have met with limited success. Recently, the results of a large genome wide association study in OA has identified a novel susceptibility locus on chromosome 3 tagged by two SNPs, rs11177 (p=1.25x10^{-10}) which lies within the coding region of GNL3 and rs6976 (p=7.24x10^{-11}) situated in the 3’UTR of GLT8D1. The GNL3 gene encodes the protein nucleostemin which is found within the nucleolus of stem cells and tumour cells. It functions to regulate cell cycle progression, embryogenesis, tumorigenesis, tissue regeneration and ribosome biogenesis but its role in the joint is unknown.

In an attempt to identify the causal variant(s) at locus 3p21.1 I conducted a mutation screen of GNL3 which identified a common non-synonymous coding variant, rs2289247, which was in strong LD (r^2=0.92) with rs11177, as well as several other variants. Localisation studies showed that GNL3 was expressed at the mRNA and protein level in several joint tissues. While levels of mRNA expression were found to be significantly higher in human articular chondrocytes from OA patients as compared with controls, levels of GNL3 protein were significantly lower in OA chondrocytes than controls. Further studies showed that cytokines which have been implicated in the pathogenesis of OA such as IL1β, IL13, TNFα and FGF2 had no effect on GNL3 mRNA in cartilage. Knockdown of GNL3 using siRNA in articular chondrocytes and the chondrosarcoma cell line, JJ012, did not alter the mRNA expression of chondrogenic markers; COL2A, ACAN, MMP3, MMP13, RUNX2 and SOX9. Cultures of mesenchymal stem cells and articular chondrocytes from patients of different rs11177 genotypes, showed no difference in chondrogenic potential. Furthermore, genotypes at rs11177 and rs2289247 did not influence the expression of p53, MDM2 or GNL3 in response to stressful stimuli, including cisplatin and hypoxia, when cloned into a melanoma cell line.

Studies of zebrafish carrying a loss of function mutation in gnl3 revealed a significant reduction in cartilage volume and an alteration in cartilage structure, as evident by a reduced number of chondrocytes, disorganised stacking and an increase in cartilage extracellular matrix in the mutant fish.

This research has shown that gnl3 plays a vital role in chondrogenesis in zebrafish and has shown evidence of dysregulation of GNL3 expression in OA human articular chondrocytes. The in vitro studies failed to identify any specific effects of the variants rs11177 and rs2289247 on GNL3 expression, chondrogenesis or p53 stress response although, it remains possible that the variants may have modest effects that were not detected by the assays used. The zebrafish studies illustrate that gnl3 plays a critical role in normal cartilage development however further studies on GNL3 in OA would be of interest.
Osteoarthritis (OA) is a degenerative joint disease with a strong genetic component that is characterised by joint pain due to loss of articular cartilage from the affected joints, and abnormalities of bone in proximity to the affected joints. The risk of developing OA is influenced by both environmental and genetic factors. Until recently, research studies which have attempted to identify the genes that predispose individuals to OA, have yielded conflicting results. However, a recent study has pinpointed a region on human chromosome 3p21 surrounding a gene called, GNL3 or nucleostemin as a potential contributory factor in OA. This gene is known to play a vital role in the cell proliferation and growth of cancer and stem cells but its role in the joint is unknown. In this thesis several studies were performed to clarify the role of GNL3 in OA.

The first step was to analyse the DNA sequence in GNL3 and a gene nearby to determine if people with OA had variants within the gene more often than normal individuals. This identified several variants within GNL3 which were more common in OA cases as compared with unaffected people. Studies were then performed to analyse where nucleostemin (the GNL3 gene product) was expressed in the joint. This showed that nucleostemin was expressed in various joint tissues and that levels of the protein were lower in OA patients versus control individuals who did not have OA. Further studies were performed to investigate what effect reducing levels of nucleostemin had on the function of cartilage cells and also on what effect various inflammatory mediators had on levels of nucleostemin. Finally studies were performed to evaluate the effects of nucleostemin in zebrafish - an experimental model which can be used to assess the effects of various genes on bone and cartilage development. This showed that zebrafish, which lacked a functional copy of the gnl3 gene, showed significant abnormalities of cartilage structure.

Overall the research has shown that GNL3 does play a role in cartilage biology and that this may well be the candidate gene for OA on chromosome 3p21. Further studies to investigate the mechanisms by which GNL3 predisposes to OA would be of interest.
Acknowledgements

First and foremost I wish to thank my supervisors Stuart Ralston and Donald Salter for their guidance, support and enthusiasm throughout this project.

I would like to acknowledge members of the Bone and Osteoarticular research groups at the MMC, Edinburgh. Particularly, George Nuki, Christine Beadle, Simon Roberts and members of staff at Royal Infirmary, Edinburgh and Victoria Hospital, Kirkcaldy for their help in obtaining ethical approval and consent for the joints used in this study; Members of the Bone Research Group for extracting the DNA; Asim Azfer, Grigore Rischitor, Helen Caldwell, Jessica Andersson, Micaela Rios-Visconti, Natalia Harasymowicz, Noor Jamil, Sam Gray, Antonia Sophocleous, Euphemie Landau, Rob van t' Hof, Omar Albagha and Ying Zhou for their help with various techniques in the lab.

Furthermore, I wish to thank: Members of staff at the HGU services for carrying out the sequencing; Terry Gray, Euan Murray and Ted Hupp (CRUK, Edinburgh) for sharing their knowledge and guiding me through the Gateway® cloning process; Susan Harvey and Helen Caldwell (Breakthrough Unit, Edinburgh) for providing an excellent histology service; Alyson MacInnes and Paul Essers (Hubrecht Institute, Utrecht) for kindly providing the zebrafish; Carol Ward and Simon Langdon (Breakthrough Unit, Edinburgh) for their help with the hypoxia experiments; All participants and researchers of the arcOGEN study and finally, the Principal’s Career Development Scholarship (University of Edinburgh) for their funding.

Most of all, I am eternally grateful to my Mom, Dad, Gran, Bryan and Phil for their love and constant encouragement.
Presentations

Abstract presentation: Genetics of Osteoarthritis at The SNP Course VII 2010, The Erasmus Postgraduate School Molecular Medicine, Rotterdam

Poster presentation: Investigating the Role of GNL3 in Osteoarthritis at TreatOA Summer School 2011, Harris Manchester College, Oxford

Poster presentation: Investigating the Role of GNL3 in Osteoarthritis at Osteoarthritis Research Society International (OARSI) Conference 2012, Barcelona

Presentation: Genetics of Osteoarthritis as part of the Overcoming Osteoarthritis Event at Edinburgh International Science Festival 2012, Edinburgh

Publications

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>AACT</td>
<td>α₁-antichymotrypsin</td>
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<tr>
<td>ACAN</td>
<td>aggrecan</td>
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<td>ADAM(TS)</td>
<td>a disintegrin and metalloprotease (with thrombospondin)</td>
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<td>allelic expression imbalance</td>
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<td>Avon Longitudinal Study of Parents and Children</td>
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<td>Arthritis Research UK OA Genetics</td>
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<td>EGCUT</td>
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<td>ENU</td>
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<td>eQTL</td>
<td>expression quantitative trait loci</td>
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<td>ER</td>
<td>oestrogen receptor</td>
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<td>exostosin 2</td>
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<td>FTO</td>
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<tr>
<td>GAPDH</td>
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<td>GWAS</td>
<td>genome-wide association study</td>
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<td>HA</td>
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<td>human articular chondrocyte</td>
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<td>HBPI1</td>
<td>high mobility group (HMG) box transcription factor 1</td>
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<td>HIF</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>horseradish peroxidase</td>
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<td>Hardy-Weinberg equilibrium</td>
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<td>IGF-binding protein 5</td>
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<td>IHC</td>
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<td>integration host factor</td>
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<td>indian hedgehog</td>
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<td>Iscove’s Modified Dulbecco's Medium</td>
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<td>IMPDH</td>
<td>inosine-5'-monophosphate dehydrogenase</td>
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<td>interphotoreceptor matrix proteoglycan 1</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<td>ITIH</td>
<td>inter-alpha trypsin inhibitor heavy chain</td>
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<td>joint space narrowing</td>
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<td>kb</td>
<td>kilo base pairs</td>
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<td>kDa</td>
<td>kilo dalton</td>
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<td>micro RNA</td>
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<td>matrix metalloprotease</td>
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<td>MRC</td>
<td>Medical Research Council</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>MrOS</td>
<td>Osteoporotic Fractures in Men</td>
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<td>mitochondrial DNA</td>
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<td>mesenchymal stem cell</td>
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<td>MUSTN1</td>
<td>musculoskeletal embryonic nuclear protein 1</td>
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<td>Myc</td>
<td>myelocytomatosis oncogene</td>
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<td>nuclear factor</td>
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p53          tumour suppressor protein 53
pA           polyadenylation
PAPPA        pappalysin-1
PBRM1        polybromo 1
PBS          phosphate buffered saline
PCR          polymerase chain reaction
PCSK6        serine protease paired amino converting enzyme 4 (PACE)
PGE2         prostaglandin E2
piRNA        piwi-interacting RNA
PML          promyelocytic leukaemia
PoBI         People of the British Isles
PolyPhen     Polymorphism Phenotyping
PRKAR2B      protein kinase-cAMP-dependent-regulatory-type IIβ
P2RX7        purinergic receptor (P2X) ligand-gated ion channel 7
PTGS2        prostaglandin endoperoxide synthase 2
PTHLH        parathyroid hormone-like hormone
PVDF         polyvinylidene difluoride
RANKL        receptor activator of nuclear factor kappa-B ligand
RIPA         radio immuno precipitation assay
RNA          ribonucleic acid
ROS          reactive oxygen species
RPS          ribosomal subunits
rRNA         ribosomal RNA
RSL1D1       ribosomal L1-domain-containing 1
RT           reverse transcription
RUNX2        runt-related transcription factor 2
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<td>standard deviation</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>sentrin specific peptidase 6</td>
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<td>SLRP</td>
<td>small leucine-rich proteoglycan</td>
</tr>
<tr>
<td>SMAD3</td>
<td>SMAD family member 3</td>
</tr>
<tr>
<td>snoRNA</td>
<td>small nucleolar RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SOD2</td>
<td>superoxide dismutase 2</td>
</tr>
<tr>
<td>SOF</td>
<td>Study of Osteoporotic Fractures</td>
</tr>
<tr>
<td>(L-)SOX</td>
<td>(long form) SRY-type HMG box</td>
</tr>
<tr>
<td>SPCS1</td>
<td>signal peptidase complex subunit 1</td>
</tr>
<tr>
<td>SREB2</td>
<td>sterol regulatory element binding protein 2</td>
</tr>
<tr>
<td>STAB1</td>
<td>stabilin 1</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-related modifier</td>
</tr>
<tr>
<td>SUPT3H</td>
<td>suppressor of Ty3 homolog</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>T</td>
<td>tween 20</td>
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<tr>
<td>T1DGC</td>
<td>Type 1 Diabetes Genetics Consortium</td>
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<tr>
<td>TBE</td>
<td>tris/borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
</tbody>
</table>
TGF    transforming growth factor
TNA    tetranectin
TNF    tumour necrosis factor
THR    total hip replacement
TILLING target induced local lesions in genomes
TJR    total joint replacement
TKR    total knee replacement
TMEM   transmembrane protein
TP63   tumour protein 63
TreatOA Translational Research in Europe Applied Technologies for OA
TRF1   telomere repeat binding factor 1
TRPV1  transient receptor potential cation channel subfamily V member
TRIM32 tripartite motif containing 32
TSI    Tuscans in Italy
UK     United Kingdom
USA    United States of America
UTR    untranslated region
VDR    vitamin D receptor
VEGF   vascular endothelial growth factor
WTCCC2-1958BC Wellcome Trust Case Control Consortium 2-1958 Birth Cohort
WTCCC2-UKBS Wellcome Trust Case Control Consortium 2-UK Blood Donor Service
ZFP161 zinc finger protein 161 homolog (mouse)
Chapter 1: Introduction

1.1 Osteoarthritis

1.1.1 Definition

Osteoarthritis (OA) (OMIM #165720) is defined by OA Research Society International (OARSI) (Lane et al. 2011) as,

‘... a progressive disease of synovial joints that represents failed repair of joint damage that results from stresses that may be initiated by an abnormality in any of the synovial joint tissues, including articular cartilage, subchondral bone, ligaments, menisci (when present), periarticular muscles, peripheral nerves, or synovium. This ultimately results in the breakdown of cartilage and bone, leading to symptoms of pain, stiffness and functional disability.’

1.1.2 Epidemiology

OA is a late onset disease, which rarely occurs before 40 years of age (Arthritis Research Campaign 2004). It is the most common form of arthritis in the world and reported by Arthritis Research UK (2013) to affect 8.75 million people in the United Kingdom (UK). In theory, any synovial joint in the body can be affected however this study has focused on two of the most clinically important sites, the hip and knee which affect an estimated 8% (2.1 million people) and 18% (4.7 million people) of the UK population over the age of 45, respectively (Arthritis Research UK 2013).

A rapidly aging population due to advances in modern medicine and a subsequent increase in life expectancy has resulted in a consequent increase in spending on diagnosis and treatment of OA ((Zeggini et al. 2012) and as reviewed in (Ge et al. 2006)). Currently there are no disease-modifying drugs for treating the disease ((Davidson 2010) and as reviewed in (Pulsatelli et al. 2013)) and in severe cases of OA, characterised by unmanageable pain and disability, arthroplasty is often considered ((Zeggini et al. 2012 and as reviewed in (Ge et al. 2006)). The latter, is responsible for 93% and 98% of total hip and knee replacement (THR, TKR) surgeries respectively, in the UK (Arthritis Research UK 2013).
1.1.3 Aetiology

OA is termed primary or idiopathic in nature when there is no known cause (as reviewed in (Ge et al. 2006)). Secondary OA occurs as a result of joint degeneration due to a combination of predisposing events including, inflammatory, metabolic, developmental or neurological disorders and previous joint injury (as reviewed in (Buckwalter et al. 2005; Hoaglund 2013)).

The risk of developing OA is influenced by both environmental and genetic factors (as reviewed in (Valdes & Spector 2010b)). This risk increases with age and BMI and is more common among post-menopausal females. Furthermore, excessive mechanical loading, joint laxity and injury, greater bone density and predisposing genetics variants have also been associated with the pathogenesis of OA ((Beary & Luggen 2006) and as reviewed in (Dieppe & Lohmander 2005; Ge et al. 2006)).
1.2 Joint Tissue

The diarthrodial joint (Figure 1.1) is comprised of subchondral bone covered with a layer of articular cartilage, surrounded by synovial fluid enclosed in the joint capsule and attached to the muscle by ligaments. The meniscus is also present in the knee (as reviewed in (Poole 2011)). OA occurs as a result of an imbalance in the degradation and synthesis of joint tissues (as reviewed in (Ge et al. 2006)), culminating in the deterioration of articular cartilage (Jones et al. 2008).

![Diagram of a diarthrodial synovial joint, the human knee](image)

Figure 1.1: Diagrammatic representation of a diarthrodial synovial joint, the human knee

1.2.1 Synovium and Synovial fluid

The synovium is derived from mesenchymal stem cells (MSCs) and results in a dual layer membrane which lines the joint capsule (as reviewed in (Ge et al. 2006)). The subintima or deep layer is a hypocellular membrane composed of fibrous, areolar and adipose tissue ((Castor 1960) and as reviewed in (Ge et al. 2006)). The intima or inner layer is composed of two cell types. Type A synovial cells display macrophage-like characteristics and type B are fibroblastic. A third cell type which displays characteristics of both A and B synovial cells is also reported ((Wooley et al. 2005) and as reviewed in (Athanasou 1995; Ge et al. 2006)). Type A synoviocytes protect the joint from bacterial infection by phagocytosis. Type B synoviocytes are
Investigating the Role of GNL3 in Osteoarthritis

responsible for the synthesis and secretion of the joint lubricants, hyaluronic acid (HA) and lubricin ((Wooley et al. 2005) and as reviewed in (Poole 2011)).

The synovial fluid is a plasma ultra-filtrate with high viscosity due to the presence of HA and lubricin which allow the joint to articulate frictionless movement ((Rhee et al. 2005; Wooley et al. 2005) and as reviewed in (Ge et al. 2006)). The synovial fluid also contains derived inflammatory cytokines, proteases and debris from matrix turnover within the joint (as reviewed in (Huber et al. 2000; Martel-Pelletier et al. 2008)) and MSCs, thought to be involved in tissue repair ((Jones et al. 2008) and as reviewed in (Poole 2011)). Nutrients which supply the fuel for metabolic activities of the chondrocyte are also filtered through the synovial fluid from blood vessels present in the deep layer of synovium (as reviewed in (Huber et al. 2000; Ge et al. 2006; Poole 2011)).

1.2.2 Human Articular Cartilage
Articular cartilage is composed of chondrocytes embedded within an extracellular matrix (ECM) rich in collagen and proteoglycans. It is an aneural and avascular connective tissue found in diarthrodial joints. Its main function is to absorb impact and ensure frictionless movement within the joint thereby allowing efficient load bearing (as reviewed in (Martel-Pelletier et al. 2008)).

1.2.2.1 Chondrocytes
Human articular chondrocytes (HACs) are the only cells found in articular cartilage and make up approximately 2-5% of the total volume (as reviewed in (Goldring 2006)). These cells vary in diameter and the shape is dependent on the zonal origin in the cartilage (Section 1.2.2.3) (as reviewed in (Huber et al. 2000)). HACs are encased in lacunae, a glycanalyx-filled fibrillar pericellular capsule and are hence isolated from the surrounding ECM (as reviewed in (Poole 1997)). Chondrocytes are responsible for the biosynthesis of both catabolic and anabolic factors which play a vital role in maintaining a homeostatic balance (Section 1.2.2.4) in the ECM (as reviewed in (Martel-Pelletier et al. 2008)).
MSCs, found in the bone marrow and synovial fluid (Jones et al. 2008), are multipotent progenitor cells which give rise to osteoblasts, chondrocytes and adipocytes (as reviewed in (James 2013)). Chondrogenesis is regulated by bone morphogenic proteins (BMPs) which belong to the transforming growth factor (TGF)-β superfamily ((Wozney et al. 1988; Phimphilai et al. 2006) and as reviewed in (Erlebach et al. 1995)). Chondrogenesis is initiated by SRY-type HMG box 9 (SOX9) which interacts with collagen II A (COL2A) to regulate chondrocyte development (Bell et al. 1997; Lefebvre et al. 1997). A long form (L-) of SOX5 and SOX6 are also reported to contribute to the chondrogenic process in this manner (Lefebvre et al. 1998).

1.2.2.2 Extracellular Matrix
The ECM consists of tissue fluid and macromolecules, namely a collagen fibre network entangled in proteoglycans and non-collagenous proteins (as reviewed in (Huber et al. 2000; Martel-Pelletier et al. 2008)). The structure, density and orientation of these molecules differ throughout the cartilage zones (Section 1.2.2.3) (as reviewed in (Huber et al. 2000)).

1.2.2.2.1 Collagens
Collagens provide cartilage with strength and tensile stiffness required in order to withstand mechanical joint stress (as reviewed in (Martel-Pelletier et al. 2008)). Collagen II is the most abundant collagen (approximately 80% of total collagen) and is made up of triple helix of α1(II) chains (as reviewed in (Bruckner & Vanderrest 1994; Huber et al. 2000)). Other collagens found in articular cartilage include collagen XI, a fibrillar collagen with a similar structure to collagen II; collagen IX, a non-fibrillar collagen which associates with types II and XI; collagen X, expressed by hypertrophic chondrocytes (Section 1.2.3.1) and collagen VI, associated with the pericellular matrix (Section 1.2.2.1) (as reviewed in (Bruckner & Vanderrest 1994; Poole 1997; Huber et al. 2000; Ge et al. 2006; Martel-Pelletier et al. 2008)).

1.2.2.2 Proteoglycans
Proteoglycans give the ECM its hydrophilic properties and are vital in providing resistance to compressive loading. The most abundant proteoglycan (approximately
90%) in articular cartilage is aggrecan (ACAN). Aggregates of aggrecan molecules surround a central HA protein backbone. Aggrecan is a core protein which consists of three globular proteins connected by interglobular and glycosaminoglycan regions. The latter are formed of keratan- and chondroitin-sulphate chains. Other smaller, less abundant proteoglycans which aid in maintaining stability and tissue integrity such as fibromodulin, biglycan, decorin, lumican and versican are also present ((Bayliss et al. 1983) and as reviewed in (Huber et al. 2000; Buckwalter et al. Martel-Pelletier et al. 2008)).

1.2.2.2.3 Non-Collagenous Glycoproteins
Non-collagenous glycoproteins such as, anchorin, cartilage matrix protein (CRTM), tenascin, fibronectin and cartilage oligomeric matrix protein (COMP) which serve to further conserve matrix stability, are also found in articular cartilage (as reviewed in (Huber et al. 2000; Buckwalter et al. 2005)).

1.2.2.3 Zonal Architecture of Articular Cartilage
Articular cartilage has several zones which from the surface to the subchondral bone are termed the superficial, middle and deep zones (Figure 1.2). The deep zone is separated by a ‘tidemark’ from calcified cartilage and subchondral bone ((Rolauffs et al. 2008) and as reviewed in (Huber et al. 2000; Ge et al. 2006)). Each zone differs with regard to chondrocyte morphology and matrix composition. In the superficial zone, chondrocytes appear elongated and flat becoming progressively more rounded and ellipsoid in the middle and deep zones ((Aydelotte & Kuettner 1988) and as reviewed in (Huber et al. 2000; Ge et al. 2006)). Chondrocyte volume increases and cell density decreases, the nearer the cells are to the subchondral bone (Stockwell 1967; Wong et al. 1996). The anaerobic metabolic activity of the cells is also higher in the deeper articular cartilage zones which survive under hypoxic conditions (typically 1%) due to the avascular structure of articular cartilage ((Wong et al. 1996) and as reviewed in (Goldring 2006; Pfander & Gelse 2007)). Collagen fibres are present in densely packed layers which run parallel to the joint surface in the superficial zone becoming gradually thicker and more sparingly distributed in the middle zone and the orientation perpendicular to the subchondral bone in the deep zone ((Minns & Steven 1977; Aydelotte & Kuettner 1988) and as reviewed in
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

(Bruckner & Vanderrest 1994; Huber et al. 2000; Archer et al. 2003; Ge et al. 2006)). Proteoglycans are less dense in the upper superficial zone and the deeper zones also have a higher ratio of keratan sulphate ((Stockwell & Scott 1967; Venn & Maroudas 1977; Aydelotte et al. 1988) and as reviewed in (Huber et al. 2000)).

In addition to the laterally defined zones of articular cartilage, each chondrocyte is surrounded by a circumferential microenvironment which includes the pericellular, territorial and inter-territorial matrices (Figure 1.2:). These too display matrix heterogeneity regarding collagen and proteoglycan structure. While the composition of these regions does differ between the lateral zones, overall the thickest collagen fibres are found in the inter-territorial matrix and are presented in a radial pattern from the chondrocyte. Collagen fibres of the territorial matrix are thinner but also display a radial configuration. The finest collagen fibres are found in a compact network adjacent to the cell in the pericellular matrix which also contains proteoglycans and glycoproteins. The greatest concentration of keratan- and chondroitin-sulphate proteoglycans is found in the inter-territorial and territorial matrices respectively ((Poole et al. 1982) and as reviewed in (Poole 1997; Huber et al. 2000)).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Figure 1.2: The zonal architecture of articular cartilage (adapted from (Jackson et al. 2001 (review); Poole 2011 (review)) Grey box inset showing the chondrocyte microenvironment, namely the pericellular (dark grey), territorial (medium grey), inter-territorial (light grey) matrices. Please refer to text for details (Section 1.2.2.3).

1.2.2.4 Cartilage Homeostasis

The chondrocyte is responsible for the biosynthesis and degradation of the surrounding ECM. This occurs in response to mechanical stresses, cytokines and growth factors on the joint (as reviewed in (Goldring 2006)).

Anabolic factors such as BMP-2, -4, -6, -7, -9 and -13, insulin-like growth factor (IGF)-1, TGFβ-1, -2 and -3 and fibroblast growth factor (FGF)-2, -9 and -18 act to increase the synthesis of the ECM components (collagen and proteoglycans), TGFβ and FGF18 repair damaged cartilage, IGF1 encourages chondrocyte survival, BMP-2 and -7, growth differentiation factor (GDF)-5, FGF-2, -4, -8, -9, -10 and -18 coordinate chondrogenic differentiation and proliferation and BMP7 can reverse the effect of proteolytic enzymes (as reviewed in (Goldring 2006; Martel-Pelletier et al. 2008; Pulsatelli et al. 2013)).

In contrast, catabolic processes degrade the ECM. Matrix metalloproteases (MMPs) are collagen specific proteinases. MMP-1, -3, -8 and -13 cleave intact collagen fibres, targeting collagens III, IX, I and II respectively ((Reboul et al. 1996) and as reviewed in (Cawston & Wilson 2006; Goldring 2006; Martel-Pelletier et al. 2008)).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Collagen fragments, gelatin and collagens IV and V are substrates for MMP-2 and-9. MMP-3,-10 and -11 degrade a variety of proteoglycans including, elastin, laminin and fibronectin (as reviewed in (Cawston & Wilson 2006; Martel-Pelletier et al. 2008)). Proteoglycan loss in articular cartilage is predominantly mediated by aggrecanases, a disintegrin and metalloprotease with thrombospondin (ADAMTS)-4 and -5 ((Song et al. 2007) and as reviewed in (Cawston & Wilson 2006; Goldring 2006; Martel-Pelletier et al. 2008)). These aggrecanases target specific sites located in the interglobular domain of aggrecan resulting in cleavage of glycosaminoglycan residues (Tortorella et al. 2000; Song et al. 2007).

Furthermore, some anabolic factors are also important in regulating matrix turnover. Namely, TGFβ mediates the degradation of aggrecan and collagen by stimulating the expression of ADAMTS4 (Moulharat et al. 2004) and MMP13, respectively (Moldovan et al. 2000) and FGF2 inhibits BMP7 and IGF1 (Loeser et al. 2005).

Interleukin (IL)-1β and tumour necrosis factor (TNF)-α are the key inflammatory cytokines in articular cartilage which alter the production of proteolytic enzymes (MMPs and aggrecanases), prostaglandin E₂ (PGE₂), nitric oxide (NO), oncostatin M and other cytokines such as IL-4, -6, -10, -13, -17 and -18 (as reviewed in (van den Berg 1999; Goldring 2006; Martel-Pelletier et al. 2008)).

1.2.2.5 Grading of Osteoarthritis

The severity of OA can be graded radiographically, based on joint space narrowing (JSN) and osteophyte formation, according to the Kellgren-Lawrence (KL) scale, from 0 (healthy) through IV (severe OA) (as reviewed in (Schiphof et al. 2008)). Cartilage abnormalities may also be graded according to the Collins-McElligott system, a pathoanatomical method which measures sulphate uptake by chondrocytes (hypertrophic late stage OA chondrocytes being the highest) and correlates this with cartilage architecture (Collins & McElligott 1960; Pritzker et al. 2006). Mankin et al. (1971) introduced a grading system that involves assessment of glycosaminoglycan staining of cartilage and architectural changes in cartilage including the tidemark. However, this involved a complicated system which lacked consensus among OA studies and hence OARSI published cohesive guidelines for OA scoring using the
histopathological staining described by Mankin et al. to grade cartilage and stage the degree to which the joint surface was affected. Details of the OARSI scoring systems are outlined in Figure 1.3 (Pritzker et al. 2006).

![Histopathology Staging](image)

**Figure 1.3: OARSI cartilage histopathology grading and staging** Sections are stained with safranin-O and a light green counterstain. Staging % refers to the percentage of the length, volume or area of cartilage involved. ECM, Extracellular matrix; HAC, Human articular chondrocyte (Pritzker et al. 2006).

### 1.2.3 Subchondral Bone

The subchondral bone is a porous calcified organ which provides mechanical strength, structure and protection for the body and contributes to maintaining calcium/phosphate homeostasis (as reviewed in Hadjidakis & Androulakis 2006)).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

1.2.3.1 **Bone Structure**

Subchondral bone is divided into cortical and trabecular bone (as reviewed in (Burr 2004; Hadjidakis & Androulakis 2006; Goldring & Goldring 2010)). These are separated from the hyaline cartilage by a layer of calcified cartilage delineated by a ‘tidemark’ abut the cartilage deep zone (Figure 1.2:) (as reviewed in (Burr 2004; Goldring & Goldring 2010)).

Calcified cartilage is mineralised with little proteoglycan and also contains hypertrophic chondrocytes which produce collagen X and are responsible for endochondral ossification (as reviewed in (Martel-Pelletier et al. 2008; Poole 2011)). The thin layer of cortical subchondral bone is a hypovascular, non-porous, calcified tissue (as reviewed in (Hadjidakis & Androulakis 2006; Goldring & Goldring 2010)) which covers and is supported by the less dense cancellous trabecular bone, a spongy tissue which contains the bone marrow (as reviewed in (Köse & Hasirci 2005; Hadjidakis & Androulakis 2006; Sophocleous 2009)).

1.2.3.2 **Bone Matrix**

The bone matrix, otherwise known as the osteoid, constitutes a network of collagen I fibres associated with hydroxyapatite crystal deposits. Osteoblasts, osteocytes and osteoclasts are the cells present in mature bone and play a key role in its remodelling ((Widmaier et al. 2004) and as reviewed (Hadjidakis & Androulakis 2006)).

Osteoblasts are derived from MSCs and are responsible for bone formation ((Widmaier et al. 2004) and as reviewed (Hadjidakis & Androulakis 2006)). BMPs induce osteoblast differentiation by stimulating the expression of transcription factors namely, runt-related transcription factor 2 (*RUNX2*) and osterix ((Wozney et al. 1988; Phimphilai et al. 2006) and as reviewed in (Erlebacher et al. 1995; Witkowska-Zimny et al. 2009)). Mature osteoblasts, which become surrounded by the calcified osteoid are termed osteocytes ((Widmaier et al. 2004) and as reviewed (Hadjidakis & Androulakis 2006)). Osteoclasts are hematopoietic in origin. The primary function of these cells is bone resorption (as reviewed (Hadjidakis & Androulakis 2006)). Promyeloid precursors initiate osteoclastogenesis in response to RANK-ligand, osteoprotegerin (OPG)-ligand (Section 1.2.3.3) or osteoclast differentiation factor
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

(O DF) (as reviewed in (Vaananen et al. 2000)). Initially osteoclasts secrete hydrogen ions which dissolve the crystalline hydroxyapatite. Subsequently, proteolytic enzymes are released which digest the collagen fibres ((Widmaier et al. 2004) and as reviewed in (Vaananen et al. 2000)).

1.2.3.3 Regulation of Bone Remodelling
Bone remodelling is activated by nuclear factor (NF)-κB (receptor activator of nuclear factor kappa-B ligand (RANKL)) which interacts with the RANK receptor to stimulate osteoclastogenesis and bone resorption. OPG can block the effects of RANKL, thereby inhibiting its effects and inducing osteoclast apoptosis (as reviewed in (Vaananen et al. 2000; Hadjidakis & Androulakis 2006)).

Systemic factors are also important regulators of bone matrix turnover. Parathyroid hormone, calcitriol and vitamin D3 regulate calcium uptake. Thyroid hormones and glucocorticoids can influence both bone formation and degradation. Osteoclastogenesis is inhibited and osteoblastogenesis stimulated in the presence of oestrogen (as reviewed in (Hadjidakis & Androulakis 2006)).

Furthermore, growth factors such IGF, FGF, TGFβ, BMPs and vascular endothelial growth factor (VEGF) play an important regulatory role in bone remodelling (as reviewed in (Hadjidakis & Androulakis 2006; Witkowska-Zimny et al. 2009)). Cytokines, IL1β and TNFα, reportedly stimulate osteoclastogenesis via the OPG pathway ((Hofbauer et al. 1999) and as reviewed in (Hadjidakis & Androulakis 2006)) and IL6 regulates both bone formation and resorption ((Moonga et al. 2002) and as reviewed in (Hadjidakis & Androulakis 2006)).

1.2.4 Effects of Osteoarthritis on the Joint
OA affects all joint tissues. Although predominantly these manifest as fibrillation and a loss of articular cartilage and eburnation and sclerosis of subchondral bone, the synovium, ligaments and meniscus (when present) are also affected (as reviewed in (Aigner & McKenna 2002; Buckwalter et al. 2005; Ge et al. 2006; Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)).
These changes are brought about by an imbalance in the anabolic and catabolic processes (Section 1.2.2.4) involved in maintaining the joint tissues (as reviewed in (Ge et al. 2006)).

The chondrocytes, responsible for maintaining the homeostatic balance (Section 1.2.2.4) in cartilage, are low in number and quiescent in mature cartilage with low levels of matrix turnover (as reviewed in (Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). The regenerative ability is poor however in OA, chondrocytes proliferate forming small replicative clone clusters surrounded by a pericellular matrix rich in collagen VI, primarily in the superficial layer (Section 1.2.2.3) (as reviewed in (Poole 1997; Huber et al. 2000; Aigner & McKenna 2002; Buckwalter et al. 2005; Goldring & Goldring 2010; Salter et al. 2011)). This is seen throughout the cartilage however the number of cells which die by apoptosis but also necrosis increases, resulting in empty lacunae closer to the subchondral bone (as reviewed in (Sandell & Aigner 2001; Aigner & McKenna 2002; Salter et al. 2011; Zamli & Sharif 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). There is also an increase in collagen X production by hypertrophic chondrocytes, especially in the calcified cartilage layer (as reviewed in (Sandell & Aigner 2001; Aigner & McKenna 2002; Loeser et al. 2012; Pulsatelli et al. 2013)).

The ability of chondrocytes to synthesise anabolic factors decreases with age however in response to OA, this does increase (as reviewed in (Huber et al. 2000; Buckwalter, Mankin & Grodzinsky 2005; Salter et al. 2011)). Despite these efforts, the catabolic activity predominates and the level of ECM degradation exceeds that of its production, resulting in OA (as reviewed in (Sandell & Aigner 2001; Goldring & Goldring 2010; Loeser et al. 2012; Pulsatelli et al. 2013)). These include the production of MMPs, aggrecanases and cytokines resulting in the loss of proteoglycans (largely keratan sulphate) and collagen fibre network ((Venn & Maroudas 1977) and as reviewed in (Huber et al. 2000; Sandell & Aigner 2001; Aigner & McKenna 2002; Buckwalter, Mankin & Grodzinsky 2005; Goldring & Goldring 2010; Salter et al. 2011; Loeser et al. 2012)). MMP inhibitor levels are also decreased in OA (as reviewed in (Sandell & Aigner 2001)).
Articular cartilage, normally smooth and shiny in appearance is soft and yellow in colour with localised swelling and fibrillation as a result of OA processes (as reviewed in (Huber et al. 2000; Salter et al. 2011)). As the disease progresses these fibrillations extend deeper into the cartilage matrix and can result in complete erosion or eburnation exposing the underlying subchondral bone (Figure 1.3) (as reviewed in (Huber et al. 2000; Buckwalter, Mankin & Grodzinsky 2005)). The layer of calcified cartilage (Figure 1.2:) also progresses as a result of an increase in endochondral ossification which in turn, contributes to the thinning of articular cartilage (as reviewed in (Burr 2004; Goldring & Goldring 2010; Salter et al. 2011; Loeser et al. 2012)). Endochondral ossification is also responsible for the formation of osteophytes which are fibrocartilaginous outgrowths brought about by TGFβ and may help to stabilise the joint (as reviewed in (Sandell & Aigner 2001; Buckwalter, Mankin & Grodzinsky 2005; Goldring & Goldring 2010; Salter et al. 2011; Loeser et al. 2012)).

The subchondral bone volume increases resulting in thickening of the subchondral plate and microcracks (Figure 1.2: and Figure 1.3) (as reviewed in (Huber et al. 2000; Burr 2004; Buckwalter, Mankin & Grodzinsky 2005; Martel-Pelletier et al. 2008; Goldring & Goldring 2010; Salter et al. 2011; Loeser et al. 2012)). The latter are penetrated by small capillaries which extend into the calcified cartilage. These capillaries are thought to transport catabolic factors from the bone and aggravate degradation of the ECM. Furthermore, there is an increase in trabecular bone volume (Figure 1.2:), which occurs as a result of high bone turnover and consequent hypomineralisation (Section 1.2.3.2 and Section 1.2.3.3) (as reviewed in (Burr 2004; Ge et al. 2006; Martel-Pelletier et al. 2008; Goldring & Goldring 2010; Loeser et al. 2012)). Atypical collagen type I molecules which arise due to abnormal osteoblast functioning, most likely contribute to these structural changes (Bailey et al. 2002). There is also evidence of osteonecrosis and subchondral bone cysts as well as bone marrow fibrosis and necrosis. Late stage OA patients can often present with altered bone shape (Figure 1.3) (as reviewed in (Huber et al. 2000; Buckwalter, Mankin & Grodzinsky 2005; Salter et al. 2011; Loeser et al. 2012)).
The synoviocytes (Section 1.2.1) of the intima layer show signs of hyperplasia resulting in multiple layers, fibrosis and a villous synovium profile ((Benito et al. 2005) and as reviewed in (Salter et al. 2011; Loeser et al. 2012)). The subintima layer is hypervascular (as reviewed in (Salter et al. 2011; Loeser et al. 2012)). Synovitis can occur, although the inflammation is less than that of other arthritic conditions (as reviewed in (Buckwalter, Mankin & Grodzinsky 2005; Salter et al. 2011; Loeser et al. 2012)). There is also an increase in the concentration of detritic waste from degradation of the joint tissue and inflammatory cytokines (as reviewed in (Buckwalter, Mankin & Grodzinsky 2005; Salter et al. 2011)).
1.3 Role of Genetics in Osteoarthritis

OA is a complex disease and as discussed above (Section 1.1.3), the risk of developing OA is influenced by environmental, systemic and genetic factors (as reviewed in (Felson & Zhang 1998; Loughlin 2001; Valdes & Spector 2010b; Loughlin 2011a)). The contribution of the latter is thought to be polygenic, following the widely accepted principle for many other complex diseases which suggests that several common variants with moderate effect sizes all contribute to the onset of the disease (as reviewed in (Dai & Ikegawa 2010; Meulenbelt 2012; Panoutsopoulou & Zeggini 2013)). The study of the genetic architecture of OA is further complicated by the fact that while end-stage OA appears to manifest similar symptoms, predominantly in the articular cartilage (Section 1.2.4), there are in fact a variety of phenotypes such as pain, joint shape and JSN, termed ‘endophenotypes’, which may have different genetic aetiologies (as reviewed in (Loughlin 2011a; van Meurs & Uitterlinden 2012; Panoutsopoulou & Zeggini 2013)). Furthermore, there may be differences in the genetic contributions between different ethnic groups, gender and joints (as reviewed in (Loughlin 2001; Loughlin 2011a; Hoaglund 2013; Panoutsopoulou & Zeggini 2013)).

1.3.1 Heritability of Osteoarthritis

In 1941 Stecher identified a familial inheritance of Heberden’s nodes, a feature frequently associated with hand OA, was more frequent among females and thought to follow a pattern of dominant Mendelian inheritance ((Stecher 1955; Kellgren et al. 1963) and as reviewed in (MacGregor & Spector 1999; Spector & MacGregor 2004; Fernandez-Moreno et al. 2008; Valdes & Spector 2008)). An epidemiological study carried out by Kellgren et al. (1963) in the UK, also reported a genetic contribution to the development of Heberden’s nodes and generalised OA (i.e. at multiple sites). The authors also suggested that OA inheritance may be polygenic ((Kellgren et al. 1963) and as reviewed in (MacGregor & Spector 1999; Spector & MacGregor 2004; Fernandez-Moreno et al. 2008; Valdes & Spector 2008)). The Baltimore Longitudinal Study of Aging ((Hirsch et al. 1998) and the Framingham Offspring Study (Felson et al. 1998) confirmed the increased risk of hand and knee OA among individuals with affected family members and showed that OA is a multifactorial
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

disease with a recessive Mendelian pattern of inheritance (as reviewed in (MacGregor & Spector 1999; Spector & MacGregor 2004)).

Sibling studies in the UK (Chitnavis et al. 1997; Lanyon et al. 2000; Neame et al. 2004), Sweden (Lindberg 1986) and Iceland (Ingvarsson et al. 2000) calculated that individuals with a sibling, who had undergone joint arthroplasty, were at an increased risk of developing OA themselves when compared to controls (as reviewed in (MacGregor & Spector 1999; Spector & MacGregor 2004; Valdes & Spector 2008)).

However, familial clustering can be confounded by shared environmental factors. Twin studies are able to determine the genetic contribution to the disease independently of environmental factors (as reviewed in (MacGregor & Spector 1999; Spector & MacGregor 2004; Valdes & Spector 2008)). To this end, Spector et al. (1996) carried out a classic twin study on mono- and di-zygotic female twins in the UK. They reported the heritability for radiographic hand and knee OA to be between 39-65%. Using an extended group from the same cohort, MacGregor et al. (2000) identified ~60% heritability for radiographic hip OA. All genetic liability estimates were corrected for age and BMI and also correlated with JSN and osteophytes (as reviewed in (MacGregor & Spector 1999; Loughlin 2001; Spector & MacGregor 2004; Valdes & Spector 2008; Valdes & Spector 2010b)). Page et al. (2003) reported an estimate of 61% heritability for radiographic hip OA in mono- and di-zygotic male twins in the United States of America (USA).

These studies have revealed that OA is not inherited as a Mendelian trait (as reviewed in (Loughlin 2001)) and justified further investigation into the genetic architecture in the form of linkage, candidate gene and association studies. These efforts have contributed the discovery of OA susceptibility genes, identified several of the variants responsible, determined the magnitude of their effect and elucidated to the mechanisms by which they influence the pathogenesis of OA.

CHAPTER 1: INTRODUCTION
1.3.2 Linkage Studies

Linkage studies have used genetic markers such as single nucleotide polymorphisms (SNPs) and short tandem repeats (microsatellites), to determine regions of genetic linkage which co-segregate with the OA phenotype (as reviewed in (Stein & Elston 2009)). The results of these studies (Table 1.1) have suggested several potential loci which may house OA susceptibility genes, some of which have been followed-up in candidate gene studies (Section 1.3.3) (as reviewed in (Valdes & Spector 2008)).
<table>
<thead>
<tr>
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<th>Cytogenetic location</th>
<th>Population</th>
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INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 1: INTRODUCTION

<table>
<thead>
<tr>
<th>Chr.</th>
<th>Cytogenetic location</th>
<th>Population</th>
<th>Joint</th>
<th>Gender</th>
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Table 1.1: Chromosomal loci for primary OA of the hand, hip and knee, identified by linkage analysis (as reviewed in (Loughlin 2001; Valdes & Spector 2008)) UK, United Kingdom; USA, United States of America.

1.3.3 Candidate Gene Studies

Candidate gene studies require a prior knowledge and understanding of the disease pathogenesis in order to enable viable genes to be selected for analysis. Clues as to the location of these genes can be found in linkage studies (as reviewed in (Valdes &
Other studies on rare and extreme phenotypes which manifest in skeletal malformations and secondary OA conditions, such as spondyloepiphyseal dysplasia and chondrodysplasias, could provide insight as to which pathways might be involved in the OA process (as reviewed in (Spector & MacGregor 2004; van Meurs & Uitterlinden 2012)). These pathways include those involved in skeletal development (BMPs), Wnt signalling, cartilage ECM homeostasis, chondrocyte and osteocyte differentiation, inflammation, apoptotic, mitochondrial, hormonal (thyroid and oestrogen) and pain sensitivity (Fernandez-Moreno et al. 2008; Valdes & Spector 2008; Valdes & Spector 2010a). A summary of the genes in these pathways reported to contribute to OA are listed in Table 1.2.

Although many candidate genes studies have yielded results that were nominally significant, very few of the candidate genes shown in Table 1.2 were associated with OA at a genome-wide significant level (p≥5x10^{-8}). An example of a candidate gene which has been associated with OA at a level of genome-wide significance is \textit{GDF5}. This gene lies on 20q11.2 (NCBI 2014a) and encodes growth differentiation factor 5, a member of the TGFβ-superfamily. \textit{GDF5} was originally studied as a candidate gene for OA due to its role in skeletogenesis (Miyamoto et al. 2007). In this study, a SNP in the 5’ untranslated region (UTR), rs143383, was associated with hip OA (p=1.8x10^{-13}, odds ratio (OR) 1.79, 95% confidence interval (CI) 1.53-2.09) in the Japanese population and knee OA in the Japanese (p=0.0021) and Chinese (p=0.00028) populations (Miyamoto et al. 2007). A meta-analysis study of European and Asian cohorts, later reported an association with knee OA which was consistent across the populations (p=9.4x10^{-7}, OR 1.15, 95% CI 1.09-1.22) (Evangelou et al. 2009). Another meta-analysis study on knee OA achieved genome-wide significance (p=4.1x10^{-11}, OR 1.18, 95% CI 1.12-1.23) in Europeans (Valdes et al. 2011b).
## Chapter 1: Introduction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Population</th>
<th>Joint</th>
<th>Gender</th>
<th>Function</th>
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<td>Stimulates ECM production by chondrocytes</td>
<td>Meulenbelt et al. 1998</td>
</tr>
<tr>
<td><strong>IL1a, IL1b, IL1RN</strong></td>
<td>UK</td>
<td>Knee</td>
<td></td>
<td></td>
<td>Loughlin et al. 2002a</td>
</tr>
<tr>
<td></td>
<td>Netherlands</td>
<td>Hip</td>
<td></td>
<td><strong>IL1</strong>: Pro-inflammatory cytokine; <strong>IL1RN</strong>, <strong>IL1</strong> receptor antagonist, MMP regulation</td>
<td>Meulenbelt et al. 2004</td>
</tr>
<tr>
<td></td>
<td>UK</td>
<td>Knee</td>
<td></td>
<td></td>
<td>Smith et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Europe</td>
<td>Hand</td>
<td></td>
<td></td>
<td>Moxley et al. 2007</td>
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<tr>
<td></td>
<td>Japan</td>
<td>Knee</td>
<td></td>
<td></td>
<td>Kanoh et al. 2008</td>
</tr>
<tr>
<td><strong>IL4R</strong></td>
<td>UK</td>
<td>Hip</td>
<td>Female</td>
<td>Regulates chondrocyte function in response to mechanical stimulation</td>
<td>Forster et al. 2004</td>
</tr>
<tr>
<td><strong>IL6</strong></td>
<td>USA</td>
<td>Knee</td>
<td></td>
<td>Pro-inflammatory cytokine</td>
<td>Nicklas et al. 2005</td>
</tr>
<tr>
<td></td>
<td>Italy</td>
<td>Hip</td>
<td></td>
<td></td>
<td>Pola et al. 2005</td>
</tr>
<tr>
<td>Gene</td>
<td>Population</td>
<td>Joint</td>
<td>Gender</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>---------</td>
<td>--------</td>
<td>--------------------------------------------------------</td>
<td>--------------------------------</td>
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<tr>
<td>IL6</td>
<td>Finnish</td>
<td>Hand</td>
<td></td>
<td>Anti-inflammatory cytokine</td>
<td>Kämäräinen et al. 2008</td>
</tr>
<tr>
<td>IL10</td>
<td>Greece</td>
<td>Knee</td>
<td></td>
<td></td>
<td>Fytiri et al. 2005a</td>
</tr>
<tr>
<td>LRP5</td>
<td>UK</td>
<td>Knee</td>
<td></td>
<td>Wnt signalling, Regulates bone mass</td>
<td>Smith et al. 2005</td>
</tr>
<tr>
<td>MATN3</td>
<td>Iceland</td>
<td>Hand</td>
<td></td>
<td>Non-collagenous ECM protein</td>
<td>Stefansson et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Netherlands</td>
<td>Hand</td>
<td></td>
<td></td>
<td>Min et al. 2006</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Spain</td>
<td>Knee</td>
<td></td>
<td>Adenosine triphosphate (ATP) energy metabolism</td>
<td>Rego-Perez et al. 2008</td>
</tr>
<tr>
<td>OPG</td>
<td>UK</td>
<td>Knee</td>
<td>Female</td>
<td>TNFα factor receptor superfamily, Osteoclastogenesis inhibitor</td>
<td>Valdes et al. 2004; Valdes et al. 2006</td>
</tr>
<tr>
<td>SMAD3</td>
<td>UK &amp; Estonia</td>
<td>Hip/Knee</td>
<td></td>
<td>Cartilage maintenance, TGFβ-signalling</td>
<td>Valdes et al. 2010</td>
</tr>
<tr>
<td>SREB2</td>
<td>Greece</td>
<td>Knee</td>
<td></td>
<td>Lipid metabolism transcription factor</td>
<td>Kostopoulou et al. 2012</td>
</tr>
<tr>
<td>TNA</td>
<td>UK</td>
<td>Knee</td>
<td>Female</td>
<td>Plasminogen-binding protein, Bone mineralisation, Osteogenesis, ECM degradation</td>
<td>Valdes et al. 2004; Valdes et al. 2006</td>
</tr>
<tr>
<td></td>
<td>UK</td>
<td>Knee</td>
<td>Male</td>
<td></td>
<td>Valdes et al. 2006</td>
</tr>
<tr>
<td>TNFα</td>
<td>USA</td>
<td>Knee</td>
<td></td>
<td>Proinflammatory cytokine</td>
<td>Nicklas et al. 2005</td>
</tr>
<tr>
<td>VDR</td>
<td>UK</td>
<td>Knee</td>
<td>Female</td>
<td></td>
<td>Keen et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Netherlands</td>
<td>Knee</td>
<td></td>
<td></td>
<td>Uitterlinden et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Finland</td>
<td>Hand</td>
<td></td>
<td></td>
<td>Solovieva et al. 2006</td>
</tr>
<tr>
<td></td>
<td>UK</td>
<td>Knee</td>
<td>Male</td>
<td></td>
<td>Valdes et al. 2006</td>
</tr>
</tbody>
</table>

Table 1.2: Genes reported to contribute to hand, hip and knee OA in candidate gene studies (as reviewed in (Loughlin 2001; Fernandez-Moreno et al. 2008; Valdes & Spector 2008; Valdes & Spector 2010a; Reynard & Loughlin 2013)) ECM, Extracellular matrix; UK, United Kingdom; USA, United States of America.
1.3.4 Genome-Wide Association Studies

Genome-wide association studies (GWAS) are a hypothesis-free approach to identify the susceptibility loci associated with complex diseases such as OA (as reviewed in (Chapman & Valdes 2012)). This high-throughput technique enables researchers to genotype millions of SNPs across tens of thousands of individuals from both case and control groups (as reviewed in (Stein & Elston 2009)). Initially, genotyping is carried out in a discovery sample set. Following this, promising SNPs are then prioritised for replication in a second larger sample set (as reviewed in (McCarthy et al. 2008; Chapman & Valdes 2012)).

By taking advantage of the linkage disequilibrium (LD) structure (i.e. correlation between nearby alleles), researchers are able to genotype fewer SNPs yet still detect up to ~85% of the Caucasian genome ((Altshuler et al. 2010) and as reviewed in (Stein & Elston 2009; Dai & Ikegawa 2010; Valdes & Spector 2010b)). Importantly, the functional variant may not be directly genotyped hence further investigation into variation at the significantly associated loci is required in order to identify the true causal variant(s) associated with the disease (Altshuler et al. 2010). Imputing information from data sets such as HapMap (http://hapmap.ncbi.nlm.nih.gov/) and 1000 Genomes projects (http://www.1000genomes.org/) can help to define linkage regions ((Altshuler et al. 2010) and as reviewed (Ioannidis et al. 2009)). Fine mapping of LD blocks tagged by variants of genome wide significance is then undertaken, in order to identify the causal variant(s) and functional studies executed, in order to validate potential susceptibility genes in the pathogenesis of OA (as reviewed in (Ioannidis et al. 2009; Gonzalez 2013; Reynard & Loughlin 2013)). The hypothesis-free nature of GWAS has enabled the discovery of new disease pathways, providing further insight into the mechanisms involved in musculoskeletal biology and shown that developmental pathways are as important in the study of OA as joint maintenance (as reviewed in (Loughlin 2011a; Reynard & Loughlin 2013)).

Studies which have identified loci associated, at a level of genome-wide significance ($p \geq 5 \times 10^{-8}$), with the prevalence and progression of OA, are discussed below.
1.3.4.1 **Double Von Willebrand Factor Type A (DVWA)**

A GWAS conducted by Miyamoto *et al.* (2008) reported the *DVWA* gene, on chromosome 3p24.3, to be significantly (p=7.3x10^{-11}) associated with knee OA in Japanese and Chinese cohorts. The tagging SNP, rs7639618 is in strong LD with rs11718863 and both are thought to alter binding to β-tubulin consequently having a detrimental effect on intracellular transport in chondrocytes. Studies in the European population did not report any significant associations with hip or knee OA (Meulenbelt *et al.* 2009; Valdes *et al.* 2009b).

1.3.4.2 **Human Leukocyte Antigen (HLA) and Butyrophilin-like 2 (BTNL2)**

Nakajima *et al.* (2010) carried out a GWAS and reported a significant association with knee OA susceptibility in the Japanese population. This region on chromosome 6 is tagged by SNPs, rs7775228 (p=2.43x10^{-8}, OR 1.34, 95% CI 1.21-1.49) and rs10947262 (p=6.73x10^{-8}, OR 1.32, 95% CI 1.19-1.46) corresponding to HLA class II/III genes (*HLA-DQA2* and *HLA-DQB1*) and *BTNL2* which are involved in the immune response and T-cell activation, respectively. In addition, combining analysis of the Japanese, Greek and Spanish populations found rs10947262 to be significantly associated (p=5.10x10^{-9}, OR 1.31, 95% CI 1.20-1.44). However, these signals were not replicated in subsequent European (Valdes *et al.* 2011c) or Chinese cohorts (Shi *et al.* 2010).

1.3.4.3 **Locus 7q22**

Kerkhof and colleagues (2010) performed a GWAS in the Dutch population and performed a subsequent meta-analysis in several other Caucasian European cohorts. They reported an OA susceptibility locus at 7q22. This locus, tagged by rs3815148, was found to be significantly associated (p=8x10^{-8}, OR 1.14, 95% CI 1.09-1.19) with hand and/or knee OA. In an additional meta-analysis study, Evangelou *et al.* (2011) confirmed an association (p=9.2x10^{-9}, OR 1.17, 95% CI 1.11-1.24) with knee OA at this locus, tagged by rs4730250 (in LD with rs3815148 ($r^2=0.63$ (HapMap-CEU^1))).

The 7q22 locus includes six genes in a large >500 kilo base pairs (kb) LD block.

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^1 CEU - Utah residents with ancestry from Northern and Western Europe
namely, G protein-coupled receptor 22 (GPR22), a component of oligomeric golgi complex 5 (COG5), high mobility group (HMG) box transcription factor 1 (HBP1), dihydouridine synthase 4-like (DUS4L), B-cell receptor-associated protein 29 (BCAP29) and protein kinase-cAMP-dependent-regulatory-type IIβ (PRKAR2B) (Kerkhof et al. 2010; Evangelou et al. 2011). Messenger ribonucleic acid (mRNA) expression was confirmed in all joint tissues for all genes (Evangelou et al. 2011).

1.3.4.4 arcOGEN study

The Arthritis Research UK OA Genetics (arcOGEN) consortium investigated the genetics of hip and knee OA in a large two-stage GWAS. All primary OA cases displayed radiographic evidence (KL≥2) of advanced disease and approximately 80% had also undergone total joint replacement (TJR). All cases were genotyped on the Illumina® 610-Quad BeadChip. Population controls, used in the discovery stages, were ascertained from publicly available data sets (Table 1.4). OA-free controls and KL<2 controls were used in some analysis in order to eliminate phenotype misclassification (Panoutsopoulou et al. 2011; Zeggini et al. 2012).

1.3.4.4.1 Stage I

During stage I, 3177 cases and 4894 controls from the UK were genotyped across 514898 SNPs. The association signals were then validated and selected for replication in silico in 4124 cases and 37581 controls. Meta-analysis of the discovery and replication data highlighted 36 SNPs which were taken forward for de novo replication in 6188 cases and 8280 controls and in silico replication in 213 cases and 2531 controls. All individuals were of European ² descent. While no statistically significant variants were identified, the strongest associations are listed in Table 1.3 (Panoutsopoulou et al. 2011).

² arcOGEN (UK), deCODE (Iceland), Rotterdam Study (Netherlands), Framingham Study (USA), Twins UK (UK), Estonian Genome Centre of University of Tartu (EGCUT) (Estonia), Genetics Osteoarthritis and Progression (GARP) (Netherlands), Santiago (Spain), Santander (Spain), Greece, Osteoporotic Fractures in Men (MrOS) (USA), Study of Osteoporotic Fractures (SOF) (USA), Oxford controls (UK)
<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele</th>
<th>Gene</th>
<th>Locus</th>
<th>Stratum</th>
<th>OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2277831</td>
<td>G</td>
<td>MICAL3</td>
<td>22q11.21</td>
<td>Hip/Knee</td>
<td>1.07 (1.04-1.11)</td>
<td>2.3x10^-5</td>
</tr>
<tr>
<td>rs11280</td>
<td>C</td>
<td>C6orf130</td>
<td>6p21.1</td>
<td>Knee</td>
<td>1.10 (1.05-1.16)</td>
<td>3.2x10^-5</td>
</tr>
<tr>
<td>rs2615977</td>
<td>A</td>
<td>COL11A1</td>
<td>1p21</td>
<td>Hip</td>
<td>1.10 (1.05-1.15)</td>
<td>1.1x10^-5</td>
</tr>
</tbody>
</table>

Table 1.3: Strongest association signals for hip and/or knee OA from stage I of arcOGEN GWAS. MICAL3, microtubule associated mono-oxygenase calponin and LIM domain containing 3; C6orf130, O-acyl-ADP-ribose deacylase 1; COL11A1, collagen type XI alpha 1 (NCBI 2014a). Chromosome locations were identified using NCBI (2014a). p values are combined discovery and replication values (Panoutsopoulou et al. 2011).

1.3.4.4.2 Stage II

In stage II, 7410 cases and 11009 controls (Table 1.4) were analysed across 485491 SNPs. *In silico* replication of 129 SNPs in 5064 cases and 40619 controls from European^3^ cohorts and *de novo* replication of 26 promising SNPs in 2409 cases and 2319 controls from UK based cohorts, identified eight loci, five of which achieved a level of genome-wide significance (Table 1.5) (Zeggini et al. 2012).

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Platform</th>
<th>No. Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTCCC2-U2KBS</td>
<td>Illumina 1.2M Duo</td>
<td>2501</td>
</tr>
<tr>
<td>WTCCC2-1958BC</td>
<td>Illumina 1.2M Duo</td>
<td>2699</td>
</tr>
<tr>
<td>T1DGC</td>
<td>Illumina HumanHap550K</td>
<td>2530</td>
</tr>
<tr>
<td>ALSPAC</td>
<td>Illumina Infinium 670k</td>
<td>743</td>
</tr>
<tr>
<td>PoBI</td>
<td>Illumina 1M</td>
<td>2536</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11009</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.4: Details of UK population-based controls WTCCC2-U2KBS and -1958BC, Wellcome Trust Case Control Consortium 2-UK Blood Donor Service and -1958 Birth Cohort; T1DGC, Type 1 Diabetes Genetics Consortium; ALSPAC, Avon Longitudinal Study of Parents and Children and PoBI, People of the British Isles (Zeggini 2010).

^3 arcOGEN (UK), deCODE (Iceland), EGcut (Estonia), GARP (Netherlands), Rotterdam Study (Netherlands) and Twins UK (UK)
## INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

**CHAPTER 1: INTRODUCTION**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele</th>
<th>Gene(s)</th>
<th>Locus</th>
<th>Stratum</th>
<th>OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
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<td>rs6976</td>
<td>T</td>
<td>GLT8D1</td>
<td>3p21.1</td>
<td>TJR</td>
<td>1.12 (1.08-1.16)</td>
<td>7.24x10^{-11}</td>
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<tr>
<td>rs11177</td>
<td>A</td>
<td>GNL3</td>
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<td>TJR</td>
<td>1.12 (1.08-1.16)</td>
<td>1.25x10^{-10}</td>
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<td>rs4836732</td>
<td>C</td>
<td>ASTN2</td>
<td>9q33.1</td>
<td>Female THR</td>
<td>1.20 (1.13-1.27)</td>
<td>6.11x10^{-10}</td>
</tr>
<tr>
<td>rs9350591</td>
<td>T</td>
<td>FILIP1, SENP6</td>
<td>6q14.1</td>
<td>Hip</td>
<td>1.18 (1.12-1.25)</td>
<td>2.42x10^{-9}</td>
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<tr>
<td>rs10492367</td>
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<td>KLHDC5, PTHLH</td>
<td>12p11.22</td>
<td>Hip</td>
<td>1.14 (1.09-1.20)</td>
<td>1.48x10^{-8}</td>
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<td>rs835487</td>
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<td>CHST11</td>
<td>12q23.3</td>
<td>THR</td>
<td>1.13 (1.09-1.18)</td>
<td>1.64x10^{-8}</td>
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<td>rs12107036</td>
<td>G</td>
<td>TP63</td>
<td>3q28</td>
<td>Female TKR</td>
<td>1.21 (1.13-1.29)</td>
<td>6.71x10^{-8}</td>
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<td>rs8044769</td>
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<td>FTO</td>
<td>16q12.2</td>
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<td>1.11 (1.07-1.15)</td>
<td>6.85x10^{-8}</td>
</tr>
<tr>
<td>rs10948172</td>
<td>G</td>
<td>SUPT3H, CDC5L</td>
<td>6p21.1</td>
<td>Male</td>
<td>1.14 (1.09-1.20)</td>
<td>7.92x10^{-8}</td>
</tr>
</tbody>
</table>

Table 1.5: Strongest association signals for hip and/or knee OA from stage II of arcOGEN GWAS Chromosome locations were identified using UCSC Genome Browser (Kent et al. 2002). p values are combined discovery and replication values. Please refer to text for gene names (Section 1.3.4.2) TJR, Total Joint Replacement; THR, Total Hip Replacement; TKR, Total Knee Replacement (Zeggini et al. 2012).
The strongest signal associated with TJR is tagged by two SNPs at locus 3p21.1, rs6976 and rs11177 (r^2=1) which lie in the 3'UTR of glycosyltransferase 8 domain containing 1 (GLT8D1) and coding region of guanine nucleotide binding protein-like 3 (GNL3), respectively (Zeggini et al. 2012). This region is discussed in detail in Section 1.4.

The other four loci reported to be of genome-wide significance are associated with hip strata. Astrotactin 2 (ASTN2), tagged by rs4836732, is reported to regulate neuronal migration in the brain ((Wilson et al. 2010; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Other genes in this region include a metalloproteinase, pappalysin-1 (PAPPA) and an E3 ubiquitin protein ligase, tripartite motif containing 32 (TRIM32) (as reviewed in (Reynard & Loughlin 2013)). Sentrin specific peptidase 6 (SENP6) and filamin A interacting protein 1 (FILIP1) are positioned either side of rs9350591 (Zeggini et al. 2012). This SNP is ~326kb from collagen XII (α1 chain) (COL12A1), a fibril-associated collagen reported to regulate bone formation ((Izu et al. 2011; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Transmembrane protein 30A (TMEM30A), cytchrome C oxidase subunit 7A2 (COX7A2), interphotoreceptor matrix proteoglycan 1 (IMPG1) and myosin VI (MYO6) are also present at this locus (as reviewed in (Reynard & Loughlin 2013)). Parathyroid hormone-like hormone (PTHLH) which lies ~96kb from rs10492367, is a promising candidate gene for OA as it is known to be involved in chondrocyte maturation, endochondral ossification and up-regulation of COL2A1 via SOX9 in hypoxic HACs ((Zeggini et al. 2012; Pelosi et al. 2013) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Rs10492367 is also ~59kb downstream of kelch domain containing 5 (KLHDC5) ((Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Carbohydrate (chondroitin-4)-sulfotransferase (CHST11), which houses rs835487, is responsible for glycosaminoglycan formation and plays an important role in growth factor signalling, bone formation and chondrocyte development ((Kluppel et al. 2005; Karlsson et al. 2010; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). RNA expression levels are also elevated in OA cartilage.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

compared to normal articular cartilage ((Karlsson et al. 2010; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)).

Three additional loci were close to genome-wide significance. Intron 12 of tumour protein 63 (TP63) contains rs12107036 and was associated with TKR in females. This gene is essential for limb, epithelial and craniofacial development ((Mills et al. 1999; Yang et al. 1999; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Female OA was associated with rs8044769 which lies within intron 1 of the fat mass and obesity associated (FTO) gene. The latter is known to influence the risk of obesity, a well documented risk factor for OA ((Lohmander et al. 2009; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)). Male OA was associated with rs10948172 which lies between suppressor of Ty3 homolog (SUPT3H) and cell division cycle 5-like (CDC5L). The role of these genes in OA is unclear however this locus could be correlated with RUNX2 (~500kb away), a vital transcription factor in skeletogenesis (Section 1.2.3.2) ((Stein et al. 2004; Zeggini et al. 2012) and as reviewed in (Panoutsopoulou & Zeggini 2013)).

1.3.4.5 Nuclear Receptor Co-Activator 3 (NCOA3)

NCOA3 is a nuclear receptor co-activator thought to play a role in hormone-mediated transcriptional activity. In a recent meta-analysis on European subjects, rs6094710 which tags NCOA3 at locus 20q13, reached genome-wide significance (p=7.9x10^{-9}, OR 1.28, 95% CI 1.18-1.39) in association with hip OA. This study also reported lower mRNA expression of NCOA3 in OA cartilage (Evangelou et al. 2013b).

1.3.4.6 Additional Susceptibility Loci

Although some susceptibility loci for OA have been identified, as mentioned above, a complete understanding of the genetic architecture of OA has yet to be achieved. Some authors refer to this as ‘missing heritability’. There may be a number of reasons why the genetic risk factors for complex common diseases, such as OA, have yet to be identified. Firstly, many risk alleles are rare (minor allele frequency (MAF) <1%) and not captured by current genotyping arrays (as reviewed in (Loughlin 2011b; Chapman & Valdes 2012)). Secondly, susceptibility variants may have low OR (≤1) and therefore small individual effects (Figure 1.4:) which require large
study sizes in order to be identified (as reviewed in (Loughlin 2011b; Chapman & Valdes 2012; van Meurs & Uitterlinden 2012)). And lastly, polymorphisms which alter more than a single nucleotide, such as copy number variants (CNVs), may be at play however the number of these variants represented on current arrays is low (as reviewed in (McCarroll & Altshuler 2007; Loughlin 2011b; Chapman & Valdes 2012)). Furthermore, discrepancies in disease phenotype definition and the subsequent errors in case-control classification, particularly when population-based controls are used, have reduced the chance of identifying significant associations (as reviewed in (Chapman & Valdes 2012; van Meurs & Uitterlinden 2012; Panoutsopoulou & Zeggini 2013)).

To overcome these obstacles the ongoing efforts of consortia such as arcOGEN and Translational Research in Europe Applied Technologies for OA (TreatOA), have

**Figure 1.4: Disease variant distribution** (adapted from Koeleman et al. 2013 (review)).
increased study power through the recruitment of large numbers of subjects and international meta-analysis ((Chapman et al. 2008; Evangelou et al. 2011; Zeggini et al. 2012; Evangelou et al. 2013b) and as reviewed in (McCarthy et al. 2008)).

Phenotype heterogeneity may be reduced by classifying cases according specific symptoms referred to as ‘endophenotypes’ (Section 1.3). A member of the Wnt signalling pathway involved in chondrogenesis, DOT1-like histone H3K79 methyltransferase (DOTIL) at locus 19p13.3 contains rs12982744 which was recently reported to be associated with hip JSN (a representation of cartilage thickness) (p=1.1x10^{-11}) (Betancourt et al. 2012) and male hip OA in a European meta-analysis (p=7.8x10^{-9}, OR 1.17, 95% CI 1.11-1.23) (Evangelou et al. 2013a). Hip morphology was shown to be correlated (p=0.005) with rs12885300, which lies within iodothyronine-deiodinase enzyme 2 (DIO2) (Waarsing et al. 2011). Symptomatic knee OA in the Caucasian population was reported in a candidate gene study to be associated with rs8065080 (p=0.00039, OR 0.75, 95% CI 0.64-0.88 (vs. healthy controls); p=0.0136, OR 0.73, 95% CI 0.57-0.94 (vs. asymptomatic knee OA)), which lies in transient receptor potential cation channel subfamily V member 1 (TRPV1), a gene involved in pain sensitivity (Valdes et al. 2011a). Further investigation into symptomatic OA-related pain identified rs900414, an intronic SNP in the serine protease paired amino converting enzyme 4 (PACE) gene (PCSK6) (p=4.3x10^{-5}, OR 1.35, 95% CI 1.17-1.56 (vs. asymptomatic knee OA)) in a meta-analysis of the Caucasian population (Malfait et al. 2012) and rs7958311 (p=3.3x10^{-4}) of purinergic receptor (P2X) ligand-gated ion channel 7 (P2RX7) in a Canadian meta-analysis which investigated chronic pain in a combined cohort of OA and post-mastectomy patients compared to unaffected controls (Sorge et al. 2012).

Furthermore, next generation sequencing (NGS) which can aid in fine-mapping causal variants and the detection of rare variants (as reviewed in (Marchini & Howie 2010; van Meurs & Uitterlinden 2012; Gonzalez 2013)), can also improve upon the resolution of LD structure, as demonstrated by imputing data from the 1000 Genomes Project in a follow-up of the arcOGEN stage I study (Panoutsopoulou et al. 2011) to establish rs11842874 (p=2.1x10^{-8}, OR 1.17, 95% CI 1.11-1.23) on chromosome 13q34 as an OA susceptibility locus for hip and/or knee joints in the
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

European population (Day-Williams et al. 2011). This intronic variant lies within the guanine nucleotide exchange factor, MCF.2 cell-line-derived transforming sequence-like (MCF2L), a regulator of nerve growth (Day-Williams et al. 2011).

1.3.5 Functional Studies

A number of susceptibility loci discovered thus far are expression quantitative trait loci (eQTL). An eQTL is a polymorphism which identifies transcriptional regulatory regions affecting gene expression at a genome-wide level. This is important as it may be used as a tool for prioritising certain genes at disease-associated susceptibility loci (as reviewed (Michaelson et al. 2009)). However, few variants have been assessed in joint tissue and hence the relevance to OA is debatable (as reviewed in (van Meurs & Uitterlinden 2012)). Transcriptomic research is now beginning to compare the normal and OA cartilage expression profile using microarrays and soon RNA-sequencing, will allow for novel transcripts to be detected as well (as reviewed in (van Meurs & Uitterlinden 2012; Reynard & Loughlin 2013)). Allelic expression imbalance (AEI) studies which can detect whether there are any differences in the transcription of each allele in heterozygote individuals are also being used in an attempt to prioritise genes for further investigation.

Several groups (Aigner et al. 2006; Sato et al. 2006; Fukui et al. 2008; Geyer et al. 2009; Sanchez-Sabate et al. 2009; Karlsson et al. 2010; Del Rey et al. 2012; Xu et al. 2012) have performed genome-wide RNA microarray analysis for OA and revealed significantly altered expression in cartilage, subchondral bone and synovial tissues.

Follow-up studies on GWAS loci revealed, rs143383, in the 5’UTR of GDF5 alters the mRNA expression of this gene in joint tissues ((Miyamoto et al. 2007; Southam et al. 2007; Egli et al. 2009) and as reviewed in (Reynard & Loughlin 2013)). Further studies revealed it was under the influence of an adjacent SNP, rs143384. The authors also reported AEI for rs143383 and another SNP in the 3’UTR, rs56366915 ((Egli et al. 2009) and as reviewed (Reynard & Loughlin 2013)). GPR22 harbours an eQTL in strong LD ($r^2=0.95$) with the tagging SNP at locus 7q22 (Kerkhof et al. 2010). This eQTL was reported in a lymphoblast cell line and further investigation revealed differential expression of GPR22 in normal and OA mouse chondrocytes.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

(Kerkhof et al. 2010) however it was not detected in human cartilage (Raine et al. 2012). Raine et al. (2012) showed that the other genes at this locus were differentially expressed, with HBPI (rs4730250, rs3815148) showing significantly reduced expression in OA cartilage and synovium and an AEI too. Differential expression has also been reported for DIO2 in human cartilage and an AEI was observed for rs225014 (Bos et al. 2012).

1.3.6 Epigenetic Studies

Epigenetic mechanisms are heritable changes which alter the expression of a gene in response to development, differentiation and the environment without causing changes to the deoxyribonucleic acid (DNA) (as reviewed in (Reynard & Loughlin 2012)). In OA these changes can occur in response to injury, mechanical loading and aging (as reviewed in (Barter & Young 2013)). There are three forms of epigenetic alterations which can occur namely, DNA methylation, chromatin alterations and non-coding RNAs.

DNA methylation can inhibit transcription by targeting CpG islands in gene promoter or enhancer regions (as reviewed in (Reynard & Loughlin 2012; Gonzalez 2013)). Reynard et al. (2011) reported modified expression of GDF5 was due to methylation at the 5'UTR SNP, rs143383. DNA methylation has also been shown to contribute to the OA process by altering the expression of inducible NO synthase gene (iNOS), SOX9, MMP-3, -9, -13, IL1β, ADAMTS4, leptin, BMP7 and superoxide dismutase 2 (SOD2) (as reviewed in (Reynard & Loughlin 2012; Barter & Young 2013; Gonzalez 2013)).

Histone methylation, acetylation, phosphorylation, sumoylation and ubiquitination can modify the chromatin structure and thereby alter transcription factor access (as reviewed in (Reynard & Loughlin 2012)). DOT1L for example, is a histone methyltransferase which can activate members of the Wnt signalling pathway (Betancourt et al. 2012). The expression of COL-2A1, -9A1, ACAN, RUNX2, ADAMTS-5, -9, COMP, MMP-1, -13 are regulated by histone deacetylases (HDACs).

\footnote{SUMO – Small ubiquitin-related modifier (NCBI 2014a)}
Investigating the Role of GNL3 in Osteoarthritis

Methyltransferase, *SET-1A* regulates the activity of cyclo-oxygenase 2 (*COX2*) and *iNOS* promoters. Sirtuin 1 (*SirT1*) inhibits NFκB-induced apoptosis in response to inflammatory cytokines (as reviewed in (Reynard & Loughlin 2012; Barter & Young 2013)).

Non-coding RNAs include long non-coding RNAs (lncRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), which bind to target RNAs causing post-transcriptional degradation or translation repression. *BMP7* is targeted by miR-22, *SMAD2/3* by miR-455-3p, *SOX9* is regulated by miR-101, -140, -675 and -194 (which also regulates *SOX5*), *COL2A* by miR-29b and -675 and *MMP13* by miR-9, -146, -27b and -140. miRNAs, such as miR-675 are influenced by hypoxia and inflammation but may also act on IL1β to influence gene expression of for example *COX2* by miR-199a-3p and -101.3, *MMP13* by miR-27b, *COL2A1* and *iNOS* by miR-34 and miR-140 which regulates IGF-binding protein 5 (*IGFBP5*), *ADAMTS5* and *ACAN*. LncRNA, DA125942 regulates the expression of *SOX9* and *PTHLH*. SnoRNAs, U38 and U48 may hamper ribosomal (r)RNA processing in response in injury (as reviewed in (Reynard & Loughlin 2012; Barter & Young 2013; Gonzalez 2013)).
1.4 Locus 3p21.1

Locus 3p21.1 was found to be the most significant (Table 1.5) OA-associated locus in the arcOGEN study. This locus is tagged by two strongly correlated ($r^2=1$) (Figure 2.4) SNPs rs11177 and rs6976 which lie within GNL3 and GLT8D1 respectively (Zeggini et al. 2012). In addition to the latter, several other genes are also found at this locus namely, stabilin 1 (STAB1), polybromo 1 (PBRM1), signal peptidase complex subunit 1 (SPCS1), inter-alpha trypsin inhibitor heavy chain (ITIH)-1, -3 and -4 (ITIH-1, -3, -4), 5’-nucleotidase domain containing 2 (NT5DC2), NIMA (never in mitosis gene a)-related kinase 4 (NEK4), musculoskeletal embryonic nuclear protein 1 (MUSTN1) and transmembrane protein 110 (TMEM110) (NCBI 2014a) (Figure 1.5). Reverse transcription (RT)-polymerase chain reaction (PCR) confirmed all of these genes, except STAB1, NEK4 and ITIH-1, -3, -4 were expressed in normal and OA cartilage. Furthermore mRNA expression of all genes was also seen in other joint tissues namely, tendon, ligament, meniscus, fat pad and osteophytes except ITIH1 which was only observed in the tendon, meniscus and synovium (Zeggini et al. 2012). Three C/D box snoRNAs are also encoded at this locus namely, SNORD-19, -19B and -69 (NCBI 2014a).

Expression of the transmembrane receptor protein, STAB1 in sinusoidal endothelial cells and tissue macrophages is induced by carcinogenic and chronic inflammatory processes (as reviewed in (Kzhyshkowska 2010)). PBRM1 is an important regulator of transcriptional activity and chromatin remodelling. It functions as tumour suppressor and is associated with the progression of clear cell renal cell carcinoma (Pawlowski et al. 2013; NCBI 2014l). The serine protease inhibitors, ITIH-1, -3, -4 are serum glycoproteins which can be covalently linked to HA and are reported to be important in expansion of the cumulus oophorus and inflammation in diseases such as rheumatoid arthritis (Zhuo et al. 2004; Baranova et al. 2013; NCBI 2014f; NCBI 2014g; NCBI 2014h). The serine/threonine kinase, NEK4 is a mitotic regulator which has been suggested to play a role in the response to double-stranded DNA damage and cellular senescence in human fibroblasts ((NCBI 2014j; Nguyen et al. 2014) and as reviewed in (Reynard & Loughlin 2013)). MUSTN1 is a nuclear protein involved in developmental and regenerative processes of the musculoskeletal system.
The functions of *SPCS1* (NCBI 2014s), *NT5DC2* (NCBI 2014k) and *TMEM110* (NCBI 2014t) and snoRNAs (NCBI 2014p; NCBI 2014q; NCBI 2014r) in this region have yet to be elucidated.

*GLT8D1* belongs to the family of glycosyltransferase proteins (NCBI 2014b). The specific function of this protein has yet to be determined however a decrease in glycosaminoglycan content or an altered glycosylation pattern of proteoglycans and glycoproteins could impair the structure and stability of these proteins and/or glycosylate proteins which have an important function in chondrocyte metabolism, resulting in reduced load-bearing efficiency, an adverse increase in protease secretion by chondrocytes and subsequent cartilage loss. Therefore, *GLT8D1* may be of great functional importance in maintaining the material properties of cartilage and the surrounding synovial fluid.

While a number of these genes appear viable candidates in the study of OA, my research focused on *GNL3*, not only because it houses one of the index SNPs for this locus but there was considerable functional evidence (Section 1.4.1) reported in the literature which I speculated could contribute to the pathogenesis of OA.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 1: INTRODUCTION

Figure 1.5: Regional association plot for locus 3p21.1 showing the statistically significant rs6976 associated with TJR. Chromosome 3 positions (mega base pairs (Mb) (NCBI build 36) are plotted against discovery set case-control association values (\(-\log_{10}(p \text{ value})\)) for each SNP. \(r^2\) values are the estimated correlation coefficients for each SNP in relation to rs6976 in the CEU\(^3\) population (International HapMap Project 2005). Red line, recombination rates (centimorgan (cM)/Mb); Purple diamond, p value for the discovery set; Purple square, p value for combined discovery and replication meta-analysis (Zeggini et al. 2012).

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\(^3\) CEU - Utah residents with ancestry from Northern and Western Europe
1.4.1 Nucleostemin (\(GNL3\))

1.4.1.1 Structure

In \(Homo sapiens\) (GRCh37/hg19), \(GNL3\) (NM_014366.4) (NCBI 2014c) consists of 15 exons and is known to encode the 549 amino acid (Ensembl 2014), \(\sim 62\)kDa protein (Stothard 2000) nucleostemin (Tsai & McKay 2002) (NP_055181.3 (NCBI 2014c), CCDS2861.1 (NCBI 2014n)). Other transcripts, (NM_206825/6.1) (NCBI 2014d; NCBI 2014e) which encode a smaller 537 amino acid (Ensembl 2014), \(\sim 60.5\)kDa (Stothard 2000) protein (NP_996561.1 (NCBI 2014d; NCBI 2014e), CCDS43100.1 (NCBI 2014o)) have also been reported.

Nucleostemin belongs to the family of YlqF/YawG GTPases (as reviewed in (Tsai & Meng 2009)). It consists of a N-terminal basic domain which houses \(rs11177\), coiled-coil domain, MMR_HSR1 guanosine-5'-triphosphate (GTP)-binding site domain (G1-G5), intermediate region and C-terminal acidic domain (Figure 1.6) (Tsai & McKay 2002; Tsai & McKay 2005; Meng et al. 2007).

![Figure 1.6: Schematic representation of nucleostemin domains and protein binding sites N, N-terminus; G4 and G1, GTP-binding sites (G5, G2 and G3 not shown); C, C-terminus. Red bar, \(rs11177\). The intermediate domain lies between the GTP-binding sites and the acidic (adapted from Ma & Pederson 2008b (review)).](image)

1.4.1.2 Nucleolar Localisation

Nucleostemin is a nucleolar protein which shuttles bi-directionally between the nucleolus and nucleoplasm in a GTP-dependent manner in response to intra- and extra-cellular signals (Tsai & McKay 2005). The N-terminal is vital in directing nucleostemin to the nucleolus and the GTP-binding motifs regulate the accumulation of protein aggregates (Tsai & McKay 2002; Tsai & McKay 2005). These two regions interact with ribosomal L1-domain-containing 1 (\(RSL1D1\)) which dictates the
subnucleolar distribution of nucleostemin (Meng et al. 2006). The binding of GTP, releases the intermediate domain from the nucleoplasm thereby preventing it from suppressing the nucleolar localisation of the basic domain (Tsai & McKay 2005; Meng et al. 2006). The intermediate region and acidic domain are necessary if nucleostemin is to exit the nucleolus and dissociation of GTP results in expulsion of nucleostemin to the nucleoplasm (Tsai & McKay 2005).

1.4.1.3 Expression

Originally identified in the nucleoli of rat embryonic neural stem cells, cell lines (HEK293⁶, CHO⁷, H1299⁸, U2OS⁹ and Saos-2¹⁰) and primitive bone marrow cells (Tsai & McKay 2002), nucleostemin expression has since been confirmed in several other stem and cancer cells, injured or regenerating tissues and cell lines. Nucleostemin expression was confirmed in adult bone marrow MSCs of human (Kafienah et al. 2006), mouse (Baddoo et al. 2003) and panda (Liu et al. 2013), mouse embryonic stem cells (Politz et al. 2005), mouse spermatogonia and spermatocytes (Ohmura et al. 2008). Human brain tumours (Malakootian et al. 2010) and human liver, gastric, pancreatic, bladder, renal and oesophagus squamous carcinomas (Liu et al. 2004) also displayed nucleostemin expression. Other cancer cell lines expressing nucleostemin include prostate cancer (PC-3¹¹) (Liu et al. 2010), Burkitt’s lymphoma (Raji¹²) (Huang et al. 2008), leukaemia (CCRF-CEM¹³) (Huang

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⁶ HEK293 - Human embryonic kidney cells (Sigma-Aldrich Co. 2014a)
⁷ CHO - Chinese hamster ovary (Sigma-Aldrich Co. 2014b)
⁸ H1299 - Human non-small cell lung carcinoma derived from a 43 year old Caucasian male (ATCC 2014f) (p53 null) (Tsai & McKay 2002)
⁹ U2OS - Osteosarcoma cells derived from the tibia sarcoma of a 15 year old female (Sigma-Aldrich Co. 2014d) (wildtype p53) (Tsai & McKay 2002)
¹⁰ Saos-2 - Osteosarcoma cells derived from an 11 year old Caucasian female (Sigma-Aldrich Co. 2014c) (p53 null) (Tsai & McKay 2002)
¹¹ PC-3 - Prostate adenocarcinoma derived from a 62 year old Caucasian male (ATCC 2014g)
¹² Raji – B lymphocytes derived from the maxilla of an 11 year old Black male (ATCC 2014h)
¹³ CCRF-CEM - T lymphoblasts derived from peripheral blood of a 4 year old Caucasian with acute lymphoblastic leukaemia (ATCC 2014a)
et al. 2008), HL-60\textsuperscript{14} (Huang et al. 2011), NB4\textsuperscript{15}, K562\textsuperscript{16} (Huang et al. 2009), breast adenocarcinoma (MCF-7\textsuperscript{17}) (Huang et al. 2009) and cervical cancer (HeLa\textsuperscript{18}) (Ma & Pederson 2007; Romanova et al. 2009). With regard to joint tissues, nucleostemin expression has been confirmed in mouse primary chondrocytes (Gossan et al. 2013) and human chondrosarcoma cell line (SW-1353\textsuperscript{19}) (Gossan et al. 2013), rat (Politz et al. 2005) and mouse skeletal myoblasts (Hirai et al. 2010), human ligament stem cells (Zhang et al. 2011) and rabbit (Zhang & Wang 2010) and human tendon stem cells (Zhang & Wang 2013). The latter have also shown increased expression under hypoxic conditions in vitro (Zhang & Wang 2013). Nucleostemin is expressed in rat and mouse cardiomyocytes. The latter also showed an increase in the expression of nucleostemin in response to injury (Siddiqi et al. 2008). This was also observed in mouse hepatocytes in response to liver injury (Shugo et al. 2012). Furthermore, limb and lens regeneration in the newt also showed an increased accumulation of nucleostemin in the nucleolus (Maki et al. 2007).

1.4.1.4 Biological Function

Nucleostemin has been implicated in the processes of proliferation and cell cycle progression (Tsai & McKay 2002) which contribute to its role in embryogenesis, organogenesis, regeneration and tumorigenesis (see below and Section 1.4.1.3). It has also been suggested to play a role in rRNA processing and ribosome biogenesis (Romanova et al. 2009).

\textsuperscript{14} HL-60 – Promyeloblasts derived from peripheral blood of a 36 year old Caucasian female with acute promyelocytic leukaemia (ATCC 2014c)

\textsuperscript{15} NB4 – Acute promyelocytic leukaemia cell line derived from a 20 year old female (Lanotte et al. 1991)

\textsuperscript{16} K562 – Chronic myelogenous leukaemia cell line derived from a 53 year old female (ATCC 2014d)

\textsuperscript{17} MCF-7 – Mammary gland epithelial cells derived from a 69 year old Caucasian female (ATCC 2014e)

\textsuperscript{18} HeLa - Cervical adenocarcinoma cell line derived from a 31 year old Black female (ATCC 2014b)

\textsuperscript{19} SW-1353 - Chondrosarcoma cells derived from the humerus of a 72 year Caucasian female (ATCC 2014i)
Nucleostemin expression levels rapidly decrease prior to cell cycle exit and it is through this mechanism, that the proliferative ability of several cancer cells and stem cells are maintained. Accordingly, expression of GNL3 is low in differentiated cells (Tsai & McKay 2002; Beekman et al. 2006).

Several studies have observed cell cycle arrest in response to alterations in nucleostemin, suggesting it may play a role in maintaining G1-S (Beekman et al. 2006; Ma & Pederson 2007; Dai et al. 2008) or G2-M (Tsai & McKay 2002; Meng et al. 2008) progression. The shuttling of nucleostemin is altered as mitosis progresses. During interphase, nucleostemin is predominantly localised in the nucleolus although, some may be present in the nucleoplasm. Nucleostemin then progressively dissociates from the nucleolar region, primarily during prophase and appears more diffusely distributed in later stages of the cell cycle (Tsai & McKay 2002).

Both overexpression of nucleostemin or a loss thereof may have an adverse effect on the cell. Successful nucleostemin knockdown experiments have employed small interfering RNAs (siRNAs) to produce a non-proliferative phenotype in cortical stem cells (Tsai & McKay 2002) and osteosarcoma cell line (U2OS) (Tsai & McKay 2002; Ma & Pederson 2007; Dai et al. 2008; Meng et al. 2008). The latter also presented a similar loss-of-function phenotype when gene dosage was increased (Tsai & McKay 2002; Dai et al. 2008; Meng et al. 2008).

Tsai and McKay (2002) observed the N-terminal basic domain (Figure 1.6) of nucleostemin binds to the tumour suppressor protein 53 (p53). Ma and Pederson (2007) investigated the extent to which p53 was responsible for cell cycle arrest in response to alterations in nucleostemin. They reported that in vitro depletion of nucleostemin only arrested cell cycling in the presence of p53 and that nucleostemin itself could be down-regulated by another tumour suppressor protein, ARF. Dai et al. (2008) and Meng et al. (2008) subsequently showed that the coiled-coiled domain (and possibly the acidic domain too (Meng et al. 2008)) of nucleostemin in fact bound to the central acidic domain of mouse double minute 2 (MDM2), inhibiting its action as an E3 ubiquitin ligase thus preventing the proteasomal degradation of p53 (Dai et al. 2008). Nucleostemin may also undergo MDM2-mediated ubiquitination...
and proteasomal degradation in the nucleoplasm (Huang et al. 2009). Nucleostemin depletion could trigger a nucleolar stress response, releasing ribosomal protein L5, L11 and 5S into the nucleoplasm where they could bind MDM2 thus abrogating p53 ubiquitination (Dai et al. 2008; Donati et al. 2013). Overexpression of nucleostemin is thought to result in its nucleoplasmic accumulation where it can bind to MDM2 (Dai et al. 2008; Meng et al. 2008), this may occur in competition with ribosomal protein, L23 (Meng et al. 2008).

Nucleolar stress results in nucleolar disassembly, releasing nucleostemin and other ribosomal proteins from the nucleolus and results in p53 activation ((Rubbi & Milner 2003; Avitabile et al. 2011) and as reviewed (Lo & Lu 2010)). It can be triggered by number of factors such as injury (Avitabile et al. 2011), heat shock, nutrient depletion, DNA damage and with particular relevance to OA, hypoxia (Rubbi & Milner 2003; Ma & Pederson 2007). However, the nucleolus is reported to be intact in nucleostemin deficient mice (Beekman et al. 2006) and siRNA knockdown U2OS cells (Dai et al. 2008). Furthermore, Politz et al. (2005) showed that nucleostemin is localised to a nonribosomal subnucleolar location. In contrast, Romanova et al. (2009) used HeLa cells to demonstrate that nucleostemin is part of a large nucleolar ribosomal protein unit which contains several ribosomal proteins and proteins known to be involved in pre-rRNA processing of the 60S ribosomal subunit as well as translation initiation factors. Mutant nucleostemin homologues also disrupted large ribosomal assembly in Drosophila (Rosby et al. 2009) and zebrafish (Essers et al. 2014) studies. Nucleostemin may act to anchor these factors in the nucleolus and regulate cell proliferation by acting as a chaperone during ribosomal biogenesis. Ribosomal protein shuttling and protein/RNA complex conformation may therefore be dependent on GTP hydrolysis (as reviewed in (Lo & Lu 2010)). Huang et al. (2008; 2009) reported that in vitro depletion of guanine nucleotides results in reduced rRNA synthesis, nucleolar disassembly and nucleostemin efflux to the nucleoplasm causing MDM2-mediated degradation of nucleostemin and cell cycle arrest. In another in vitro study, Lo et al. (2012) reported that the proteasomal degradation of nucleostemin triggered by GTP depletion was most likely due to its dissociation from other nucleolar ribosomal proteins and could occur independently.
of MDM2 and ubiquitination. The authors also reported that nucleostemin mRNA levels were unaffected.

Nucleostemin knockout mice (Gnl3−/−) are embryonic lethal and did not survive beyond day 4 (Beekman et al. 2006; Zhu et al. 2006) due to the fact that the blastocyst did not enter S phase of the cell cycle which resulted in a reduction in cell proliferation (Beekman et al. 2006). Overexpression of nucleostemin could rescue the null embryos (Gnl3−/−) (Zhu et al. 2006). However, it was not possible to rescue the latter by p53 null mice (Beekman et al. 2006). Heterozygous mice (Gnl3+/−) showed no growth or fertility abnormalities (Zhu et al. 2006). Embryonic fibroblasts of heterozygous mice (Gnl3+/−) were however shown to have decreased levels of nucleostemin, reduced proliferative capacity and increased cellular senescence compared to the wildtype mice (Gnl3+/+) (Beekman et al. 2006; Zhu et al. 2006). Overexpression of nucleostemin was shown to improve upon this phenotype (Zhu et al. 2006).

Both of these studies have demonstrated the role of nucleostemin in embryogenesis. Zhu et al. (2006) proposed the reason for the increase in the number of senescent cells could be attributed to the fact that nucleostemin inhibits the telomeric association of telomere repeat binding factor 1 (TRF1) thereby preventing telomere attrition (Meng et al. 2011) or by enhancing the recruitment of DNA repair factor (RAD51) via enhanced association of sumoylated TRF1 with promyelocytic leukaemia (PML)-IV (Hsu et al. 2012). The authors (Zhu et al. 2006) also suggested nucleostemin could manipulate cellular aging of human cells in this manner.

Furthermore, both mouse knockout studies reported that despite previous reports of interaction with p53 and an increase in the number of apoptotic cells, the fact that expression of p53 was unaltered in heterozygous embryonic fibroblasts (Zhu et al. 2006) and embryonic lethality could not be rescued by removing functional p53 (Beekman et al. 2006), suggests that nucleostemin may also function independently of p53 during embryogenesis (Beekman et al. 2006; Zhu et al. 2006).
1.4.1.5 Interactions and Regulation

Nucleostemin interacts with a number of proteins involved in the regulation of biological processes such as proliferation and growth, ribosome biogenesis, cellular senescence, apoptosis, aging, endocrine functioning and response to cellular stress.

Cell cycle progression may be regulated via the ability of nucleostemin to interact with MDM2 and p53 (Section 1.4.1.4) (Tsai & McKay 2002; Dai et al. 2008; Meng et al. 2008). Pim1 kinase regulates cell survival via the Akt pathway. Nucleostemin accumulated in the nuclei of mouse cardiomyocytes when pim1 kinase was overexpressed. Furthermore, pim1 kinase and nucleostemin expression co-localised in mouse myocardium in response to infarction (Siddiqi et al. 2008). The mitogenic factor, FGF2 (Section 1.2.2.4 and Section 1.2.3.3) has been shown to increase nucleostemin expression in adult mouse cardiomyocytes (Siddiqi et al. 2008) and human bone marrow stem cells (Kafienah et al. 2006). The oncogenic protein and ribosome biogenesis regulator, myelocytomatosis oncogene, (Myc), has also been shown to enhance the expression of nucleostemin in human and rat fibroblast cell lines and in vivo (mice) by binding to the promoter region and upregulating cell proliferation, independently of p53 (Zwolinska et al. 2012).

The role of nucleostemin in pre-rRNA processing and ribosome biogenesis (Section 1.4.1.4) is supported by the fact that it is reported to be part of a large ~700kDa ribosomal complex in HeLa cells. This includes additional nucleolar proteins, Pes1, DDX21, and EBP2, ribosomal subunits (RPS-6, -8, -13, -14 and -24) and a translation initiation factor, EIF2B1α (Romanova et al. 2009).

Proteins which bind to nucleostemin in the nucleolus include ARF, nucleophosmin (or B23) and RSL1D1 (as reviewed in (Pederson & Tsai 2009)). Upstream regulation of nucleostemin is thought to occur via ARF (Section 1.4.1.4) which can also activate p53 via MDM2 (Ma & Pederson 2007). Nucleophosmin is a regulator of ribosome biogenesis and cell proliferation which interacts with the N-terminal domain of nucleostemin (Ma & Pederson 2008a; Avitabile et al. 2011). Nucleophosmin is co-localised with nucleostemin in the nucleoli of U2OS cells (Ma & Pederson 2008a). In rat cardiomyocytes and progenitor cells, stressful stimuli
disrupt the nucleolus which results in the nucleoplasmic translocation of nucleostemin and nucleophosmin (Avitabile et al. 2011). RSL1D1 (Section 1.4.1.2) interacts with the N-terminal and GTP-binding regions of nucleostemin and aids in defining its subnucleolar location (Meng et al. 2006).

Nucleostemin regulates telomere length and cellular senescence via interactions with TRF1 (Zhu et al. 2006; Meng et al. 2011). As discussed previously (Section 1.4.1.4) this can also aid the recruitment of DNA repair gene, RAD51 via TRF-sumoylation to repair telomeric damage (Hsu et al. 2012). Nucleostemin-mediated RAD51 recruitment has also been demonstrated to be vital in repairing non-telomeric chromosomes of neural stem cells. This occurs independently of ribosome biogenesis or p53 (Meng et al. 2013).

In a recent study, nucleostemin expression was shown to coincide with circadian regulation in primary mouse chondrocytes and the SW1353 chondrosarcoma cell line which the authors speculated could, in conjunction with several other genes involved in cartilage homeostasis, contribute to the age-related onset of OA (Gossan et al. 2013).

Oestrogen induces the expression of nucleostemin in MCF7 cells (Charpentier et al. 2000). This may provide an explanation for the increased risk of OA among post-menopausal women (as reviewed in (Roman-Blas et al. 2009)).

Oxidative stress, in the form of reactive oxygen species (ROS) such as hydrogen peroxide ($\text{H}_2\text{O}_2$), can protect the nucleostemin from degradation. As demonstrated in U2OS cells, this is brought about by thiol-reversible disulphide bond-mediated oligomerisation which results in the nucleolar accumulation of nucleostemin and under extreme conditions, results in the formation of insoluble aggregates (Huang et al. 2011). Nucleostemin levels were also increased under hypoxic conditions when culturing human tendon stem cells (Zhang & Wang 2013).
1.5 Study Aim

The purpose of this study was to follow-up on one of the genes from the most significant region associated with TJR in the arcOGEN GWAS. Therefore the aim of this study was to investigate the role of nucleostemin (GNL3) at locus 3p21.1 in primarily hip but also knee OA. This project focused on GNL3 because it showed functional promise due to its role in cell proliferation, ribosome biogenesis and tissue regeneration. Furthermore, it was regulated by several factors known to be associated with the onset and progression of OA such as FGF2, oxidative stress and oestrogen. Accordingly, I hypothesised that nucleostemin could potentially regulate cartilage development, maintenance and/or repair, thus making it a viable candidate in the pathogenesis of OA.

To date the literature surrounding nucleostemin is focused primarily in the field of cancer cells and stem cells. In contrast, this work is focused on the expression and function of nucleostemin in cartilage and other joint tissues. As no studies (other than the arcOGEN paper which identified locus 3p21.1) have investigated this, all work presented here attempts to address the role of GNL3 in OA through the identification of associated variants, its expression in normal and OA tissues and how this might be influenced by GNL3 genotypes and OA relevant factors such as cytokines and stress, and the use of zebrafish as an in vivo model for the assessment of gnl3 on skeletal development.

Hence, the specific aims of this thesis were to:

1. Explore the genetic differences of GNL3 using Sanger sequencing in order to identify any novel and/or causal variants which may be associated with OA.
2. Confirm the expression of GNL3 in both non-cultured human chondrocytes and cultured cells from human joint tissues (cartilage, bone and synovium).
3. Examine whether GNL3 expression differed between chondrocytes of normal and osteoarthritic origin, different genotypes or under the influence of cytokines, IL-1β, -13 and TNFα, and growth factor, FGF2 in vitro.
5. Investigate the functional role of the selected \textit{GNL3} variants in the response to cellular stress via the p53 pathway \textit{in vitro}.

6. Analyse the effect of \textit{gnl3} knockout on cartilage \textit{in vivo} using zebrafish as a model.
Chapter 2: Mutation Screening of GNL3

2.1 Introduction

The overall aim of this chapter was to identify a novel causal variant(s), representative of a predisposition to OA. The most significant hits from the arcOGEN GWAS, rs11177 (p=1.25x10^{-10}) and rs6976 (p=7.24x10^{-11}), are at locus 3p21.1 (Table 1.5). While it is recognised that several potential genes may be represented at this locus (Section 1.4), experiments have focused on GNL3 which contains the rs11177 coding variant. This variant lies in the N-terminal, a region which plays a vital role in determining the subnucleolar location of GNL3 (Figure 1.6 and Section 1.4.1.4). Furthermore, GNL3 shows promise as an OA risk factor due to the functional role which it plays in cell proliferation and ribosome biogenesis, interactions with members of the p53 pathway and regulation by OA-relevant factors such as oestrogen, FGF2 and oxidative stress (Section 1.4.1.4 and Section 1.4.1.5).

Rs11177 may only be representative of the true causal variant(s), which may be any of a number of polymorphisms in LD. Hence, the coding region of GNL3 was sequenced in an attempt to uncover any further variation which might be present. Non-coding SNPs or other forms of variation may also play a role in OA however it is notable that, only the promoter region and 10 base pairs (bp) either side of each exon were examined here.
2.2 Materials and Methods

2.2.1 Samples

2.2.1.1 Patient details

The criteria for case selection were as follows; 1) OA was unlikely to have developed from any secondary predisposition (e.g. injury or obesity) and 2) The age of onset was as young as possible, thereby enriching the sample set for cases most likely due to a genetic predisposition. The youngest available primary OA cases from the arcOGEN\(^{20}\) (Section 1.3.4.4) dataset (Males (n=19); Females (n=32); mean age ± standard deviation (SD) = 56.0 ± 4.5 years) were selected and matched for gender and age to DEXA\(^{21}\) controls (Males (n=18); Females (n=33); mean age ± SD = 55.5 ± 3.8 years). Please refer to Table 2.1 for details. In total 102 chromosomes were analysed in both the case and control populations.

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>19 (37.3%)</td>
<td>18 (35.3%)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>32 (62.7%)</td>
<td>33 (64.7%)</td>
</tr>
<tr>
<td><strong>THR</strong></td>
<td>32 (62.7%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>TKR</strong></td>
<td>17 (33.3%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>THR&amp;TKR</strong></td>
<td>2 (3.9%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>56.0 ± 4.5</td>
<td>55.5 ± 3.8</td>
</tr>
</tbody>
</table>

Table 2.1: Details of the arcOGEN cases and DEXA controls sequenced across GNL3 Values are n (%). Age, mean (years) ± 1SD. THR, Total Hip Replacement; TKR, Total Knee Replacement.

2.2.1.2 DNA Extraction

DNA was extracted from blood using the QIAamp® DNA blood kit (Qiagen®) by members of the Bone Research Group (Molecular Medicine Centre (MMC), Edinburgh). Samples were stored at -80°C.

2.2.1.3 Measuring DNA Concentration and Quality

DNA concentration was measured on the NanoDrop1000 v3.7.0 (Thermoscientific) and sample purity was assessed using the A260/A280 ratio. All samples had a ratio

---

\(^{20}\) arcOGEN - OA patients who have undergone TJR

\(^{21}\) DEXA - Osteoporosis patients who have not undergone TJR

---

**CHAPTER 2: MUTATION SCREENING OF GNL3**
ranging from 1.8 to 2.0, in order to ensure that all samples used were free from contaminants e.g. proteins and/or phenol (ThermoFisher Scientific 2008).

2.2.2 PCR

2.2.2.1 Primer Design

Using the *H. sapiens* (GRCh37/hg19) reference sequence NM_014366.4 (NCBI 2014c) (GNL3) obtained from UCSC Genome Browser (Kent et al. 2002) and excluding polymorphic sites (NCBI 2013); the online tool, Primer3 v0.4.0 (Untergasser et al. 2012) was used to design primers (Invitrogen™). Primer specificity was assessed in silico using UCSC PCR (Kent 2011).

Primers for the amplification of the 15 exons and promoter (c.-1_-173G) of GNL3 are listed in Appendix Table 7.1. Please note however that some GNL3 amplicons were sequenced with alternate primers and these are listed in Appendix Table 7.2.

2.2.2.2 Amplification

All reactions (Appendix Table 7.3) were prepared on ice, using Qiagen® Taq DNA polymerase kit with a final volume of 25µl and run on the MJ Research® thermocycler. All samples were subject to the same PCR conditions (Appendix Table 7.4) except for differing annealing temperatures (Appendix Table 7.1). Water was used as a negative control in all PCR reactions.

2.2.2.3 Visualisation

All amplified products were diluted 1:1 in 1X Orange-G loading dye (Appendix Solution 7.2.1) and run alongside a low molecular weight marker (New England BioLabs®) on a 1.5% agarose (Bioline)–tris/borate/ethylenediaminetetraacetic acid (EDTA) (TBE) (1X) gel containing 1:10000 SYBR® Safe (Invitrogen™). Visualisation took place under ultraviolet light using GENEGenius (Syngene) and GeneSnap v6.05.01 (Syngene) software.

2.2.3 DNA Sequencing

Samples were sequenced in both directions by HGU services (Medical Research Council (MRC), Edinburgh). The sequence of the promoter, coding region and 10 intronic base pairs either side of each exon were then analysed using Mutation
Surveyor®. The reason 10bp was selected as the limit, is because this region contains the splice sites for each exon and any alterations in the sequence might alter exonic splicing.

2.2.4 Statistical Analysis

Equation 2.1 was used to determine whether the genotypes for each SNP were in Hardy-Weinberg equilibrium (HWE). One degree of freedom (df) was used for all tests.

\[
\chi^2 = \sum \frac{(\text{Observed}-\text{Expected})^2}{(\text{Expected})}
\]

Equation 2.1: Calculating chi-squared \((\chi^2)\) for Hardy-Weinberg equilibrium (Hartl & Clark 1997) Observed values were obtained from mutation screening results. This was then used to calculate allele frequencies \((p + q = 1)\) which were then used to determine the number of individuals expected in each genotype \((p^2 + 2pq + q^2 = 1)\) if the populations are in HWE.

Populations found to be in HWE were then tested for allelic association. Chi-squared \((\chi^2)\) tests were conducted using MiniTab® v12.23 (MiniTab Inc) to determine whether the genotype distribution observed among the case and control subjects deviated from those expected under the null hypothesis of no association. Two df were used in all tests.

\(\chi^2\) is not considered valid when \(n \leq 5\) (Albagha 2012). The null hypothesis was rejected and any deviations in the allele frequency were considered significant and unlikely to be due to chance, if \(p \leq 0.05\).

2.2.5 Bioinformatic Analysis

To further my understanding of the variants identified in this mutation screen and to help prioritise these for further investigation;

1. Haploview 4.2 (Barrett et al. 2005) was used to calculate \(r^2\) and determine whether any variants were in LD and likely to co-segregate during recombination.
2. All polymorphisms were examined across several species namely; human, chimp, rhesus monkey, cow, dog, chicken and zebrafish, in order to determine how well conserved the polymorphic sites are (EMBL-EBI 2012).

3. Any non-synonymous coding variants, insertions and deletions were assessed in silico using SIFT v.4.0.322 (Kumar et al. 2009; J. Craig Venter Institute 2011) and PolyPhen-2 v.2.1.023 (Adzhubei et al. 2010; PolyPhen-2 2011).

4. An eQTL browser (Degner et al. 2012) was used to investigate whether any of the SNPs identified in this study had previously been reported as eQTL. The latter are defined as genetic variants which are correlated with an alteration in gene regulation and ultimately a variation in gene expression in a given cell type (Gilad et al. 2008).

5. SwissRegulon Portal (Swiss Institute of Bioinformatics 2013) was used to identify transcription factor binding motifs in the promoter of GNL3. Any variants found within a binding motif could be of importance in regulating the expression of GNL3.

6. Lastly, I referred to the reported conserved functional protein domains of nucleostemin (Figure 1.6) (Tsai & McKay 2002) and assessed whether the variants identified were located in a region which was essential for nucleostemin to carry out its role in the cell.

---

22 SIFT - Sort Intolerant From Tolerant variation by assessing the conservation of the polymorphic site across homologous sequences and physical properties of amino acids.

23 PolyPhen-2 - Polymorphism Phenotyping based on sequence conservation, phylogenetic information and protein structure.
2.3 Results

The *GNL3* promoter and 15 exons were successfully amplified and sequenced (Figure 2.1 and Table 2.2) across 51 arcOGEN cases and 51 DEXA controls.

![Mutations identified in GNL3 mutation screen](image)

**Figure 2.1:** Mutations identified in *GNL3* mutation screen (Table 2.2) Black bar indicates amino acid codon. All DNA sequences are 5’ to 3’ orientation. Chromatograms from Chromas LITE v2.1.1 (Technelysium 2012); Green, Adenine; Blue, Cytosine; Black, Guanine; Red, Thymine.
Investigating the Role of GNL3 in Osteoarthritis

<table>
<thead>
<tr>
<th>GNL3 Region</th>
<th>dbSNP</th>
<th>Genotype Frequency</th>
<th>Allele Frequency</th>
<th>HWE p value</th>
<th>Allelic Association p value</th>
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</thead>
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<td></td>
<td></td>
<td>Case</td>
<td>Cntrl</td>
<td>Allele</td>
</tr>
<tr>
<td>Promoter</td>
<td>Novel</td>
<td>c.-134G&gt;A</td>
<td>GA</td>
<td>0.02</td>
<td>0.00</td>
</tr>
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<td></td>
<td>AA</td>
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<td></td>
<td>rs1108842</td>
<td>c.-29A&gt;C</td>
<td>AC</td>
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<td>0.22</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>0.27</td>
<td>0.31</td>
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<tr>
<td>Exon 3</td>
<td>rs11177</td>
<td>c.116G&gt;A p.(R39Q)</td>
<td>GA</td>
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<td>0.43</td>
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<td></td>
<td></td>
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<td>0.18</td>
</tr>
<tr>
<td>Exon 11</td>
<td>rs2289247</td>
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<td>0.39</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0.22</td>
<td>0.24</td>
</tr>
<tr>
<td>Exon 13</td>
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<td>c.1413A&gt;G p.(=)</td>
<td>AG</td>
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<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Exon 14</td>
<td>rs151237641</td>
<td>c.1506C&gt;G p.(N502K)</td>
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<td>0.02</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 2.2: Sequencing results for GNL3<sup>+</sup> Minor Allele Frequency (MAF) taken from HapMap (Barrett et al. 2005) ‘Utah residents with Northern and Western European ancestry from the CEPH collection’ (CEU) and ‘Tuscans in Italy’ (TSI) populations (International HapMap Project 2005)<sup>b</sup> HapMap data not available. Values are derived from NHLBI Exome Sequencing Project (North America) (NCBI 2013). HWE, Hardy-Weinberg equilibrium; Cntrl, Control; N/A, χ² is considered invalid.

<sup>a</sup>Minor Allele Frequency (MAF) taken from HapMap (Barrett et al. 2005) ‘Utah residents with Northern and Western European ancestry from the CEPH collection’ (CEU) and ‘Tuscans in Italy’ (TSI) populations (International HapMap Project 2005).  
<sup>b</sup>HapMap data not available.
Mutation screening of the promoter identified a novel variant, c.-134G>A, which was seen in only one heterozygous case subject. This variant results in a guanine to adenine alteration and is reported to lie within the binding site for transcription factors, aryl hydrocarbon receptor (AHR)/aryl hydrocarbon receptor nuclear translocator/-2 (ARNT/-2) and zinc finger protein 161 homolog (mouse) (ZFP161) (Figure 2.2 and Figure 2.3) (Swiss Institute of Bioinformatics 2013).

![DNA sequence and transcription factor binding sites](image1)

**Figure 2.2:** Transcription factor binding sites upstream of GNL3 at c.-134G>A (bold). Chromosome location refers to *H. sapiens* (hg19) reference sequence. DNA sequence is in 5’ to 3’ orientation. Image adapted from SwissRegulon (Swiss Institute of Bioinformatics 2013).

![Consensus binding motifs](image2)

**Figure 2.3:** Consensus binding motifs for AHR/ARNT/ARNT2 and ZFP161 transcription factors (Swiss Institute of Bioinformatics 2013). Black arrows indicate the novel polymorphic site (c.-134G>A) identified in the mutation screen.

Another promoter SNP, rs1108842 which results in the alteration of an adenine to cytosine residue 29bp upstream of the GNL3 start site was also identified. The MAF for cases and controls at rs1108842 was 0.40 and 0.42 respectively, with no allelic association found (p=0.861) however, the populations were not in HWE (case, p<0.001; control, p<0.001). Rs1108842 was the only variant reported to be an eQTL for GNL3 in fibroblasts (ρ = -0.549, p=3x10^-7) (Dimas et al. 2009; Degner et al. 2012).
No transcription factor binding sites were reported at rs1108842 (Swiss Institute of Bioinformatics 2013).

Two non-synonymous coding variants were identified in the screen and are in strong LD \( (r^2=0.92) \) (Figure 2.4). The first of these variants is the tagging SNP from the arcOGEN GWAS, rs11177 which converts the second base, in the codon for a conserved arginine residue (Table 2.3), from a guanine to an adenine resulting in a glutamine residue in exon 3. The MAF was marginally higher in case subjects (0.44) than controls (0.39). The populations were in HWE (case, \( p=0.542 \); control, \( p=0.497 \)). There was no allelic association \( (p=0.792) \). The second variant, rs2289247 found downstream in exon 11 gives rise to a methionine residue in place of a valine as a result of the guanine nucleotide at the first position in the codon being replaced by an adenine. The populations were in HWE (case, \( p=0.723 \); control, \( p=0.152 \)). There was no allelic association \( (p=0.717) \). The MAFs were 0.45 and 0.43 in case and control populations respectively. A third non-synonymous variant which lies in exon 14, rs151237641 was identified in one heterozygous control sample. The latter variant results in an asparagine to lysine conversion as a result of the third base in the codon being altered from a cytosine to a guanine residue. \textit{In silico} assessment of the coding SNPs predicted that these variants were unlikely to be functionally significant (J. Craig Venter Institute 2011; PolyPhen-2 2011).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 2: MUTATION SCREENING OF GNL3

Figure 2.4: Linkage disequilibrium plot ($r^2$) of GNL3 variants (Table 2.2 and Figure 2.1) and rs6976 (GLT8D1) at 3p21.1\(^24\) (Barrett et al. 2005).

<table>
<thead>
<tr>
<th>Variant</th>
<th>rs1108842</th>
<th>rs11177</th>
<th>rs2289247</th>
<th>rs4532127</th>
<th>rs151237641</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novel</td>
<td>A</td>
<td>C</td>
<td>Q</td>
<td>M</td>
<td>E</td>
</tr>
<tr>
<td>Human</td>
<td>G</td>
<td>A</td>
<td>R</td>
<td>V</td>
<td>E</td>
</tr>
<tr>
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<td>C</td>
<td>R</td>
<td>M</td>
<td>E</td>
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<td>T</td>
<td>R</td>
<td>M</td>
<td>E</td>
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<td>A</td>
<td>R</td>
<td>K</td>
<td>E</td>
</tr>
<tr>
<td>Cow</td>
<td>G</td>
<td>T</td>
<td>R</td>
<td>S</td>
<td>E</td>
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<td>Dog</td>
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<td>T</td>
<td>Q</td>
<td>S</td>
<td>E</td>
</tr>
<tr>
<td>Chicken</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>L</td>
<td>E</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>-</td>
<td>-</td>
<td>Q</td>
<td>T</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Amino Acid</th>
</tr>
</thead>
</table>

Table 2.3: Conserved sites for GNL3 variants identified in the mutation screen

The synonymous variant found in exon 13, rs4532127 alters the third base of the codon for glutamic acid from an adenine to guanine residue. The latter allele was only seen in heterozygous individuals and MAF scores for cases and controls were 0.01 and 0.05 respectively.

\(^24\) rs151237641 not shown because it is not part of the HapMap data set.


2.4 Discussion

The overall aim of this project was to identify a new causal variant(s), representative of a predisposition to OA. I identified several variants in addition to the arcOGEN tagging SNP, rs11177 in exon 3. These include, two polymorphisms located upstream in the promoter region namely, rs1108842 and a novel variant, c.-134G>A as well as two additional non-synonymous coding variants, rs2289247 (exon 11) and rs151237641 (exon 14). Lastly, I identified a synonymous coding variant rs4532127 in exon 13.

The novel promoter variant was only seen in one heterozygous OA patient and is therefore a rare variant with unknown effect which alters a highly conserved guanine nucleotide (Table 2.3). It does however lie within the transcription factor binding motif (Figure 2.2 and Figure 2.3) for AHR/ARNT/ARNT2 (Swiss Institute of Bioinformatics 2013). The latter is also able to bind to nucleophosmin (Section 1.4.1.5) (ISMARA 2014). AHR is a ligand which belongs to the basic-helix-loop-helix Period-ARNT-Single-minded (bHLH-PAS) family. It forms a complex with other members of this family in response to exogenous toxins (ARNT/-2) and hypoxia (hypoxia inducible factor (HIF)-1α, -2α and -3α). These complexes subsequently bind to promoter regions of other genes and initiate transcription via RNA polymerase II recruitment which regulates gene transcription in response to the stimulus (as reviewed in (Hankinson 2008; Hankinson 2010)). The AHR/ARNT complex may function as an ubiquitin ligase to mediate the degradation of androgen and oestrogen receptors. The transcription factor complex has been demonstrated to reduce the expression of sox9b homolog in zebrafish jaws (Section 5.1) in response to environmental contaminants (Xiong et al. 2008). AHR stimulation by environmental pollutants can also induce apoptosis in rabbit articular chondrocytes by upregulating ROS levels (Lee & Yang 2012). Furthermore, AHR is expressed in the synovial cells of OA and rheumatoid arthritis patients and can increase the expression of cytokines, IL-1β, -6 and -8, in response to environmental toxins. In addition, the latter study showed that AHR expression was stimulated by TNFα in vitro (Kobayashi et al. 2008) (Section 1.2.2.4 and Section 1.2.3.3). The novel promoter variant of GNL3 also lies within an additional transcription factor binding
site (Figure 2.2 and Figure 2.3) recognised by ZFP161 (Swiss Institute of Bioinformatics 2013). GC-rich regions in the 5' regulatory domains of several mammalian genes bind to the Kruppel-like zinc finger domains (Cys2His2-type) of ZFP161 and modulate transcription (Numoto et al. 1993; Kaplan & Calame 1997; Numoto et al. 1997; Orlov et al. 2006). ZFP161 is a homologue of the mouse protein ZF5. The latter has been demonstrated to repress the transcription of β-Actin and c-myc and overexpression inhibited growth in vitro (Numoto et al. 1993; Numoto et al. 1995; Lee et al. 2004). ZF5 expression has been detected in mouse skeletal muscle (Numoto et al. 1997; Lee et al. 2004). To date, there are no reports on the function of the ZFP161 or ZF5 in skeletal development or OA. There are also no publications on the interactions of either, ZFP161 or AHR/ARNT/ARNT2 with GNL3 (NCBI 2014m).

Rs1108842 is a common polymorphism which lies 29bp upstream of GNL3. The MAF was only marginally lower amongst the OA cases than the controls however, there was no allelic association (p=0.861). It should also be noted that the populations were not found to be in HWE (case, p<0.001; control, p<0.001) which could be confounding the allelic association results. There are several possible reasons for deviations in HWE. There may have been genotyping error, patients may have been related and therefore not selected from an outbred population or other unknown factors in the patient selection may have enriched for one genotype over the other. The rs1108842 variant was in LD (Figure 2.4) with the tagging SNP, rs11177 however the likelihood of co-segregation was not very strong (r2=0.61). Having investigated the eQTL in this region, rs1108842 was found to be a cis-acting regulator of GNL3 expression in fibroblasts, which occur when chondrocyte dedifferentiation is induced (Sandell & Aigner 2001; Degner et al. 2012). All other eQTL were identified in lymphoblastoid cell lines and are thought to be irrelevant here (Degner et al. 2012). Furthermore, in a recent meta-analysis on type 2 diabetes mellitus, a condition known to be associated with adiponectin levels (an adipocyte-secreted hormone); Dastani et al. (2012) reported rs1108842 to be of genome-wide significance. This may be interest here, given the strong correlation between obesity and the increased risk of developing OA (Section 1.1.3).
The non-synonymous coding variant in exon 3, rs11177, is in perfect LD (Figure 2.4) with rs6976, which lies downstream of GNL3 in the 3’UTR of GLT8D1. These two polymorphisms represent the 3p21.1 tagging SNPs from the arcOGEN GWAS (Section 1.4). The MAF for rs11177 amongst the cases (0.44) was higher than that seen in the controls (0.39) and HapMap populations (0.42). The case and control populations were both in HWE (case, p=0.542; control, p=0.497) however, no allelic association was found (p=0.792). It may be of interest that the MAF amongst the controls (0.39) was similar to that of the controls (MAF for cases not given) used in the discovery set of arcOGEN study (0.38) (Zeggini et al. 2012). The latter also showed a difference of genome-wide significance (p=1.25x10^{-10}) between cases and controls (Table 1.5). One possible explanation for these discrepancies in significance may be that OA is a complex disease and is therefore susceptible to errors in phenotype classification which could reduce the significance of these findings. However, every effort was made to avoid such errors, and a more likely explanation for these differences is that, the data set used here is relatively small and of limited power compared to that of the sample size used in the arcOGEN study. Rs11177 lies within the codon for an arginine residue which is highly conserved across several species (Table 2.3) hence one would assume that an alteration to the amino acid residue is likely to have a detrimental effect. Furthermore, this variant lies within the N-terminal basic domain (Figure 1.6) which Tsai and McKay (2002) reported to be vital for nucleostemin to, not only carry out the function of cell cycle progression and help to localise the protein to the nucleolus but also mediate the interaction with p53 and other proteins (Section 1.4.1.2, Section 1.4.1.4 and Section 1.4.1.5). However, the in silico tools PolyPhen-2 (Adzhubei et al. 2010; PolyPhen-2 2011) and SIFT (Kumar et al. 2009; J. Craig Venter Institute 2011) predicted that rs11177 was unlikely to be functionally significant.

Downstream in exon 11 I identified rs2289247, another non-synonymous coding variant for which the MAF was only marginally greater amongst the OA cases (0.45) than the controls (0.43) and like rs11177, it was also greater than the MAF in the HapMap population (0.44). Both populations were in HWE (case, p=0.723; control, p=0.152) however, no allelic association was found (p=0.717). Bearing in mind the
lack of power in the sample size used in this study and the strong correlation \( r^2 = 0.92 \) with rs11177 (Figure 2.4), it is plausible that were rs2289247 to be genotyped in a larger, more power sample set, that the difference between cases and controls would be significantly different. There is a lack of consensus (Table 2.3) across other species at rs2289247 and *in silico* tools predicted the functional effects of this variant to be benign (Kumar et al. 2009; Adzhubei et al. 2010; J. Craig Venter Institute 2011; PolyPhen-2 2011).

The third missense variant identified in this screen is that of rs151237641 in exon 14. This is a rare variant which was genotyped in only one heterozygous control and hence the MAF is low. This variant was not genotyped in the HapMap study and therefore no statistics are available for the MAF among the CEU and Tuscans in Italy (TSI) populations (Barrett et al. 2005; International HapMap Project 2005). It was genotyped in the NHLBI exome sequencing project however the minor allele was not detected among the North American population (NCBI 2013). No linkage data was available for this variant and it does not appear to be conserved across species (Table 2.3). *In silico* predictions were benign (Kumar et al. 2009; Adzhubei et al. 2010; J. Craig Venter Institute 2011; PolyPhen-2 2011). It should be noted, that rs151237641 does lie within the acidic domain of nucleostemin (Figure 1.6) which as discussed (Section 1.4.1.2), mediates the expulsion of nucleostemin from the nucleolus upon GTP dissociation (Tsai & McKay 2005).

Considering the shortcomings in using computer based prediction methods, PolyPhen-2 (PolyPhen-2 2011) and SIFT (J. Craig Venter Institute 2011) scores suggest these non-synonymous variants would be tolerated on their own but may have a more deleterious effect as part of a larger haplotype of predisposing variants.

Lastly, I identified a synonymous variant in exon 13, rs4532127. While the MAF does appear to be greater among controls it was not possible to conduct a \( \chi^2 \) test here as \( n \leq 5 \). It was not possible to determine the linkage of this variant to any of those mentioned above. The fact that the amino acid residue is unchanged means that this site is conserved across all species investigated here (Table 2.3). However this does not rule out the possibility of an imbalance in allelic transcription and/or aberrant
RNA expression. The functional significance of rs4532127 is unknown however it is situated within the acidic domain of GNL3 (Figure 1.6) which, was reported to aid in determining the nucleolar binding affinity of nucleostemin (Section 1.4.1.2) (Tsai & McKay 2005).

I recognise the limitations of this work include the relatively small number of samples sequenced and acknowledge that other areas of the genome not analysed here, such as intronic sequences and epigenetic regulation sites, may also have a deleterious effect on the expression of GNL3. Furthermore, if a region beyond the primer binding site boundary were to be deleted on one allele, such as in the case of CNVs, this would not be detected with the Sanger sequencing techniques utilised in this study. The initial decision to sequence GNL3 was based on the fact that rs11177 was not only the strongest signal from the arcOGEN study but it lay within the coding region of GNL3 itself and although the literature supported a strong argument for the functional role of GNL3 in the pathogenesis of OA, the true gene could be any of those which lie within the recombination hotspots at locus 3p21.1 (Figure 1.5). To this end, it should be noted that GLT8D1 (NM_001010983.1 (GRCh37/hg19)) was also screened, as rs6976 lies within the 3’UTR of the latter. However, GNL3 showed promising functional significance in parallel experiments and hence work on GLT8D1 was discontinued and all future screening efforts were focused on GNL3.

In summary, the mutation screening of GNL3 identified several SNPs and a novel polymorphism in an attempt to uncover the true causal variant(s) tagged by rs11177 in the arcOGEN study. While variants in the promoter region seem promising, the future work of this project focused on the non-synonymous and strongly correlated coding variants rs11177 and rs2289247. Particularly, as rs11177 is known to be significantly different between cases and controls (Zeggini et al. 2012) and result in the alteration of a highly conserved amino acid residue. Not forgetting the fact that it is most likely functioning as part of a larger haplotype of predisposing variants, both coding and non-coding, all contributing varying individual effects. Henceforth, the functional role of GNL3, in cartilage and other joint tissues, and the influence which variants rs11177 and rs2289247, have on this and interactions with other pathways, are investigated further in this study.
While the initial aim of this study to identify the potential causal variants at locus 3p21.1 was achieved by sequencing GNL3 which housed the tagging SNP, rs11177, it is recognised that in addition to sequencing other genes in this region, further genotype analysis could be carried out on the variants identified here. Using a Taqman assay to genotype more samples at these SNPs would increase the power of this study, reducing population selection bias and eliminating any ambiguities which may arise in genotype calling when using sequencing methodologies. With regard to the latter perhaps genotyping by means of restriction enzyme digestion or heteroduplex analysis could also be used to confirm the reported genotypes.

Functional work was carried out for rs11177 and rs2289247 for reasons described above however additional functional analysis might be carried out for the other variants identified in this screen to elucidate their role in the pathogenesis of OA. It would be interesting to conduct an electrophoretic mobility shift assay in order to determine whether the novel variant which lies upstream of GNL3 is recognised by transcription factors AHR/ARNT/ARNT2 and ZFP161 or a luciferase assay to determine whether these transcription factors are able to influence GNL3 expression. It may also be of interest to determine whether alleles at this novel variant are able to influence the binding of AHR, in complex with HIF1α or ARNT/-2, to the GNL3 site in response to factors such as hypoxia or TNFα in HACs and cells from other human joint tissues. Studies in other species have shown that the expression of oestrogen receptors, cytokines (IL-1β, -6, -8) and apoptosis can be affected by AHR/ARNT. Therefore, RNA expression analysis should be carried out to determine whether these novel variants upstream of GNL3 are able to influence transcription of oestrogen receptors and cytokines. Annexin V staining could be used to determine whether there are any differences in apoptotic cell numbers between transcription factor binding site alleles.

RNA expression profiling in OA relevant human tissues and/or cell lines would be useful in determining whether variants identified in this screen are eQTL or prone to AEI. Protein localisation experiments such as fluorescent confocal microscopy would aid in determining whether the variants, particularly those located in the N-
terminal, acidic and intermediate domains, are able to influence the location of the GNL3 protein in various joint tissues.
Chapter 3: Expression of GNL3 in Joint Tissue

3.1 Introduction

OA is a disease which results predominantly in the degradation of articular cartilage. However, its onset and progression affect all joint tissues, including the bone and synovium (Section 1.2.4) (as reviewed in (Aigner & McKenna 2002; Buckwalter et al. 2005; Ge et al. 2006; Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)).

The arcOGEN study (Zeggini et al. 2012) identified locus 3p21.1, which houses GNL3 (among other genes), to be associated with OA so severe that it resulted in TJR of the hip or knee. These individuals also had KL≥2 (Section 1.2.2.5). While this project originated based on these finding, the difficulties encountered in obtaining non-OA knee joints has resulted in the majority of in vitro experiments being carried out on HACs extracted from femoral head cartilage.

The GNL3 gene (Section 1.4.1) encodes the nucleolar protein, nucleostemin which is found in the nucleoli of proliferating stem and cancer cells (Tsai & McKay 2002). It is reported to be involved in cell cycle progression, tumorigenesis, (Tsai & McKay 2002) embryogenesis (Beekman et al. 2006; Zhu et al. 2006), tissue regeneration (Maki et al. 2007; Siddiqi et al. 2008) and ribosome biogenesis (Romanova et al. 2009). Nucleostemin expression has been observed in several joint tissues, such as chondrocytes (Gossan et al. 2013), ligaments (Zhang et al. 2011), tendons (Zhang & Wang 2010; Zhang & Wang 2013) and skeletal muscle (Politz et al. 2005; Hirai et al. 2010) however, to date, its expression has not been reported in non-cultured HACs.

HACs play an active role in producing both catabolic and anabolic factors which are vital in maintaining the homeostatic balance of the surrounding ECM (as reviewed in (Goldring 2006; Martel-Pelletier et al. 2008)). The production and degradation of ECM molecules is altered in response to cytokines and growth factors (Section 1.2.2.4). Of these, IL1β is known to up-regulate the production of catabolic factors (such as MMPs) and suppress proteoglycan production by chondrocytes which aggravates the OA process ((Tetlow et al. 2001) and as reviewed in (van den Berg 1999; Cawston & Wilson 2006)). TNFα is another major catabolic cytokine which
stimulates ECM destruction (as reviewed in (van den Berg 1999; Goldring 2006)). IL13 is a regulatory cytokine which can inhibit the production of IL1β and TNFα (as reviewed in (van den Berg 1999)). FGF2 stimulates the production of structural ECM proteins and is a powerful mitogen for adult articular chondrocytes (as reviewed in (Martel-Pelletier et al. 2008)). In contrast, it has also been confirmed to hinder anabolic factors, osteogenic protein 1 and IGF1, in HACs (Loeser et al. 2005). FGF2 is released in response to mechanical injury which mediates ECM remodelling (Vincent et al. 2002). FGF2 has also been shown to up-regulate the expression of nucleostemin in human (Kafienah et al. 2006) and murine (Baddoo et al. 2003) bone marrow stem cells and murine cardiomyocytes (Siddiqi et al. 2008).

Nucleostemin, although predominantly a nucleolar protein (Section 1.4.1.2), is capable of shuttling between the nucleolus and nucleoplasm as the cell cycle progresses or in response to nucleolar stress ((Tsai & McKay 2002; Huang et al. 2008) and as reviewed in (Lo & Lu 2010)). This shuttling is mediated by GTP (Tsai & McKay 2005). In its unbound state nucleostemin is translocated to the nucleoplasm where it is degraded. This can be brought about by ubiquitination and MDM2 or may occur independently of the latter (Huang et al. 2009; Lo et al. 2012).

Evidently, the nuclear location of nucleostemin is vital if it is to function efficiently and so to this end it should be noted that rs11177 lies within the N terminal domain (Figure 1.6) which plays a fundamental role in directing nucleostemin to the nucleolus (Section 1.4.1.2) (Tsai & McKay 2002; Tsai & McKay 2005). However, the intermediate domain which houses rs2289247 may prohibit the latter if not bound to GTP (Tsai & McKay 2005; Meng et al. 2006). Furthermore, these variants could alter protein conformation which could affect interactions with other RNAs and proteins as well as nucleolar stability.

The experiments carried out in this section aim to confirm the expression of GNL3 in both non- and cultured human joint tissues, examine whether GNL3 expression differed between chondrocytes of normal and osteoarthritic origin, different genotypes or under the influence of cytokines IL-1β, -13 and TNFα and growth factor, FGF2, in vitro.
3.2 Materials and Methods

3.2.1 Human Joint Samples

3.2.1.1 Patient Samples
Cartilage samples were obtained from TJR surgeries undergone at the Royal Infirmary in Edinburgh and Victoria Hospital in Kirkcaldy and above knee amputations at Kings Mill Hospital in Nottingham. Initially, samples were collected from both hip and knee surgeries. However, the difficulties incurred in acquiring non-osteoarthritic knee cartilage meant, for the majority of experiments, HACs were extracted from articular cartilage taken from the femoral head. With regard to the experiments carried out here, all individuals who had undergone a hip or knee replacement due to severe OA are classified as ‘OA’ and all individuals who underwent joint replacement due to a neck of femur fracture (NOF) or had an above knee amputation, are hereafter referred to as ‘normal’.

3.2.1.2 Ethical Approval
The Lothian Regional Ethics Committee approved this study (Reference no: 04/S1102/41). All necessary consent forms were completed by the relevant parties and all patients remained anonymous throughout the course of this study.

3.2.1.3 Primary Human Articular Chondrocytes

3.2.1.3.1 Sample Processing
All cartilage samples were processed within 24 hours of surgery, in a class 2 microbial safety cabinet (Envair) under sterile conditions.

Cartilage was removed from the bone and cut into 1-2mm³ pieces. These were soaked in antimicrobial solution (5% fungizone (Gibco®), 5% L-glutamine (Sigma®), 5% penicillin-streptomycin (Gibco®) in phosphate buffered saline (PBS) (1X) (Invitrogen™)) for 1hr. The sample was then washed with PBS (1X) and immersed in 0.25% trypsin-EDTA (Sigma®) for 30min at 37°C in the 5% CO₂ incubator (Sanyo). Following this, the trypsin-EDTA was removed and the sample incubated overnight at 37°C in collagenase (Gibco®).
The following day, the digested tissue was passed through a sterile sieve into a 50ml polypropylene tube (BD Falcon™) and washed thrice by re-suspension in PBS (1X) (Invitrogen™) and centrifugation (Sigma® 4K15) at 2000rpm for 5min. Approximately 1-5ml seeding medium (Iscove’s Modified Dulbecco's Medium (IMDM, PAA) containing 10% foetal calf serum (FCS, Sigma®), 1% fungizone (Gibco®), 1% L-glutamine (Sigma®), 1% penicillin-streptomycin (Gibco®)) was then added to the cells and filtered through a 70μm nylon cell strainer (BD Falcon™).

### 3.2.1.3.2 Cell Counting
The concentration of HACs in the seeding medium was determined with the aid of a haemocytometer (Neubauer chamber). Firstly, 10μl of the cell solution was applied to either side of the haemocytometer, in between the glass slide and coverslip. Cells were then counted at 10X magnification under an Olympus® CK2 microscope. Cell viability was assessed using Trypan Blue (Life Technologies™).

### 3.2.1.3.3 Seeding Human Articular Chondrocytes
HACs were then seeded using at an optimal density of 5x10⁴ cells/ml in 20-25ml seeding medium (Section 3.2.1.3.1) in a 75cm² flask (Cellstar®, Greiner Bio-One). Seeding density was adjusted accordingly for different culture vessels.

### 3.2.1.3.4 Human Articular Chondrocyte Culture
The cells were grown in monolayer culture for 10-14 days at 37°C in a 5% CO₂ incubator (Sanyo), replacing the feeding medium (IMDM containing 10% FCS (Sigma®), 1% L-glutamine (Sigma®), 1% penicillin-streptomycin (Gibco®)) every 2-3 days. Culturing chondrocytes for longer periods resulted in dedifferentiation from the characteristic polygonal shape to the spindle-shaped fibroblast phenotype and expression of type I collagen.

### 3.2.1.4 Primary Human Bone Cell Cultures
Complementary DNA (cDNA) from osteoblast-like cell cultures, extracted from female OA bone, was prepared and donated by Grigore Rischitor (MMC, Edinburgh).
3.2.1.5 **Primary Human Synovial Cell Cultures**
cDNA from synovial cell cultures was prepared and donated by Samuel Gray (MMC, Edinburgh).

3.2.1.6 **Freezing Human Articular Cartilage**
Transverse sections of cartilage were snap frozen in liquid nitrogen for at least 1 min and stored at -80°C for later use in immunohistochemistry (IHC) experiments.

3.2.2 **Non-Human Joint Samples**

3.2.2.1 **Murine Cartilage Samples**
RNA from STR/ort25 and CBA mice cartilage at 26 (early OA) and 40 (late OA) weeks was donated by Andrew Pitsillides (Royal Veterinary College, London).

cDNA from the untreated, TNFα- and IL1β-stimulated cartilage from *Cb2* (cannabinoid receptor type 2) knockout (*Cb2*<sup>-/-</sup>) and C57-B16 wildtype (*Cb2*<sup>+/+</sup>) mice was prepared and donated by Asim Azfer (MMC, Edinburgh). RNA from these mice was donated by Antonia Sophocleous (MMC, Edinburgh). There is no significance in the fact that *Cb2*<sup>-/-</sup> mice were used.

3.2.2.2 **Canine Cartilage Samples**
cDNA from untreated and IL1β-stimulated canine cartilage was prepared and donated by Asim Azfer (MMC, Edinburgh).

3.2.2.3 **Bovine Cartilage Samples**
cDNA from untreated, IL1β- and IL13-stimulated bovine cartilage was prepared and donated by Asim Azfer (MMC, Edinburgh).

3.2.3 **Cell Line Culture**
All tissue culture work was carried out under sterile condition in a class 2 microbial safety cabinet (Envair).

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25 STR/ort mice spontaneously develop OA (Kyostio-Moore et al. 2011)
3.2.3.1 **Chondrosarcoma Cell Line (JJ012)**
JJ012 cells\(^{26}\), received from Joel Block (Rush University Medical Center, Chicago), were grown in Dulbecco’s Modified Eagle Medium (DMEM) (1X) (Gibco\(^{®}\)) containing 10% FCS (Sigma\(^{®}\)) and 1% penicillin-streptomycin (Gibco\(^{®}\)), at 37°C in a 5% CO\(_2\) incubator (Sanyo). The media was changed every 2-3 days.

cDNA used in initial experiments to prove the expression of *GNL3* (Section 3.3.3) and protein used as a positive control in western blots (Section 3.2.13) were kindly prepared by Noor Jamil (MMC, Edinburgh).

3.2.3.2 **Osteosarcoma Cell Line (U2OS)**
The U2OS osteosarcoma cells, donated by Bill Earnshaw (Wellcome Trust Centre for Cell Biology, Edinburgh), were grown in DMEM (PAA), containing 10% FCS (Sigma\(^{®}\)), 1% L-glutamine (Sigma\(^{®}\)), 1% penicillin-streptomycin (Gibco\(^{®}\)), for 5 days at 37°C in a 5% CO\(_2\) incubator (Sanyo). The medium was replaced once during this period.

3.2.3.3 **Chondrocyte Cell Lines (C20A4 and C28I2)**
The chondrocyte cell lines C20A4\(^{27}\) and C28I2\(^{28}\), received from Mary Goldring (Hospital for Special Surgery, Weill Cornell Medical College, New York), were cultured in DMEM (1X) (Gibco\(^{®}\)) containing 10% FCS (Sigma\(^{®}\)), 1% penicillin-streptomycin (Gibco\(^{®}\)), at 37°C in a 5% CO\(_2\) incubator (Sanyo). The media was changed every 2-3 days.

cDNA (C20A4) used in initial experiments to establish the expression of *GNL3* (Section 3.3.3) was kindly prepared by Grigore Rischitor (MMC, Edinburgh).

3.2.4 **Passaging Cells**

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\(^{26}\) The JJ012 cell line is derived from a human chondrosarcoma (Clark et al. 2010)

\(^{27}\) The C20A4 cell line is derived from the human costal chondrocytes of a 5 year old male (Goldring et al. 1994)

\(^{28}\) The C28I2 cell line is derived from the human costal chondrocytes of a 15 year old female (Goldring et al. 1994)
At ~80% confluence, cells were washed twice with PBS (1X) (Invitrogen™) and 1ml TrypLE™ Express (1X) (Life Technologies™) was added per 75cm² flask and incubated at 37°C for 5min. Cells were confirmed to be detached under an Olympus® CK2 microscope. The appropriate feeding media (10ml) was then added to the cells to inhibit the trypsin activity. This solution was homogenised using a P1000 pipette (Eppendorf®) and 1ml added to a new flask containing the same feeding media. TrypLE™ Express (Life Technologies™) and culture media volumes were adjusted accordingly for different culture vessels.) Cells were then returned to the CO₂ incubator at 37°C. The media was changed every 2-3 days until optimal confluence was reached.

3.2.5 Freezing Cells
Cells were detached as above (Section 3.2.4) and centrifuged (Sigma® 4K15) at 2000rpm for 5min at room temperature. The supernatant was removed and the pellet re-suspended in 10ml Bambanker™ (Lymphotec Inc.). The mixture was homogenised using a P1000 pipette (Eppendorf®) and divided in 1ml aliquots which were dispensed into CryoTubes® (Nunc®) and placed in an isopropanol freezing container (Nalgene™) at -80°C. This was to ensure the rate of cooling was optimal at -1°C/minute. Cells were then removed from the storage container after a minimum of 24hrs and transferred to liquid nitrogen storage.

3.2.6 Histology
3.2.6.1 Sectioning
All frozen cartilage (Section 3.2.1.6) was sectioned at 4µm by Helen Caldwell (Breakthrough Unit, Edinburgh) using a Leica CM1850 Cryostat. All sections were placed on glass slides (VWR International™ SuperFrost® Plus) and fixed with 100% acetone (Fisher Scientific). Slides were stored at -20°C and brought to room temperature prior to use.

3.2.6.2 Cytospins
Primary HACs from OA cartilage were used either immediately after overnight collagenase treatment of the cartilage or cultured for 10-14 days (Section 3.2.1.3). Cultured cells were lifted using 0.05% trypsin–EDTA (Gibco®) at 37°C for 5min,
washed twice with PBS (1X) (Invitrogen™) and re-suspended in FCS (Sigma®). Approximately 2x10^5 cells in 150µl FCS (Sigma®) were centrifuged through a filter (EZ single cytofunnel, ThermoScientific) at 1000rpm (CytoSpin™ 4 Cytocentrifuge) for 3min and fixed with 1:1 acetone:methanol (Fisher Scientific) or 100% acetone (Fisher Scientific), left to air dry and stored at -20°C.

3.2.6.3 Immunohistochemistry for GNL3

3.2.6.3.1 Immunohistochemistry

In all samples (Sections 3.2.6.1 and Section 3.2.6.2), signals were visualised using Dako EnVision®+ System-horseradish peroxidase (HRP) diaminobenzidine (DAB). Slides were washed with 0.05% PBS (1X) (Invitrogen™)-tween 20 (T) (Sigma®), treated with 0.9% H_2O_2 (Sigma®) and peroxidase-blocked for 10min. Samples were incubated for 1hr at room temperature with polyclonal goat anti-nucleostemin (AF1638, R&D Systems®) at an optimal dilution of 1 in 50 in antibody diluent (Dako). Primary antibodies were not added to negative controls. Slides were then washed with 0.05% PBS-T and incubated for 30min at room temperature with the HRP-linked secondary antibodies, polyclonal rabbit anti-goat at 1 in 200 (P0449, Dako). Excess antibodies were then removed with 0.05% PBS-T. Liquid DAB^+ substrate-chromogen solution was used to visualise the antibody binding.

3.2.6.3.2 Counterstaining and Mounting

Samples were counterstained with haematoxylin blue (ThermoFisher Scientific) for 5-20sec, rinsed in Scott’s tap water solution, dehydrated through increasing industrial methylated spirits (Genta Medical) concentrations and xylene (Genta Medical). A glass coverslip was then mounted using, DPX mounting medium (Fisher Scientific) and the slide left to air-dry.

3.2.6.3.3 Analysis

Haematoxylin stained slides were visualised on a Zeiss Axio Imager A1 microscope.

3.2.7 DNA Extraction

3.2.7.1 DNA Extraction using TRI Reagent® (Ambion®)

DNA was extracted from cell cultures with TRI Reagent® (Ambion®) using DNase-free equipment in a class 2 microbial safety cabinet (Envair). Please refer to Section
3.2.8.1 (below) for details of the steps required to achieve the opaque interphase and pink phenol phase from which the DNA was precipitated. To the latter 300µl of 100% ethanol (Sigma®) was added and mixed by inversion. Samples were then incubated at room temperature for 3min and centrifuged (Eppendorf® 5415D) at 2000 x g for 5min at 4°C. After which, the supernatant (containing the protein) was discarded.

Precipitated DNA was then washed twice by incubating the DNA pellet in 1ml DNA wash solution (Appendix Solution 7.2.2) at room temperature for 30min and mixing periodically. After each incubation period, the sample was centrifuged (Eppendorf® 5415R) at 2000 x g for 5min at 4°C and the supernatant removed. After the final wash, 1.5ml 75% ethanol (Sigma®) was added and the sample incubated for 20min with periodic mixing. After which, centrifugation took place as before.

The ethanol wash was removed and the DNA pellet air dried for 3min at room temperature. A milk alkaline solution was then added to solubilise the DNA pellet. This was achieved by dissolving the pellet in 50-200µl 8mM sodium hydroxide (NaOH) (Appendix Solution 7.2.3), followed by centrifugation (Eppendorf® 5415R) at 12000 x g for 10min to remove any insoluble material from the solution and transferring the supernatant (containing the DNA) to a new tube. The pH of the solution was adjusted to pH 7.2 by adding 1M hepes (Appendix Solution 7.2.4). Finally, EDTA (Appendix Solution 7.2.5) was added to achieve a final concentration of 1mM. DNA was stored at -20°C.

3.2.7.2 Measuring DNA Concentration and Quality
DNA concentration and quality were measured using the NanoDrop1000 v3.7.0 (Thermoscientific) as described previously (Section 2.2.1.3).

3.2.7.3 Genotyping at rs11177 and rs2289247
Samples were genotyped as described for exon 3 (rs11177) and exon 11 (rs2289247) in Section 2.2.2 and Section 2.2.3.
3.2.8 Total RNA Extraction

Prior to RNA extraction, all media was removed from cells in culture, at ~80% confluence or after the desired time point was reached. Cells not in culture were centrifuged and the supernatant was discarded. All protocols were conducted using RNase-free equipment in a class 2 microbial safety cabinet (Envair).

3.2.8.1 RNA Extraction using TRI Reagent® (Ambion®)

For a 75cm$^2$ flask, attached cells were lysed in the culture vessel, using 1ml TRI Reagent® (Ambion®) (volume was adjusted according to culture vessel) and transferred to a 1.5ml microcentrifuge tube (Eppendorf®). Alternatively, 1ml TRI Reagent® (Ambion®) was added to each of the non-cultured cell pellets. The solution was homogenised using a 1ml syringe (BD Plastipak™) with a 21G needle (BD Microlance™ 3) attached. After 5min at room temperature the tube was stored at -80°C for a maximum of 1 month.

On the day of extraction, samples were left to defrost at room temperature and the extraction process carried out according to manufacturer’s guidelines. In brief, 100µl 1-bromo-3-chloropropane (Sigma®) was added to the solution and homogenised using a P1000 pipette (Eppendorf®). After 15min at room temperature, the solution was centrifuged (Eppendorf® 5415R) at 12000 x g for 15min at 4°C and the upper clear aqueous phase (containing the RNA) transferred to a clean 1.5ml tube (Eppendorf®).

To this aqueous phase, 500µl isopropanol (Acro Organics) was added and vortexed at maximum speed for 10sec. After incubation at room temperature for 10min, the solution was centrifuged (Eppendorf® 5415R) at 12000 x g for 8min at 4°C. This resulted in a gel-like RNA pellet at the bottom of the tube. The supernatant was removed and the pellet washed in 1ml 75% ethanol (Sigma®), and centrifuged (Eppendorf® 5415R) at 7500 x g for 5min at 4°C. All ethanol was removed and the pellet air-dried for 3min and re-suspended in DEPC-treated water (Invitrogen™). RNA was stored at -80°C.
3.2.8.2 RNA Extraction using GenElute™ Mammalian Total RNA Kit (Sigma®)

Using the GenElute™ Mammalian Total RNA kit (Sigma®), RNA was extracted from a 75cm$^2$ flask, according to manufacturer’s instructions. In short, media was removed from the flask and cells were washed twice with PBS (1X) (Invitrogen™). A total of 1.5ml lysis solution was prepared, containing 10µl of β-mercaptoethanol per 1ml. The lysis solution contains guanidine thiocyanate to lyse the cells and the β-mercaptoethanol is necessary to inactivate RNases which may be present. This mixture was homogenised using a P1000 pipette (Eppendorf®), left at room temperature for approximately 1min or until cells were confirmed detached under an Olympus® CK2 microscope. The lysate was then divided into 500µl aliquots and spun through the filter column. This step destroys DNA and removes cellular debris. The lysate was then spun through a silica column. This binds the RNA so that it can be washed several times with wash solutions containing ethanol (Sigma®). The RNA was stored at -80°C.

3.2.8.3 RNA Extraction using RNeasy® RNA Extraction Kit (Qiagen®)

The RNeasy® RNA extraction kit (Qiagen®) was used, according to manufacturer protocol. Cells were then lysed in a buffer, containing β-mercaptoethanol and guanidine thiocyanate, which also inactivates RNases. On some occasions, the lysate was added to a QIAshredder spin column to ensure complete homogenisation. Through a series of ethanol washes and centrifugation the RNA bound to the membrane and RNAs (<200 nucleotides) and contaminants were removed. The membrane-bound RNA was eluted in RNase-free water (Qiagen® 2010). The RNA was stored at -80°C.

3.2.8.4 Measuring RNA Concentration and Quality

RNA concentration was measured on the NanoDrop1000 v3.7.0 (Thermoscientific) and sample purity was assessed using the A260/A280 ratio. All samples had a ratio of ~2.0, in order to ensure that all samples used were free from contaminants e.g. proteins and/or phenol (ThermoFisher Scientific 2008).
3.2.9 Reverse Transcription
All reactions were prepared on ice in a laminar flow hood (Envair) using RNase-free tubes and pipettes. All incubation steps were carried out on a G-Storm GS1 thermocycler.

3.2.9.1 Reverse Transcription using Qiagen® LongRange 2Step RT-PCR Kit
RNA was reverse transcribed using the LongRange 2Step RT-PCR Kit (Qiagen®). All reactions were prepared as described in Appendix Table 7.5. All incubation steps were as per manufacturer’s guidelines.

3.2.9.2 Reverse Transcription using Epicentre® MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit
RNA was reverse transcribed using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®). All reactions were prepared as described in Appendix Table 7.6 and Appendix Table 7.7. All incubation steps were as per manufacturer’s guidelines.

3.2.9.3 Measuring cDNA Concentration
cDNA concentration was measured on the NanoDrop1000 v3.7.0 (Thermoscientific).

3.2.10 cDNA Amplification
3.2.10.1 cDNA Amplification using Qiagen® LongRange 2Step RT-PCR Kit
3.2.10.1.1 Primer Design
One pair of intron-spanning primers was designed to amplify 1587bp of GNL3 using Primer3 v.0.4.0 (Untergasser et al. 2012) (Section 2.2.2.1). These primers are able to bind all GNL3 transcripts (NM_014366.4, NM_206825.1 and NM_206826.1) (Section 1.4.1.1). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was simultaneously amplified in order to assess cDNA quality. The latter was amplified with primers designed and donated by Grigore Rischitor (MMC, Edinburgh). All primers (Invitrogen™) are listed in Appendix Table 7.8.
3.2.10.1.2 Amplification
The cDNA was amplified using the LongRange 2Step RT-PCR kit (Qiagen®), as per manufacturer’s guidelines for a 25µl reaction (Appendix Table 7.9). Water was used as a negative control. All reactions were run on G-Storm GS1 thermocycler according to the conditions stated in Appendix Table 7.10.

3.2.10.1.3 Visualisation
All amplified products were visualised as previously described (Section 2.2.2.3) on a 1.5% agarose (Bioline)–TBE (1X) gel. Except, the 1587bp GNL3 amplicon was run alongside a 1kb ladder (New England BioLabs®).

3.2.10.1.4 Sequencing
Initial amplicons were sequenced in both directions, using the primers described in Appendix Table 7.8, by HGU services (MRC, Edinburgh). Products were confirmed to be of the correct sequence using Mutation Surveyor®.

3.2.10.2 cDNA Amplification using Qiagen® Taq DNA Polymerase Kit
3.2.10.2.1 Primer Design
3.2.10.2.1.1 Human cDNA
Primers described in the recent arcOGEN paper (Zeggini et al. 2012) were used to amplify the human GNL3 transcript. Primers for the amplification of chondrogenic markers and the housekeeping gene, β-Actin, from human cDNA were designed by Asim Azfer (MMC, Edinburgh). All primers (Invitrogen™) are listed in Appendix Table 7.11.

3.2.10.2.1.2 Non-Human cDNA
Intron-spanning primers for the amplification of non-human GNL3 transcripts were designed using Primer3 v.0.4.0 (Untergassser et al. 2012). GNL3 reference sequences for murine (NM_153547.5), canine (XM_003432798.2) and bovine (XM_002697016.2) transcripts, were obtained from NCBI (NCBI 2014a). GAPDH primers were designed by Asim Azfer (MMC, Edinburgh). All primers (Invitrogen™) are listed in Appendix Table 7.12.
3.2.10.2.2 Amplification
The cDNA was amplified using the *Taq* DNA Polymerase Kit (Qiagen®) as per manufacturer’s guidelines for a 25µl reaction. Water was used as a negative control. All reactions were run on G-Storm GS1 thermocycler according to the conditions stated in Appendix Table 7.13 and Appendix Table 7.14.

3.2.10.2.3 Visualisation
All amplified products were visualised as previously described (Section 2.2.2.3) on an agarose (Bioline)–TBE (1X) gel alongside a molecular weight marker. Please refer to text for details.

3.2.10.2.4 Sequencing
As all human primers (Appendix Table 7.11) had been used previously these were presumed to amplify the correct cDNA transcript and hence, were not sequenced here. Initial canine and bovine amplicons were sequenced in both directions, using the primers described in Appendix Table 7.12, by HGU services (MRC, Edinburgh). Products were confirmed to be of the correct sequence using Mutation Surveyor®. Murine amplicons were too small to be sequenced.

3.2.11 Quantitative PCR (qPCR)

3.2.11.1 Primer and Probe Design
Intron-spanning primers (Invitrogen™) were designed to be common for all human *GNL3* transcripts (Section 1.4.1.1). FAM™-labelled probes from the Universal ProbeLibrary (Roche Diagnostics) were selected using the ProbeFinder v.2.45 (Roche Diagnostics) (Appendix Table 7.15). The expression of housekeeping gene *18S* rRNA was also quantified in all samples using the Applied Biosystems® *18S* rRNA Endogenous Control (VIC™-MGB). Water was used as a negative control.

Associated probe-quencher molecules are released when PCR products are transcribed. This results in the emission of a quantifiable fluorescent signal by the unquenched probe (Sophocleous 2009). *GNL3* amplification was detected using FAM™-labelled probes which have an excitation wavelength of 492nm and emission wavelength of 516nm. *18S* amplification was detected using VIC™-labelled probes.
which have an excitation wavelength of 535nm and emission wavelength of 555nm (Sigma 2008).

3.2.11.2 **Amplification**

cDNA was amplified using the SensiFAST™ Probe kit (Bioline). Reactions were prepared on ice according to the conditions stated in Appendix Table 7.16 and Appendix Table 7.17. Thermocycling (Appendix Table 7.18) was carried out on the MJ Chromo4™. All samples were run in triplicate. Data was recorded and analysed using MJ Opticon Monitor™ analysis software v.3.1 (Bio-Rad).

3.2.11.3 **Standard Curve**

RNA from U2OS cultures (Section 3.2.3.2 and Section 3.2.8.2) was used as a template to produce cDNA as described in Section 3.2.9.2. The latter was amplified according to the conditions described above (Section 3.2.11.1 and Section 3.2.11.2). PCR products were purified, to remove any unwanted probes, primers and non-specific products, using QIAquick® PCR Purification Kit (Qiagen®). This was then run a on a 2% gel (Section 2.2.2.3) at 80V for 1hr alongside a 100bp ladder (New England BioLabs®). The amplicon was then extracted from the gel using QIAquick® Gel Extraction Kit (Qiagen®). The concentration of this purified amplicon was determined (Section 3.2.9.3) and the number of molecules/µl calculated using **Equation 3.1.** A 10-fold serial dilution was prepared using the purified amplicon and run on the same plate as the samples.

\[
\text{Equation 3.1: Calculating molecules/µl} \quad \text{Avogadro's number is defined as the number of molecules in one mole. bp, base pair; avg, average; nt, nucleotide Da, dalton.}
\]

\[
\text{Equation 3.1: Calculating molecules/µl} = \frac{\text{concentration (g/µl)}}{\text{copy number (g/molecule)}}
\]

\[
\text{copy number (g/molecule)} = \frac{\text{size (bp) x avg nt weight (330Da) x 2(nt/bp)}}{\text{Avogadro's number (6.022x10^{23})}}
\]

Standard curves were generated using Opticon Monitor™ v.3.1. Fluorescent signals generated after each PCR cycle are plotted on a log graph. Linear plots were created using the average C(t) value for each dilution. The C(t) value is the cycle threshold at
which log graph enters the exponential phase. It should be noted that while not all samples were run on the same plate, the same standard dilution series was run on each plate. The standard curve was calculated for each plate.

3.2.11.4 Analysis

3.2.11.4.1 Quantification
All samples were run in triplicate. Any samples which differed (≥0.5) from replicate C(t) values were removed. Average C(t) values were then compared to the standard curve and the number of molecules/µl, for each sample, calculated using Opticon Monitor™ v.3.1. Blank values were then subtracted from all samples and GNL3 normalised to 18S.

3.2.11.4.2 Statistical Analysis
The average GNL3 expression (corrected for 18S) was calculated for each sample. Samples were then grouped by disease status (i.e. normal or OA) or in vitro treatment and any values which exceeded the group average ± 3SD, were removed. P values were calculated using MiniTab® v12.23 (MiniTab Inc). A general linear model ANOVA was employed to determine whether samples differed significantly (p≤0.05) from one another in the mRNA expression of GNL3. When assessing three of more groups, a Tukey comparison test (95% confidence interval) was used to assess individual differences between the groups. Where appropriate, statistics were adjusted for age, gender, disease status and/or whether the samples were run together on the same plate or in separate experiments. Unless stated otherwise, all graphs display uncorrected averages and error bars.

3.2.12 Total Protein Extraction

3.2.12.1 Total Protein Extraction
Upon reaching approximately 80% confluence or after the desired time point was reached, all culture media was removed and cells were washed twice with PBS (1X) (Invitrogen™) containing 0.1mM sodium vanadate (Na3VO4, Sigma®). Total protein extraction was then carried out on ice. The cells were covered with lysis buffer (Protease inhibitor cocktail tablet (Roche Diagnostics), 1.0mM Na3VO4, 1% igepal (Sigma®) in 10ml PBS (1X)) for 15-30min. (Igepal (or nonident P-40 is a
detergent which has previously been used in other experiments (Lo et al. 2012) to successfully extract nucleostemin). Cells were then scraped from the flask, placed in a 1.5ml tube (Eppendorf®) and centrifuged (Eppendorf® 5415R) at 13000rpm for 15min at 4°C. The supernatant, containing the protein, was transferred to a clean tube and frozen at -80°C.

3.2.12.2 Quantification
A Pierce protein assay was used to determine the protein concentration of the samples. In a 96 well plate, 10µl of each sample, diluted 1:4 and 1:1 in water (H2O) (Gibco®), was aliquoted in duplicate alongside a set of pre-diluted bovine serum albumin (BSA) standards (Thermoscientific). Copper (II) sulphate was then diluted 1:50 in bicinchoninic acid (Sigma®) and 200µl was added to each well. Plates were then sealed and incubated at 37°C for 15min. The absorbance of each well at 562nm was measured using a BIO-TEK™ SynergyHT plate reader. Protein concentration was then calculated, using Gen5™ software, by colorimetric comparison of each sample to the standard curve.

3.2.13 Western Blot
3.2.13.1 Sample Loading and Electrophoresis
Prior to loading each sample on the gel, 25µg of protein was denatured by incubating samples at 95°C for 5min in 5X loading buffer (Appendix Solution 7.2.6). Protein extracted from JJ012 chondrosarcoma cells was used as a positive control (Section 3.2.3.1). Two molecular weight standards, Magic Mark™ XP (Life Technologies™) and Kaleidoscope (Bio-Rad), were also incubated. Samples and standards were then loaded on a Criterion™ XT precast 12% bis-tris gel immersed in XT MOPS (1X) running buffer (Bio-Rad) in an electrophoresis tank (Bio-Rad). Gels were then run at 120V for 50min.

3.2.13.2 Electrophoretic Transfer
A polyvinylidene difluoride (PVDF) membrane (GE Healthcare Amersham Hybond™ P) was activated in 100% methanol (Sigma®) for 5min and then equilibrated in transfer buffer (Appendix Solution 7.2.7) for at least 5min. The gel (Section 3.2.13.1) containing the proteins was removed from the cassette and also
submerged in transfer buffer. Extra thick blot paper, soaked in transfer buffer, was
then placed on the positive electrode of the transfer apparatus (Hoeffer TE77XP).
Successively, the PVDF membrane, gel and another blot paper were then stacked on
top. The lid of the transfer apparatus (Hoeffer TE77XP) was then closed, bringing
the negative electrode into contact with the upper blot paper. The transfer of protein
from the gel to the PVDF membrane took place at 90mA per gel for 2hrs.

3.2.13.3 Immunostaining
The protein-bound membrane (Section 3.2.13.2) was then incubated for 30min at
room temperature in 5% (w/v) skimmed milk (Marvel) – tris buffered saline (TBS)
(1X)-tween 20 (T) (Appendix Solution 7.2.8.3) to block all non-specific binding
sites. Following this the membrane was then rinsed with TBS (1X)-T and incubated
overnight at 4°C in the primary antibody solution. All primary antibodies were
diluted in 5% (w/v) milk-TBS (1X)-T solution as per Table 3.1. The following day,
membranes were washed thrice in TBS (1X)-T for a total of 1hr and then incubated
for 1hr at room temperature in the secondary HRP-linked antibody solution diluted
1:10000 in 5% (w/v) milk –TBS (1X)-T. The membrane was then washed as before.
All wash and incubation steps took place on a rocker (Biometra®) to ensure even
coverage over the membrane.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Protein</th>
<th>Host</th>
<th>Clonality</th>
<th>Conc.</th>
<th>Secondary Antibody Host</th>
<th>Band size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB4311</td>
<td>GNL3</td>
<td>mouse</td>
<td>monoclonal</td>
<td>1:1000</td>
<td>Donkey</td>
<td>62</td>
</tr>
<tr>
<td>A5060</td>
<td>β-Actin</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:1000</td>
<td>Donkey</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 3.1: Antibodies used in western blotting to detect the protein expression of GNL3
(Millipore) and β-Actin (Sigma®) All antibodies were diluted in 5% (w/v) milk -TBS-T (1X). All
secondary antibodies (Stratech®) were diluted 1:10000. kDa, kilo Dalton.

3.2.13.4 Chemiluminescence
The membrane was washed in TBS (1X) (Appendix Solution 7.2.8.2) for 10min at
room temperature to remove any tween 20 (Sigma®) which might reduce the
efficiency of the chemiluminescence. Protein expression was detected using 1ml of
Immun-Star™ WesternC™ Chemiluminescence HRP-detection kit (Bio-Rad),
prepared as per manufacturer’s instructions. Images were captured on Syngene
Investigating the role of GNL3 in osteoarthritis

GeneGnome Bio Imaging System using GeneSnap v.7.05.02 (Syngene) image software.

3.2.13.5 Antibody Removal
If proteins of a similar size were to be detected on the same membrane, then the previous antibodies were incubated in stripping buffer (Appendix Solution 7.2.9) for 10 min at 55 °C in a waterbath (Grant). The membrane was then blocked and probed as before (Section 3.2.13.3).

3.2.13.6 Analysis
3.2.13.6.1 Densitometry
All protein expression was quantified using GeneTool v.3.06.04 (Syngene) software. Background fluorescence was corrected automatically. No images were overexposed.

3.2.13.6.2 Statistical Analysis
All protein expression was normalised to the housekeeping gene, β-Actin. Samples were then grouped according to disease status (i.e. normal or OA) or in vitro treatment and any values which exceeded the average ± 3SD of the group, were removed when applicable. Outliers were first calculated for each blot and then the average value of the remaining replicates across the three blots. P values were calculated using MiniTab® v12.23 (MiniTab Inc). A general linear model ANOVA was employed to determine whether samples differed significantly (p ≤ 0.05) from one another in the protein expression (corrected for β-Actin). When assessing three or more groups, a Tukey comparison test (95% confidence interval) was used to assess individual differences between the groups. Where appropriate, statistics were adjusted for age, gender and/or disease status. Unless stated otherwise, all graphs display uncorrected averages and error bars.

3.2.14 Cytokine Treatment of Chondrocyte Cultures
Chondrocyte cultures were treated with cytokines namely, FGF2, IL1β, IL13 and TNFα.
3.2.14.1 **FGF2**
Primary HACs and cell lines, C20A4 and C28I2, were cultured as described in Section 3.2.1.3 and Section 3.2.3.3, respectively. Upon reaching 60-70% confluency, FGF2 (PeproTech®) (25ng/ml) was added to the culture media and cells returned to the incubator overnight. The following day, RNA (Section 3.2.8.1) and/or protein (Section 3.2.12.1) were extracted. All samples were cultured in duplicate.

3.2.14.2 **IL1β**
Primary HACs and cell lines, C20A4 and C28I2, were cultured as described in Section 3.2.1.3 and Section 3.2.3.3, respectively. Upon reaching 60-70% confluency, IL1β (Roche) (10ng/ml) was added to the culture media and cells returned to the incubator overnight. The following day, RNA (Section 3.2.8.1) and/or protein (Section 3.2.12.1) were extracted. All samples were cultured in duplicate.

RNA from IL1β–stimulated murine chondrocytes was kindly provided by Antonia Sophocleous (MMC, Edinburgh) and converted to cDNA by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.1).

cDNA from IL1β–stimulated canine chondrocytes was a gift to Asim Azfer (MMC, Edinburgh) who kindly provided an aliquot for this experiment (Section 3.2.2.2).

Bovine cartilage was incubated in IL1β-media. Cartilage was then digested with collagenase. RNA was then extracted and converted to cDNA. This work was carried out by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.3).

3.2.14.3 **IL13**
Bovine cartilage was incubated in IL13-media. Cartilage was then digested with collagenase. RNA was then extracted and converted to cDNA. This work was carried out by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.3).
3.2.14.4 TNFα

RNA from TNFα–stimulated murine chondrocytes was kindly provided by Antonia Sophocleous (MMC, Edinburgh) and converted to cDNA by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.1).
3.3 Results

3.3.1 GNL3 Immunohistochemistry

IHC staining of GNL3 (Section 3.2.6.3) showed dot-positive reactivity in the nuclei of primary HACs. Positive staining was identified in both non- and cultured HACs. Negative controls showed no staining of nucleostemin in the nuclei (Figure 3.1).

![Figure 3.1](image)

Figure 3.1: Dot-positive nuclear staining for GNL3 IHC in HACs (a) Primary cultured HAC cytospin from OA cartilage (bar = 10µ, inset bar = 5µ); (b) HAC in a frozen cartilage section (bar = 2.5µ); (c) Primary non-cultured HAC cytospin from OA cartilage (bar = 10µ); (d) Negative control for primary cultured HAC cytospin from OA cartilage (bar = 20µ). Brown, Nucleostemin; Blue, Nucleus.

3.3.2 DNA Genotyping at rs11177 and rs2289247 in Primary Human Articular Chondrocytes

DNA was successfully extracted from primary HACs and genotyped (Section 3.2.7). Please refer to the relevant experiments (below) for patient sample details.

3.3.3 mRNA Expression of GNL3 in Human Joint Tissues and Cell Lines

RNA from primary cultured knee HACs (Section 3.2.1.3.4) was extracted using the RNeasy® RNA extraction kit (Qiagen®) (Section 3.2.8.3). RNA from U2OS cells (Section 3.2.3.2) was extracted using the GenElute™ Mammalian Total RNA Kit (Sigma®) (Section 3.2.8.2). All RNA was converted to cDNA using the LongRange 2Step RT-PCR Kit (Qiagen®) (Section 3.2.9.1). cDNA from osteoblast-like and C20A4 cells was donated by Grigore Rischitor (MMC, Edinburgh) (Section 3.2.1.4 and Section 3.2.3.3). cDNA from synovial cells was donated by Samuel Gray (MMC, Edinburgh) (Section 3.2.1.5). cDNA from JJ012 cells was donated by Noor Jamil (MMC, Edinburgh) (Section 3.2.3.1).
cDNA was successfully amplified in all human joint tissues and cell lines using LongRange 2Step RT-PCR Kit (Qiagen®) as described in Section 3.2.10.1. The GNL3 and GAPDH amplicons are shown in Figure 3.2.

![Figure 3.2: mRNA expression of GNL3 (1587bp) and GAPDH (451bp) in human joint tissues and cell lines](image)

Figure 3.2: mRNA expression of GNL3 (1587bp) and GAPDH (451bp) in human joint tissues and cell lines cDNA PCR products were run on a 1.5% agarose gel at 75V for 45min in TBE buffer (1X). No bands were seen in the no template control (not shown). L1, 1kb DNA ladder (New England BioLabs®); L2, Low molecular weight DNA Ladder (New England BioLabs®); 1-2, Osteoblast-like cells from OA bone; 3-6, Primary cultured OA HACs; 7, Primary cultured normal HACs; 8-9, Synovial cells; 10, C20A4 chondrocytes; 11, U2OS osteosarcoma cells; 12, JJ012 chondrosarcoma cells.

### 3.3.4 Expression of GNL3 in Primary Human Articular Chondrocytes

#### 3.3.4.1 mRNA Expression of GNL3 in Primary Non-Cultured Human Articular Chondrocytes

RNA was extracted from non-cultured HACs (Section 3.2.1.3.1) using TRI Reagent® (Ambion®) (Section 3.2.8.1). All OA HACs were extracted from knee cartilage and all normal HACs were extracted from femoral head cartilage. All RNA was converted to cDNA using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2).

cDNA (40ng) was successfully amplified using DNA Polymerase Kit (Qiagen®) as described in Section 3.2.10.2. The GNL3 and β-Actin amplicons are shown in Figure 3.3. The results show expression of GNL3 and β-Actin in non-cultured OA knee HACs and non-cultured normal femoral head HACs.
Figure 3.3: mRNA expression of GNL3 (208bp) and β-Actin (250bp) in primary non-cultured HACs taken from normal femoral head and OA knee cartilage. cDNA PCR products were run on a 2% agarose gel at 75V for 45min - 1hr in TBE buffer (1X). No bands were seen in the no template control. L, Low molecular weight DNA Ladder (New England BioLabs®); 1, No template control for GNL3; 2, No template control for β-Actin.

3.3.4.2 mRNA Expression of GNL3 in Primary Cultured Human Articular Chondrocytes

All HACs were extracted from femoral head cartilage (Section 3.2.1.3). DNA was extracted from all primary HAC cultures using TRI Reagent® (Ambion®) and genotyped at rs11177 and rs2289247 (Section 3.2.7). RNA was extracted using TRI Reagent® (Ambion®) (Section 3.2.8.1) and reverse transcribed using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2). Samples were run in a qPCR experiment to determine whether there were any differences in the expression of GNL3 (corrected for 18S) (Section 3.2.11).
3.3.4.2.1 mRNA Expression of *GNL3* in Primary Cultured Human Articular Chondrocytes from Normal and Osteoarthritic Femoral Head Cartilage

The mRNA expression of *GNL3* was successfully quantified in normal and OA primary cultured HACs. Sample details are provided in Table 3.2. The results (Figure 3.4) show a significantly (*p*=0.032; corrected for age, gender and plate, *p*=0.034) higher expression of *GNL3* (corrected for 18S) in HACs extracted from OA femoral head cartilage (0.0085% ± 0.0015) compared to normal femoral head HACs (0.0040% ± 0.0011 SEM).

<table>
<thead>
<tr>
<th>Cartilage</th>
<th>Normal</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9 (75.0%)</td>
</tr>
<tr>
<td>Age</td>
<td>71.9 ± 10.6</td>
<td>62.9 ± 12.2</td>
</tr>
</tbody>
</table>

Table 3.2: Details of the patient samples used to compare the mRNA expression of *GNL3* between primary cultured HACs from normal and OA femoral head cartilage Values are n (%). Age, mean (years) ± 1SD.

Figure 3.4: qPCR results for mRNA expression of *GNL3* in primary cultured HACs taken from normal and OA femoral head cartilage Values are average *GNL3*/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM.
3.3.4.2.2 mRNA Expression of *GNL3* in Primary Cultured Human Articular Chondrocytes with Different rs11177 Genotypes

The mRNA expression of *GNL3* was successfully quantified in primary cultured HACs in all three rs11177 genotypes. Sample details are provided in Table 3.3. The results (Figure 3.5) show no significant differences in the expression of *GNL3* (corrected for 18S) between the different rs11177 genotypes, even when statistics were corrected for age, gender, plate and disease status.

<table>
<thead>
<tr>
<th>rs11177</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Normal</td>
<td>6 (42.9%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td></td>
<td>OA</td>
<td>8 (57.1%)</td>
<td>8 (72.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>3 (21.4%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11 (78.6%)</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td>Age</td>
<td>68.4 ± 13.8</td>
<td>61.1 ± 9.4</td>
<td>63.7 ± 14.0</td>
</tr>
</tbody>
</table>

Table 3.3: Details of the patient samples used to compare the mRNA expression of *GNL3* between primary cultured HACs taken from femoral head cartilage of different rs11177 genotypes. Values are n (%). Age, mean (years) ± 1SD.

Figure 3.5: qPCR results for mRNA expression of *GNL3* in primary HACs taken from femoral head cartilage of different genotypes at rs11177. GG, GA and AA refer to rs11177 genotypes. Values are average GNL3/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM.
3.3.4.2.3 mRNA Expression of GNL3 in Primary Cultured Human Articular Chondrocytes with Different rs2289247 Genotypes

The mRNA expression of GNL3 was successfully quantified in primary cultured HACs in all three rs2289247 genotypes. Sample details are provided in Table 3.4. The results (Figure 3.6) show no significant differences in the expression of GNL3 (corrected for 18S) between the different rs2289247 genotypes, even when statistics were corrected for age, gender, plate and disease status.

<table>
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<th>rs2289247</th>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1 (14.3%)</td>
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<td>1 (25.0%)</td>
</tr>
<tr>
<td>OA</td>
<td>6 (85.7%)</td>
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<td>3 (75.0%)</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>1 (14.3%)</td>
<td>2 (100.0%)</td>
<td>1 (25.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (85.7%)</td>
<td>0 (0.0%)</td>
<td>3 (75.0%)</td>
</tr>
<tr>
<td>Age</td>
<td>63.4 ± 15.2</td>
<td>48.5 ± 6.4</td>
<td>65.5 ± 12.0</td>
</tr>
</tbody>
</table>

Table 3.4: Details of the patient samples used to compare the mRNA expression of GNL3 between primary cultured HACs taken from femoral head cartilage of different rs2289247 genotypes. Values are n (%). Age, mean (years) ± 1SD.

Figure 3.6: qPCR results for mRNA expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs2289247. GG, GA and AA refer to rs2289247 genotypes. Values are average GNL3/18S percentage calculated using molecules/µl values. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM.
3.3.5 Protein Expression of GNL3 in Primary Cultured Human Articular Chondrocytes

All HACs were extracted from femoral head cartilage (Section 3.2.1.3). DNA was extracted from all primary HAC cultures using TRI Reagent® (Ambion®) and genotyped at rs11177 and rs2289247 (Section 3.2.7). Protein was extracted as described in Section 3.2.12. Samples were run in a western blot experiment to determine whether there were any differences in the expression of GNL3 (corrected for β-Actin) (Section 3.2.13). All samples were run thrice in random combinations on a total of two western blots each (i.e. two aliquots of the same sample were run alongside each other on the same blot and a third aliquot was run on a separate blot.) This was to reduce the chance of inaccuracies due to gel loading, transfer or chemiluminescence errors. The average of each sample across the three blots was used in the statistical analysis.

3.3.5.1 Protein Expression of GNL3 in Primary Cultured Human Articular Chondrocytes from Normal and Osteoarthritic Cartilage

The protein expression of GNL3 and β-Actin was successfully quantified in normal and OA primary cultured HACs. Sample details are provided in Table 3.5. The results (Figure 3.7 and Figure 3.8) show a significantly (p=0.0001; corrected for age and gender, p=0.0003) higher expression of GNL3 (corrected for β-Actin) in HACs extracted from normal femoral head cartilage (1.61 ± 0.30 SEM) compared to HACs extracted from OA femoral head cartilage (0.43 ± 0.07 SEM).

<table>
<thead>
<tr>
<th>Cartilage</th>
<th>Normal</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5.0 (35.7%)</td>
<td>7.0 (36.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>9.0 (64.3%)</td>
<td>12.0 (63.2%)</td>
</tr>
<tr>
<td>Age</td>
<td>72.4 ± 10.08</td>
<td>62.3 ± 12.27</td>
</tr>
</tbody>
</table>

Table 3.5: Details of the patient samples used to compare the protein expression of GNL3 between primary cultured HACs from normal and OA femoral head cartilage. Values are n (%). Age, mean (years) ± 1SD.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 3: EXPRESSION OF GNL3 IN JOINT TISSUE

Figure 3.7: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression in primary cultured HACs taken from normal and OA femoral head cartilage L, Magic Mark™ XP (Life Technologies™).
3.3.5.2 Protein Expression of GNL3 in Primary Cultured Human Articular Chondrocytes with Different rs11177 Genotypes

The protein expression of GNL3 was successfully quantified in primary cultured HACs in all three rs11177 genotypes. Sample details are provided in Table 3.6. The results (Figure 3.9 and Figure 3.10) show no significant differences in the expression of GNL3 (corrected for β-Actin) between the different rs11177 genotypes, even when statistics were corrected for age, gender and disease status.

<table>
<thead>
<tr>
<th>rs11177</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>13</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (46.2%)</td>
<td>5 (38.5%)</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td></td>
<td>7 (53.8%)</td>
<td>8 (61.5%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (23.1%)</td>
<td>6 (46.2%)</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td></td>
<td>10 (76.9%)</td>
<td>7 (53.8%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Age</td>
<td>67.3 ± 12.1</td>
<td>63.5 ± 10.5</td>
<td>63.7 ±14.0</td>
</tr>
</tbody>
</table>

Table 3.6: Details of the patient samples used to compare the protein expression of GNL3 between primary cultured HACs taken from femoral head cartilage of different rs11177 genotypes Values are n (%). Age, mean (years) ± 1SD.
Figure 3.9: Western blot for GNL3 (62kDa) and \( \beta \)-Actin (42kDa) protein expression in primary HACs taken from femoral head cartilage of different genotypes at rs11177. GG, GA and AA refer to rs11177 genotypes. All samples were run on the same western blot. Samples of unknown genotype are omitted from the picture. L, Magic Mark™ XP (Life Technologies™).
Investigating the Role of GNL3 in Osteoarthritis

Chapter 3: Expression of GNL3 in Joint Tissue

Figure 3.10: Western blot results for protein expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs11177. GG, GA and AA refer to rs11177 genotypes. Values are average GNL3/β-Actin. All samples were run in triplicate. Error bars are ± SEM.

3.3.5.3 Protein Expression of GNL3 in Primary Cultured Human Articular Chondrocytes with Different rs2289247 Genotypes

The protein expression of GNL3 was successfully quantified in primary cultured HACs in all three rs2289247 genotypes. Sample details are provided in Table 3.7. The results (Figure 3.11 and Figure 3.12) show no significant differences in the expression of GNL3 (corrected for β-Actin) between the different rs2289247 genotypes, even when statistics were corrected for age, gender and disease status.

<table>
<thead>
<tr>
<th>rs2289247</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Cartilage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>OA</td>
<td>5 (100.0%)</td>
<td>2 (100.0%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1 (20.0%)</td>
<td>2 (100.0%)</td>
<td>1 (33.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (80.0%)</td>
<td>0 (0.0%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Age</td>
<td>57.6 ± 8.5</td>
<td>48.5 ± 6.4</td>
<td>70.7 ± 7.5</td>
</tr>
</tbody>
</table>

Table 3.7: Details of the patient samples used to compare the protein expression of GNL3 between primary cultured HACs taken from femoral head cartilage of different rs2289247 genotypes. Values are n (%). Age, mean (years) ± 1SD.
Figure 3.11: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression in primary HACs taken from femoral head cartilage of different genotypes at rs2289247. GG, GA and AA refer to rs2289247 genotypes. The first three samples were run on the same western blot and the last five samples on different blot. Samples of unknown genotype are omitted from the picture. L, Magic Mark™ XP (Life Technologies™).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 3: EXPRESSION OF GNL3 IN JOINT TISSUE

Figure 3.12: Western blot results for protein expression of GNL3 in primary HACs taken from femoral head cartilage of different genotypes at rs2289247. GG, GA and AA refer to rs2289247 genotypes. Values are average GNL3/β-Actin. All samples were run in triplicate. Error bars are ± SEM.

3.3.6 Expression of GNL3 in Cytokine- and Growth Factor-Treated Chondrocyte Cultures

3.3.6.1 mRNA Expression of GNL3 in IL13-, IL1β- and TNFα-Treated Non-Human Chondrocyte Cultures

RNA from STR/ort and CBA mice cartilage at 26 and 40 weeks (Section 3.2.2.1) was converted to cDNA using LongRange 2Step RT-PCR kit (Qiagen®) (Section 3.2.9.1). RNA from Cb2−/− knockout and C57-Bl6 wildtype (Cb2+/+) mice was donated by Antonia Sophocleous (MMC, Edinburgh) and converted to cDNA by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.1, Section 3.2.14.2 and Section 3.2.14.4). cDNA from canine cartilage was prepared and donated by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.2 and Section 3.2.14.2). cDNA from bovine cartilage was prepared and donated by Asim Azfer (MMC, Edinburgh) (Section 3.2.2.3, Section 3.2.14.2 and Section 3.2.14.3).

cDNA (40ng) was amplified using DNA Polymerase Kit (Qiagen®) as described in (Section 3.2.10.2). The GNL3 and GAPDH amplicons are shown in Figure 3.13.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 3: EXPRESSION OF GNL3 IN JOINT TISSUE

Figure 3.13: mRNA expression of GNL3 and GAPDH in murine (A), canine (B) and bovine (C) articular cartilage cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X). L, 100bp DNA ladder (New England BioLabs®). (A) Murine: 1-2, CBA 26 weeks; 3-4, CBA 40 weeks; 5-6, Str/ort 26 weeks; 7-8, Str/ort 40 weeks; 9, Ch2+/+ untreated; 10, Ch2+/+ TNFα-treated; 11, Ch2+/+ IL1β-treated; 12, Ch2−/− untreated; 13, Ch2−/− TNFα-treated; 14, Ch2−/−IL1β-treated; 15, No template control. (B) Canine: 1-6, Cartilage treated with IL1β; 7-12, Untreated controls; 13, No template control. (C) Bovine: 1, Untreated control; 2, IL13-treated; 3, IL1β-treated, 4, No template control.

The Gnl3 and Gapdh amplicons were successfully amplified in all mouse samples (Figure 3.13: A). There appeared to be no difference in the expression of Gnl3 between controls and cytokine-treated samples.

Both GNL3 and GAPDH were successfully amplified in cDNA from canine cartilage (Figure 3.13: B). The density of the GNL3 bands in relation to GAPDH is almost identical in control and IL11β-treated samples.
Both \textit{GNL3} and \textit{GAPDH} were successfully amplified in cDNA from non-cultured bovine cartilage (Figure 3.13: C). Both IL13- and IL1β-treated samples showed a trend towards increased expression of \textit{GNL3}.

3.3.6.2 mRNA Expression of \textit{GNL3} in FGF2- and IL1β- Treated Primary Cultured Human Articular Chondrocytes and Chondrocyte Cell Lines

RNA from FGF2- (Section 3.2.14.1) and IL1β- (Section 3.2.14.2) treated primary cultured HACs (taken from normal femoral head cartilage) (Section 3.2.1.3) and cell lines, C20A4 and C28I2 (Section 3.2.3.3), was extracted using TRI Reagent® (Ambion®) (Section 3.2.8.1) and reverse transcribed using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2). Samples were run in a qPCR experiment to determine whether there were any differences in the expression of \textit{GNL3} (corrected for \textit{18S}) (Section 3.2.11).

3.3.6.2.1 Primary Cultured Human Articular Chondrocytes

The mRNA expression of \textit{GNL3} was successfully quantified in primary cultured HACs in all three groups. All HACs (n=4) were taken from normal femoral head cartilage of female subjects (mean age ± SD = 81.8 ± 9.6 years). The results (Figure 3.14) show no significant differences in the expression of \textit{GNL3} (corrected for \textit{18S}) when chondrocytes were treated with FGF2 or IL1β, even when statistics were corrected for age.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 3: EXPRESSION OF GNL3 IN JOINT TISSUE

Figure 3.14: qPCR results for mRNA expression of GNL3 in control, FGF2- and IL1β-treated primary cultured HACs. Values are average GNL3/18S percentage calculated using molecules/µl values. Each experiment was carried out in duplicate. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM.

3.3.6.2.2 Chondrocyte Cell Lines

The mRNA expression of GNL3 was successfully quantified in C20A4 and C28I2 chondrocyte cell lines in all three groups. The results (Figure 3.15) show no significant differences in the expression of GNL3 (corrected for 18S) when chondrocytes were treated with FGF2 or IL1β.

Figure 3.15: qPCR results for mRNA expression of GNL3 in control, FGF2- and IL1β-treated chondrocyte cells lines, C20A4 (A) and C28I2 (B). Values are average GNL3/18S percentage calculated using molecules/µl values. Each experiment was carried out in duplicate. All samples were run in triplicate. Blank subtracted from all values. Error bars are ± SEM.
3.3.6.3 **Protein Expression of GNL3 in FGF2- and IL1β-Treated Chondrocyte Cell Lines**

Total protein from FGF2- ([Section 3.2.14.1](#)) and IL1β- ([Section 3.2.14.2](#)) treated cell lines, C20A4 and C28I2 ([Section 3.2.3.3](#)) was extracted as described in [Section 3.2.12](#). Samples were run in a western blot experiment to determine whether there were any differences in the expression of GNL3 (corrected for β-Actin). As described in [Section 3.2.13](#), with the exception that the polyclonal anti-GNL3 antibody (AF1638, R&D Systems®) ([Table 4.2](#)) was used instead of the monoclonal antibody (MAB4311, Millipore) ([Table 3.1](#))

The protein expression of GNL3 was successfully quantified in C20A4 and C28I2 chondrocyte cell lines in all three groups. The results ([Figure 3.16](#) and [Figure 3.17](#)) show no significant differences in the expression of GNL3 (corrected for β-Actin) when chondrocytes were treated with FGF2 or IL1β.
Figure 3.16: Western blot for GNL3 (62kDa) and β-Actin (42kDa) protein expression GNL3 in control, FGF2- and IL1β-treated chondrocyte cells lines, C20A4 and C28I2 L, Magic Mark™ XP (Life Technologies™).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 3: EXPRESSION OF GNL3 IN JOINT TISSUE

Figure 3.17: Western blot results for protein expression of GNL3 in control FGF2- and IL1β-treated chondrocyte cells lines, C20A4 (A) and C28I2 (B). Values are average GNL3/β-Actin. Each experiment was carried out in duplicate.
3.4 Discussion

The expression of GNL3 was confirmed in the nuclei of non- and cultured HACs using IHC (Figure 3.1). While GNL3 appears to be predominantly expressed in the nucleoli, as previously reported (Tsai & McKay 2002), this was not confirmed here. Future work might use an antibody against nucleolin to confirm the location of the nucleoli and use this to confirm the nucleolar location of nucleostemin. IHC might also be conducted to investigate whether there are any differences in the pattern of nucleostemin expression throughout the cartilage zones, different joint tissues or between intact and degraded cartilage.

As discussed (Section 1.4.1.4) GNL3 plays an important functional role in cell proliferation and growth in cancer cells and stem cells (Tsai & McKay 2002) whereas chondrocytes are reported to be relatively dormant in mature articular cartilage (as reviewed in (Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). Therefore, the fact that this study has shown, for the first time, that GNL3 is present in HACs, is a noteworthy achievement as it confirms GNL3 as a viable candidate in the study of OA.

It should be noted that attempts to quantify differences between normal and OA cartilage using IHC proved difficult and prone to error, particularly as nucleoli were difficult to visualise, even at high magnification. Henceforth, attempts to quantify differences between the groups, should be carried out using alternate methods such as confocal microscopy. Future efforts might use this technique in combination with fluorescent antibodies in order to quantify the differences between these two groups.

Successful amplification of GNL3 in numerous cells proved expression of nucleostemin in several joint tissues namely cartilage, synovium and bone as well as several cell lines (Figure 3.2). This provided evidence to suggest that GNL3 may contribute to the osteoarticular process via a variety of joint tissues however all future efforts were focused on chondrocytes. Future studies may wish to analyse GNL3 expression in other joint tissues using the methods described here.
Aims to investigate whether the mRNA expression of GNL3 differed between chondrocytes extracted from normal and OA cartilage were successfully accomplished by cDNA end-point PCR and qPCR. Due to the fact that GNL3 had previously been reported in proliferating cells, end-point PCR was used initially, to confirm the mRNA expression of GNL3 in non-cultured HACs from both normal and OA cartilage (Figure 3.3). These results need be interpreted with caution due to the fact that all OA HACs were extracted from knee joints whereas all normal samples were taken from femoral head tissue. Therefore, any differences in mRNA expression between non-cultured normal and OA HACs could possibly be due to differences between joint sites. Perhaps qPCR could be used to quantify the difference in GNL3 mRNA expression between these non-cultured HACs. The difference in mRNA expression of GNL3 between normal and OA HACs was quantified, using qPCR techniques, in a larger sample set of cultured HACs all of which had been extracted from femoral head tissue and showed the expression of GNL3 (corrected for 18S) to be significantly higher (p=0.032; corrected for age, gender and plate, p=0.034) in OA HACs (Figure 3.4).

Aims to investigate whether the protein expression of GNL3 differed between HACs extracted from normal and OA cartilage were successfully accomplished by western blotting. It was not possible to successfully extract enough protein from non-cultured HACs (results not shown). This may be due to the fact that there are only a small number of chondrocytes in articular cartilage (Section 1.2.2.1) and GNL3 expression levels are reportedly low in non-proliferating cells (Section 1.4.1.3) (Tsai & McKay 2002). Despite having extracted adequate RNA from non-cultured HACs for use in cDNA end-point PCR and qPCR experiments, which amplify starting material, it was not possible to achieve sufficient protein for accurate analysis here. Consequently all protein expression was analysed in primary cultured HACs. The latter showed a significantly higher expression (p=0.0001; corrected for age and gender, p=0.0003) of GNL3 in normal HACs (Figure 3.7 and Figure 3.8).

Should cartilage from normal knees become available in future, it would be of interest to determine whether normal and OA HACs extracted from knee cartilage
show similar trends in both mRNA and protein expression, to the results seen here when comparing normal and OA HACs extracted from femoral head cartilage.

The fact that the expression of *GNL3* in OA HACs is elevated at the mRNA level and decreased at the protein level and vice versa in normal HACs suggests post transcriptional and/or post-translational factors may be responsible. Perhaps an initial attempt to repair damaged OA cartilage results in a rise in transcriptional activity however downstream efforts to translate the transcript may be unsuccessful. Or perhaps the reduced level of mRNA expression seen in normal cartilage could be due to the fact that nucleostemin protein stability acts as a transcription repressor to regulate cell proliferation.

A similar pattern of decreased nucleostemin protein expression (yet did not alter mRNA levels) was observed by Lo *et al.* (2012) in response to a reduction in GTP levels. They also noted that the decrease in protein was not due to ubiquitin-mediated proteasomal degradation by MDM2. On the contrary, an earlier study by Huang *et al.* (2009) showed that MDM2 was in fact responsible for the degradation of GNL3 particularly in response to GTP depletion.

OA studies have reported a functional role for GTP in response to extracellular signals via the Rho GTPases which regulate chondrogenesis (as reviewed in (Beier & Loeser 2010)). Fluctuations in GTP levels, have also been observed in response to mechanical loading in chondrocytes (Jutila *et al.* 2014).

GTP depletion may result in a reduction in rRNA synthesis and trigger nucleolar disruption (Huang *et al.* 2008) which reduces ribosomal assembly and subsequently gene transcription. Nucleostemin, nucleophosmin (Huang *et al.* 2008) and unassembled riboproteins such as L5, L11 and 5S rRNA, may then be released into the nucleoplasm (Dai *et al.* 2008; Donati *et al.* 2013). Perhaps nucleostemin acts as a chaperone for these riboproteins and GTP hydrolysis alters its structural conformation, thus affecting the formation of RNA/protein complexes (as reviewed in (Lo & Lu 2010)). Donati *et al.* (2013) reported the L5/L11/5S complex which usually forms part of the large 60S ribosomal subunit could, when expelled to the
nucleoplasm, sequester the effect of MDM2 and stabilise p53 (Section 1.4.1.4 and Section 1.4.1.5).

Another study by Huang et al. (2011) demonstrated that in the presence of a ROS, H₂O₂, nucleostemin is immobilised in the nucleolus of U2OS cells and can form insoluble aggregates which may hinder its function but which protect it from being exported to the nucleoplasm and degraded even in the absence of GTP. Low levels of ROS can have a mitogenic affect. However, prolonged exposure to high levels of ROS can result in the accumulation of insoluble protein aggregates and ultimately, apoptosis and necrosis (Section 1.4.1.5). One example of ROS with particular relevance to OA is NO. The latter not only aggravates the OA process through increased production of MMPs and a reduction in collagen and proteoglycan formation but can induce the production of other oxidants such as H₂O₂ as well (as reviewed in (Abramson et al. 2001)). There have also been some reports that NO could also function to protect cartilage (as reviewed in (Abramson 2008)). NO is synthesised from L-arginine in a reaction catalysed by iNOS (as reviewed in (Nordberg & Arner 2001)). The latter is dependent on a rate-limiting cofactor, BH4 which is controlled by GTP (Senda et al. 1995). Inosine-5′-monophosphate dehydrogenase (IMPDH) is the enzyme responsible for de novo guanine synthesis and hence vital in regulating GTP levels (Senda et al. 1995; Kim et al. 2012). IL1β and TNFα (Section 1.2.2.4 and Section 3.1) up-regulate the production of NO by iNOS and so in OA there should be a higher level of NO (as reviewed in (Goldring 2006)). However a study by Senda et al. (1995) used rodent endothelial cells to demonstrate that inhibition of IMPDH resulted in GTP depletion which inhibited the production of iNOS and consequently NO too, in the presence of inflammatory cytokines. So according to Lo et al. (2012) and Huang et al. (2009) this reduction in GTP should result in the nucleoplasmic degradation of nucleostemin. This could in turn disrupt ribosomal complexes and inhibit the MDM2-mediated degradation of p53 (Section 1.4.1.4). However, the question of how the reduction in GTP originates is not clear. Perhaps in response to OA processes, proinflammatory cytokines, IL1β and TNFα, can up-regulate the expression of NO. This process which is dependent on iNOS, reduces GTP levels. At this point nucleostemin could be subject to
nucleoplasmic degradation however there is now a high level of ROS (NO) which according to Huang et al. (2011) should override the effect of low GTP levels and result in the accumulation of nucleolar aggregates and reduced degradation. However, my results show that in fact the level of nucleostemin protein is reduced in OA HACs (Figure 3.7 and Figure 3.8). One possible explanation for this could be that the nucleolar aggregation alters the conformation and functional activity of nucleostemin. Altered protein conformation could reduce antibody binding efficiency and an increase in molecular weight due to protein oligomerisation may have resulted in incomplete protein-membrane transfer in western blotting or reduced protein solubility causing extraction inefficiency. Perhaps sonication (Huang et al. 2011) should be carried out to ensure solubilisation of any nucleostemin aggregates in future studies. Altered protein conformation could also reduce the interaction of nucleostemin with ribosomal proteins, triggering nucleolar disruption and ultimately apoptosis. Then again, maybe NO is subsequently down-regulated due to the inhibitory effects of inflammatory cytokines on IMPDH-mediated guanine synthesis and results not only in a reduction in iNOS activity but also nucleoplasmic degradation of nucleostemin.

The study conducted by Huang et al. (2011) was carried out on in the presence of H₂O₂ on U2OS osteosarcoma cells, which display high levels of nucleostemin expression and may therefore be more dependent on it for survival (Huang et al. 2009). It may therefore be difficult to draw parallels with my study. The fact that HACs are in a constant state of hypoxia, with oxygen levels ranging from 6% (at the surface) to 1% (closer to the subchondral bone) and the fact that ROS (as reviewed in (Pfander & Gelse 2007)) such as NO, are important in regulating chondrocyte function (as reviewed in (Abramson et al. 2001; Abramson 2008)) do make the regulatory mechanism proposed by Huang et al. (2011), an attractive option in explaining the observations seen in my experiments. The involvement of GTP in regulating nucleostemin (Lo et al. 2012), also seems viable due to the role which GTP plays in response to mechanotransduction (Jutila et al. 2014) and the fact that my study showed similar inconsistencies in mRNA and protein expression. Caution need be exercised when extrapolating these results to OA cartilage and bear in mind
that while the chondrocytes are in a hypoxic and non-proliferative state in intact human cartilage, the studies carried out here were on HACs which had been cultured for a period of up to 14 days at normal atmospheric oxygen levels. Despite these discrepancies the studies by Huang et al. (2011) and Lo et al. (2012) do serve to highlight the fact that nucleostemin is a redox- and GTP-sensitive protein. This may be significant in OA due to the hypoxic environment of chondrocytes which is altered as a result of cartilage degradation due to an imbalance in ECM maintenance in response to external stimuli such as mechanical stress.

The increased mRNA expression of GNL3 in OA cultures and concurrent reduction in protein may be due to the fact that although cells may initially respond to stress by increasing the transcription of GNL3, downstream post-transcription or post-translational modifications ultimately reduce its expression at the protein level. Post-translational degradation could occur as observed in the absence of GTP (Huang et al. 2009; Lo et al. 2012). Post-transcriptional mechanisms which regulate a number of cartilage-specific genes such as IL1β, iNOS and SOX9 (Section 1.3.6) (as reviewed in (Gonzalez 2013)), may also regulate the protein expression of GNL3 however, to date, none have been reported for GNL3. Although it does contain a CpG island in the 5'UTR (Kent et al. 2002) however this is more likely to regulate the initiation of transcription (as reviewed in (Barter & Young 2013)). P53, on the other hand, is known to induce miR-34 which inhibits IMPDH (Kim et al. 2012). MiR-34 causes apoptosis in rat OA chondrocytes in vitro (Abouheif et al. 2010). MiR-34 has also been shown to repress Ras GTPase signalling in a GTP-dependent manner in several cell lines (Kim et al. 2012). Accordingly, an IMPDH-induced reduction of GTP could subsequently inhibit the expression of nucleostemin which could further stimulate p53 activation.

It is also possible that nucleostemin may act independently of GTP, p53 and ribosome biosynthesis pathways as demonstrated by Hsu et al. (2012) and Meng et al. (2013) in the recruitment of RAD51 to DNA damage sites of non- and telomeric chromosomes under normal conditions in continuously dividing cells (Section 1.4.1.5). Therefore in my results perhaps normal chondrocytes are able to better repair sites of DNA damage due to the increased level of nucleostemin protein. OA
chondrocytes may initially respond to DNA damage by increasing transcription of \textit{GNL3} however, due to the lack of protein expression the cells are unable to recruit RAD51 resulting in inadequate repair which could terminate cell cycle progression.

These suggestions are of course highly speculative and would benefit from further investigation in future studies. Having established that there are in fact significant differences in nucleostemin expression between cases and controls future work, which investigates post-transcription and/or post-translation regulatory mechanisms, may also benefit from protein localisation studies which use confocal microscopy to focus not necessarily on how much but more importantly, where the protein is in HACs when challenged by alterations in GTP, by treating cells with mycophenolic acid to inhibit IMPDH, or ROS, by exposing cells to hypoxia. In hindsight, measuring the level of GTP or NO levels in the joint tissue and surrounding synovial fluid might have proved useful in supporting this hypothesis.

Aims to investigate whether the mRNA and protein expression of \textit{GNL3}, in primary HACs, was affected by the genotype, at rs11177 or rs2289247, were successfully accomplished by qPCR (Figure 3.5 and Figure 3.6) and western blotting (Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.12), respectively. The results showed no significant differences in the mRNA or protein expression of \textit{GNL3} between the three possible genotypes at rs11177 or rs2289247. Unfortunately, it proved difficult to obtain individuals with certain genotypes for these experiments and hence the data shown here had large error bars and may be prone to statistical error. Increasing the number of individuals in each group may help to increase the reliability of the results shown here and would allow for the effect of the rs11177 and rs2289247 haplotype on \textit{GNL3} expression to be assessed. Perhaps the methods used here were not sensitive enough to detect differences in expression conferred by these SNPs and may yield more significant findings if this SNP pair were to be considered as part of a larger haplotype of variants all contributing varying individual effects. Furthermore, although corrected for in statistical calculations, age, gender and disease status may have reduced the significance of these results particularly in this small sample set. Due to the fact that rs11177 and rs2289247 lie in regions of nucleostemin which are important in determining its cellular location, fluorescent
Microscopy might be used in future work to determine whether these variants are able to influence its location.

Treating the chondrocytes of Str/ort and CBA mice at 26 and 40 weeks and Cb2 wildtype and knockout mice with TNFα and IL1β, did not affect the mRNA expression of Gnl3 (in relation to Gapdh) (Figure 3.13: A).

Control and IL1β-treated canine cartilage displayed almost identical levels of GNL3 expression (in relation to GAPDH) at an mRNA level (Figure 3.13: B).

IL1β and TNFα are known to be up-regulated in OA cartilage and contribute to its destruction ((Tetlow et al. 2001) and as reviewed in (van den Berg 1999; Goldring 2006)). It is noteworthy that other factors, other than IL1β and TNFα, could influence the expression of GNL3. Further experimentation, such as a qPCR, is needed in order to quantify the differences between the control and cytokine-treated groups, if any.

In bovine cartilage (Figure 3.13: C), both IL1β- and IL13-treated samples appeared to show a trend towards increased mRNA expression of GNL3. However, the difference was not quantified and further samples would need to be run in a qPCR experiment in order to determine whether there were any significant differences. These experiments show promise regarding the influence of these cytokines, commonly associated with OA, on the mRNA expression of GNL3 (as reviewed in (van den Berg 1999)).

It should be noted that all samples from other species were donated and therefore alternate culturing, cytokine-treatment, RNA extraction and reverse transcription protocols may have influenced the results.

Treating HACs with FGF2 or IL1β did not show any significant difference in the mRNA expression of GNL3 (Figure 3.14). FGF2 was shown to increase the proliferative activity of human bone marrow stem cells in a dose-dependent manner (Kafienah et al. 2006) which suggests that perhaps the concentration used to stimulate the chondrocytes may have affected the outcome of the results reported.
here. Similarly dosage could have affected the results of IL1β-treated experiments. The use of the more sensitive qPCR technique has eliminated any errors which might occur when using a less robust gel quantification technique. Furthermore, although corrected for in statistical calculations, the age of the subjects, may have reduced the significance of these results particularly in this small sample set.

Repeating the FGF2 or IL1β treatment experiments in human chondrocyte cell lines, C20A4 and C28I2, did not show any significant difference in the mRNA expression of GNL3 (Figure 3.15). In addition, there was no change in the expression of GNL3 at a protein level (Figure 3.16 and Figure 3.17). In cardiomyocytes treated with FGF2 the protein expression of GNL3 reportedly peaked at 2hrs after which it decreased until it reached basal levels at 8hrs (Siddiqi et al. 2008). The methods used in my study treated chondrocytes overnight. The protein expression of GNL3 in response to FGF2 treatment appears to be transient. Therefore, the timing of protein extraction could have influenced the results reported here. Similarly protein extraction timing could have affected the results of IL1β-treated experiments. Errors in statistical calculations may have occurred due to the small sample size.

In summary, there was no statistically significant difference in the expression of GNL3 between untreated chondrocytes and those treated with cytokines or growth factors. The reason for this, remains speculative and as discussed may be due to statistical errors, experimental sensitivity, dosage, extraction timing, differences in mRNA and protein expression or inter-species differences. Also it is worth noting again that the chondrocytes used here were cultured at normal atmospheric oxygen levels, which is significantly different from the hypoxic environment and dormant state which occurs in intact articular cartilage. In future, qPCR and western blots should be used to quantify any alterations in GNL3 expression which might arise in response to treatment which should be administered at different concentrations and measured at several points over the course of the experiment.

It may appear that the experiments carried out in this Chapter (and Chapter 4), use nucleostemin expression as a proxy for its nuclear location. The fact that nucleostemin is degraded in the nucleoplasm (Huang et al. 2009; Lo et al. 2012)
would suggest that nucleoplasmic nucleostemin should result in a reduction in nucleostemin overall. However, prolonged exposure to ROS may result in the accumulation of nucleolar aggregates and ultimately apoptosis. Hence, the location of nucleostemin (i.e. the nucleolus) in the form of insoluble protein aggregates may give the false impression of a reduction in nucleostemin expression overall. Furthermore, short periods of ROS may have a mitogenic effect, causing location of nucleostemin to alter during cell cycling (Huang et al. 2011). While all conditions were controlled during culture, previous conditions such as the atmospheric oxygen levels from where the primary cells were extracted cannot be excluded. The picture is far from complete when it comes to nucleostemin, particularly in relation to its regulation and functioning in chondrocytes. Hence, while it appears promising (and convenient) that nucleostemin expression should act as a proxy for its location, it is not that simple and it should be noted that a comprehensive investigation which took into account the known factors and aims of each experiment were considered when analysing the results presented in this thesis.
Chapter 4: Functional Analysis of GNL3

4.1 Introduction

The aim of this chapter was to investigate the functional significance of GNL3 in OA. To achieve this aim, three sets of studies were carried out. Firstly, GNL3 knockdown was performed in cultured HACs and JJ012 chondrosarcoma cells to determine the functional role of GNL3 in cartilage homeostasis. Secondly, the effect of the variants at rs11177 on chondrogenic differentiation was examined in patient derived HACs and MSCs of different genotypes. Thirdly, expression constructs containing the two common coding variants of GNL3 identified in exons 3 (rs11177) and 11 (rs2289247) were generated. Given that GNL3 is thought to be involved in cell proliferation through an interaction with p53, the effect of these variants on the response to hypoxia and cisplatin in cultured melanoma cells was investigated.

HACs in mature articular cartilage are in a dormant non-proliferative state yet play an active role in producing both catabolic and anabolic factors which are vital in maintaining the homeostatic balance of the surrounding ECM (Section 1.2.2.4) (as reviewed in (Goldring 2006; Martel-Pelletier et al. 2008; Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). In OA cartilage (Section 1.2.4), despite an initial increase in the expression of anabolic factors, there is ultimately an imbalance in these processes and catabolic activity dominates (as reviewed in (Sandell & Aigner 2001; Buckwalter et al. 2005; Goldring & Goldring 2010; Salter et al. 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). Chondrocytes proliferate to form clusters and also undergo apoptosis and necrosis (as reviewed in (Poole 1997; Huber et al. 2000; Sandell & Aigner 2001; Aigner & McKenna 2002; Buckwalter et al. 2005; Goldring & Goldring 2010; Salter et al. 2011; Zamli & Sharif 2011; Loeser et al. 2012; Pulsatelli et al. 2013)). MSCs (Section 1.2.1) are present in low numbers in the synovial fluid ((Jones et al. 2008) and as reviewed in (Poole 2011)). So although cartilage is an avascular aneural tissue, its ability to respond to injury and mechanical loading may be aided by the regenerative properties of MSCs which are capable of chondrogenic differentiation (as reviewed in (Martel-Pelletier et al. 2008; James 2013)).
As discussed (Section 1.2.2.1), the chondrocyte is proficient in producing both catabolic and anabolic factors which maintain the ECM. COL2A (Section 1.2.2.1) is the major collagen molecule in articular cartilage responsible for providing tensile strength and stiffness. ACAN (Section 1.2.2.2) is the major proteoglycan of articular cartilage which provides cartilage with hydrophilic properties which allow resistance to compressive loading and efficient shock-absorbency (as reviewed in (Martel-Pelletier et al. 2008)). SOX9 (Section 1.2.2.1) and RUNX2 (Section 1.2.3.2) are the key transcription factors which regulate the formation of cartilage (Wright et al. 1995; Bi et al. 1999) and bone ((Ducy et al. 1997) and as reviewed in (Stein et al. 2004)), respectively. MMP13 (Section 1.2.2.4) is a matrix metalloprotease which degrades collagen II and is found to be up-regulated in OA chondrocytes (Reboul et al. 1996). MMP3 (Section 1.2.2.4) substrates include proteoglycans, laminin, elastin and fibronectin. It may also activate latent collagenases. Both MMPs are found in OA cartilage (as reviewed in (Cawston & Wilson 2006; Martel-Pelletier et al. 2008)).

The first aim of this chapter was to determine the functional role of GNL3 in cartilage homeostasis. To do this nucleostemin knockdown was performed in cultured HACS and JJ012 cells. The mRNA expression levels of the abovementioned chondrogenic markers were then investigated by cDNA end-point PCR to determine whether these were affected by a loss of GNL3.

The SNP found to be most significantly associated with OA in the arcOGEN GWAS (Zeggini et al. 2012), rs11177 (c.116G>A p.(R39Q)) was found to be in strong LD ($r^2=0.92$) (International HapMap Project 2005) with rs2289247 (c.1099G>A p.(V367M)), which was identified in the mutation screen (Table 2.2 and Figure 2.4) of GNL3. The rs11177 SNP encodes a non-synonymous amino acid change from arginine to glutamine at codon 39 of the protein. Rs11177 lies in exon 3 of GNL3 within the N-terminal domain (Figure 1.6). This region, as discussed previously, not only plays an important role in the localising GNL3 to the nucleolus (Section 1.4.1.2) but also interacts with p53 (Section 1.4.1.4) (Tsai & McKay 2002). The rs2289247 SNP encodes a non-synonymous amino acid change from valine to methionine at codon 367 of the protein. Rs2289247 lies in exon 11 within the
intermediate domain. This domain is involved in retaining nucleostemin in the nucleoplasm in the absence of GTP binding (Section 1.4.1.2) (Tsai & McKay 2005).

The nuclear location of nucleostemin is not only vital in determining its own expression but is also altered during cell cycle progression (Tsai & McKay 2002) and in response to cellular stress (Avitabile et al. 2011) (Section 1.4.1.4). Nucleostemin is degraded in the nucleoplasm by MDM2-mediated ubiquitination (Huang et al. 2009) or possibly alternate means (Lo et al. 2012) (Section 1.4.1.4). It may also accumulate and form insoluble aggregates in the nucleolus in response to high levels of ROS exposure (Huang et al. 2011) (Section 1.4.1.5). Alterations in the expression and/or location of nucleostemin may also trigger a MDM2-mediated p53 response (Section 1.4.1.4) (Tsai & McKay 2002; Dai et al. 2008; Meng et al. 2008).

There are a number of processes which may trigger the alterations in the location and expression of nucleostemin. Nuclear shuttling of GNL3 has been observed during cell cycling, which is an essential process in embryogenesis and proliferation. GNL3 accumulates in the nucleolus during interphase and starts to disperse during prophase, becoming gradually more diffused as mitosis progresses (Section 1.4.1.4) (Tsai & McKay 2002). Hence, the second aim of this chapter was to determine the effect of the GNL3 variant, rs11177, on HACs and MSCs, in proliferation and chondrogenic differentiation respectively by determining whether markers of ECM homeostasis, ACAN, COL2A, SOX9, RUNX2 and MMP-3, -13 were present at the mRNA level.

P53 is a tumour suppressor protein which mediates the response to stress stimuli such as hypoxia, DNA damage, oncogene activation and telomere shortening, in an effort to arrest cell cycle progression at the G1 checkpoint and induce apoptosis in order to prevent inappropriate proliferation of cells containing damaged DNA (as reviewed in (Levine et al. 1991; Moll & Petrenko 2003)). The N-termini of p53 and MDM2 are able to interact with one another (Honda et al. 1997; Kubbutat et al. 1998). Upon doing so, the RING finger domain in the C-terminus of MDM2 acts as an E3 ligase which is able to ubiquitinate the C-terminal lysine residues of p53 ((Honda et al. 1997; Kubbutat et al. 1998; Fang et al. 2000) and as reviewed in (Moll & Petrenko...
Disruption in the stability and expression of p53 (and MDM2) has been implicated in numerous human malignancies (as reviewed in (Levine et al. 1991; Deb 2003)). In addition, p53 expression has been observed in both normal and OA HACs (Hashimoto et al. 2009). The expression of p53 is higher in OA HACs and this expression was positively correlated with cartilage destruction and chondrocyte apoptosis in OA (Yatsugi et al. 2000; Hashimoto et al. 2009). Chondrocytes also increased the expression of p53 in response to shear strain, hydrostatic pressure, hypoxia and NO (Islam et al. 2002; Kim et al. 2002; Hashimoto et al. 2009). Furthermore, p53 has also been shown to aid in regulating chondrocyte metabolism during OA (Iannone et al. 2005).

Stressful stimuli such as changes in the oxidative state of the chondrocyte environment can induce cell death in cartilage (as reviewed in (Zamli & Sharif 2011)). Cartilage is an avascular tissue which receives oxygen and nutrients from the surrounding synovial fluid ((Coimbra et al. 2004; Pfander et al. 2005) and as reviewed in (Pfander & Gelse 2007)) (Section 1.2.1). The chondrocytes of intact cartilage exist in a hypoxic environment with oxygen levels ranging from 6% to 1%, the closer the cells are to the subchondral bone. Oxygen levels are significantly

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**CHAPTER 4: FUNCTIONAL ANALYSIS OF GNL3**
decreased in the synovial fluid of OA joints (as reviewed in (Pfander & Gelse 2007)). Hence in OA, despite an increase in exposure to the surrounding synovial fluid by fibrillation and degradation of the cartilage, and an increase in the number of blood vessels in the synovial membrane and subchondral bone, chondrocytes and synovial cells exist in a more pronounced hypoxic environment than normal cells ((Pfander et al. 2005) and as reviewed in (Pfander & Gelse 2007)). Chondrocytes are well adapted to living in a hypoxic environment ((Yudoh et al. 2005) and as reviewed in (Pfander & Gelse 2007)). Both normal and OA chondrocytes express HIF1α, a Per-ARNT-Sim transcription factor (Section 2.4) which enables survival under prolonged hypoxic conditions ((Pfander et al. 2005; Yudoh et al. 2005) and as reviewed in (Pfander & Gelse 2007)). Hypoxia, mechanical loading, pro-inflammatory cytokines (IL1β and TNFα), ROS (H2O2 and NO), IGF-1 and -2 are known to up-regulate the expression of HIF1α which is more pronounced in areas of matrix degradation in OA cartilage ((Coimbra et al. 2004; Pfander et al. 2005; Yudoh et al. 2005) and as reviewed in (Pfander & Gelse 2007)). HIF1α is necessary in order for cells to maintain energy metabolism, matrix synthesis, neoangiogenesis, proliferation and regulate apoptosis under anaerobic conditions ((Chiarugi et al. 1999; Pfander et al. 2005; Yudoh et al. 2005) and as reviewed in (Pfander & Gelse 2007)). Accordingly, HIF1α can regulate the expression of VEGF-A, iNOS, leptin, SOX9, COL2A, ACAN and p53 ((Chiarugi et al. 1999; Pfander et al. 2005; Duval et al. 2009) and as reviewed in (Hammond & Giaccia 2005; Pfander & Gelse 2007)). Hypoxia, HIF1α and p53 have also been demonstrated to play an important role in cancer. Hypoxic tumours are aggressive and more difficult to treat. Tumours are often poorly vascularised and therefore have restricted oxygen levels (as reviewed in (Hammond & Giaccia 2005)). A reduction in oxygen levels triggers HIF1α and results in p53-mediated apoptosis ((Chiarugi et al. 1999) and as reviewed in (Hammond & Giaccia 2005)). Chondrocytes also express HIF2α in response to hypoxia and pro-inflammatory cytokines (Coimbra et al. 2004; Yang et al. 2010). HIF2α initiates the synthesis of catabolic factors such as MMP-1, -3, -9, -12, -13, ADAMTS2, COX2 and iNOS (Yang et al. 2010).
With regard to the effect of hypoxia on nucleostemin, Zhang and Wang (2013) observed an increase in the protein expression of GNL3 in human tendon stem cells when subject to a low level of oxygen tension. Furthermore, prolonged exposure to ROS, resulted in the accumulation of insoluble nucleolar aggregates of nucleostemin (Huang et al. 2011).

The experiments carried out in this chapter, investigated whether the GNL3 variants rs11177 and rs2289247 are able to influence the p53/MDM2 response to stimulation by cisplatin and oxidative stress. The latter represents an OA appropriate physiological stress. However, the experiments were designed in a melanoma cell line. While hypoxia is reported to influence cancerous cells, cisplatin was also used to induce a p53 response.

Cisplatin is a chemotherapeutic agent used in the treatment of human malignancies. It consists of two ammonia molecules and two chlorine atoms in a cis arrangement around a central platinum atom in the centre (Figure 4.1). The chlorine atoms are replaced by water molecules which can bind to DNA (preferentially purines) to form either an intra- or inter-strand cross-link which ultimately inhibits DNA synthesis and induces apoptosis (as reviewed in (Boulikas & Vougiouka 2003; Barabas et al. 2008)). MDM2 can inhibit cisplatin mediated apoptosis (as reviewed in (Deb 2003)).

In this chapter, I hypothesise that variants rs11177 and rs2289247 may compromise the translocation of GNL3 to the nucleolus and thereby hinder the ability of GNL3 to respond to the stimuli via the p53 pathway. Nucleostemin has been demonstrated to interact with both p53 and MDM2. The N-terminal domain of nucleostemin interacts with p53 (Tsai & McKay 2002) whereas the coiled-coil domain (and possibly the acidic domain too) of nucleostemin interact with the central acidic domain of MDM2.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

(Dai et al. 2008; Meng et al. 2008). Increased levels of GNL3 in the nucleoplasm may sequester the action of MDM2 on p53 and in so doing confers an increase in tumour suppression. Alternatively, a reduction in the level of nucleostemin expression due to nucleoplasmic degradation (Huang et al. 2009), may trigger a nucleolar stress response which could result in ribosomal disassembly and consequently the binding of unassembled ribosomal proteins such as L5, L11 and 5S to MDM2, which would inhibit the ubiquitination of p53 preventing it from proteasomal degradation and ultimately increase the number of apoptotic cells (Section 1.4.1.4) (Dai et al. 2008; Donati et al. 2013). Or, perhaps as was observed by Huang et al. (2011), prolonged exposure to ROS could result in the accumulation of nucleolar protein aggregates (Section 1.4.1.5), the conformation of which may be altered according to the nucleostemin variant. While it is recognised that the disulphide bond-mediated oligomerisation takes places between cysteine residues, none of which occur in either wildtype or mutant alleles of rs11177 or rs2289247, the variants which are present may alter protein conformation such that the proximity of the cysteine residues to one another is altered. As a result, an increase in the nucleolar retention of GNL3 could inhibit its degradation and/or its ability to interact with other proteins and ultimately trigger a p53 response. Henceforth, the protein modifications which arise due to these non-synonymous coding variants, how these alter the (nuclear location and) expression of nucleostemin itself and the extent to which these changes influence the p53/MDM2 stress response were investigated here at the protein level.
4.2 Materials and Methods

4.2.1 Knockdown of GNL3 using siRNA in Cultured Human Articular Chondrocytes and JJ012 Chondrosarcoma Cells

4.2.1.1 Samples

4.2.1.1.1 Primary Human Articular Chondrocyte Culture

Human articular cartilage samples were obtained from NOF surgeries. These were processed and cultured as described in Section 3.2.1.3.

4.2.1.1.2 Chondrosarcoma Cell Line (JJ012)

JJ012 chondrosarcoma cells were cultured and passaged as described previously in Section 3.2.3.1 and Section 3.2.4.

4.2.1.2 siRNA

4.2.1.2.1 GNL3 siRNA

To knockdown the expression of GNL3, nucleostemin siRNA (h) (sc45830, Santa Cruz Biotechnology®) was used. The latter consists of three GNL3-specific siRNAs, each 19-25 nucleotides in length.

4.2.1.2.2 Non-Targeting siRNA Control

The negative control, siRNA-A (sc-37007, Santa Cruz Biotechnology®) is a 20-25 nucleotide non-targeting siRNA.

4.2.1.2.3 siRNA-Free Controls

Cells were also cultured in the absence of siRNA. For every transfection, three siRNA-free controls were also cultured. These include; cells cultured in feeding media (Section 3.2.1.3.4 and Section 3.2.3.1) alone, cells cultured in Opti-MEM® (Gibco®) and cells cultured in Opti-MEM® (Gibco®) and mock transfected by adding the transfection reagent but no siRNA.

4.2.1.3 siRNA Transfection of Cultured Human Articular Chondrocytes and JJ012 Chondrosarcoma Cells

All experiments were conducted using RNase-free equipment in a class 2 microbial safety cabinet (Envair). HACs and JJ012 cells were cultured in a 12 well plate until 60-70% confluence, at which point the cells were transfected. In a 1.5ml tube
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

(Eppendorf®) Opti-MEM® reduced serum media with glutaMAX™ supplement (Gibco®) was prepared containing 100nM siRNA. In a separate 1.5ml tube (Eppendorf®), the transfection reagent was diluted (30% (v/v)) in Opti-MEM® (Gibco®). Primary HACs were transfected using Oligofectamine™ (Invitrogen™) and JJ012 cells were transfected using Lipofectamine® (Life Technologies™). Both solutions were then incubated at room temperature for 10min. Following the latter incubation period, the two solutions were combined (9:1) and incubated for a further 20min. During this time the feeding media was removed from the cells and replaced with 400µl Opti-MEM® (Gibco®). Once the incubation period was complete, 100µl of the transfection mixture was then added to each well. The plate was then returned to the 5% CO₂ incubator (Sanyo) at 37°C for 4hrs after which 250µl of anti-biotic free feeding media was added to the cells. RNA (Section 4.2.1.4.1) was extracted after 72hrs.

4.2.1.4 mRNA Expression in siRNA Transfected Human Articular Chondrocyte and JJ012 Chondrosarcoma Cell Cultures

4.2.1.4.1 RNA Extraction
RNA was extracted using TRI Reagent® (Ambion®) as described previously (Section 3.2.8.1).

4.2.1.4.2 Reverse Transcription
RNA was reverse transcribed using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2).

4.2.1.4.3 Confirming GNL3 Knockdown
Initially, GNL3 knockdown was confirmed using cDNA end-point PCR (Section 3.2.10.2) which was later quantified using qPCR techniques described in Section 3.2.11.

4.2.1.4.4 Investigating the Effect of GNL3 Knockdown on mRNA Expression of Chondrogenic Markers
The expression of chondrogenic markers in controls and GNL3 knockdown cells was investigated using cDNA end-point PCR (Section 3.2.10.2). The primers for the amplification of chondrogenic markers, GNL3 and β-Actin from cDNA, are listed in
Appendix Table 7.11. Cycling conditions are as stated in Section 3.2.10.2.2. Visualisation of the cDNA PCR products are as described in Section 3.2.10.2.3.

4.2.2 Investigating the Effect of rs11177 Variants on the Chondrogenic Potential of Human Articular Chondrocytes and Mesenchymal Stem Cells

4.2.2.1 Patient Samples

Human articular cartilage samples were obtained from NOF or THR surgeries and processed as described in Section 3.2.1.3. MSC cultures were obtained from trabecular bone core obtained from the femoral head of the same sample once the cartilage had been removed.

Samples were grown in several individual flasks. This allowed each HAC sample to be investigated as a primary culture (Section 3.2.1.3) and HACs and MSCs, to be passaged (Section 4.2.2.1.3) several times in order attain sufficient cells for culture in StemPro® chondrogenic media (Appendix Solution 7.2.10) (Section 4.2.2.1.4).

4.2.2.1.1 Primary Human Articular Chondrocyte Culture

Protocols relating to the culturing of primary HAC are described in Section 3.2.1.3.

4.2.2.1.2 Primary Mesenchymal Stem Cell Culture

Stem cells were retrieved from the spongy inner core of the femoral head bone marrow and crushed in a sterile 50ml polypropylene tube (BD Falcon™) containing 5ml DMEM (Sigma®) (with 10% FCS (Sigma®), 1% L-glutamine (Sigma®) and 1% penicillin-streptomycin (Gibco®)). An additional 20ml of this media was then added and the solution left at room temperature for 5min. The middle 5-10ml was then removed, placed in a 25cm² flask (Greiner Bio-One) and cultured under the same conditions as the HACs (Section 3.2.1.3.4) changing the media every 2-3 days until cells reached ~80% confluence. Primary MSC cultures yielded a small number of cells. For this reason, cells were not studied at the primary stage and were only ever passaged (Section 4.2.2.1.3) in order to achieve sufficient numbers for downstream experiments. Markers of MSC pluripotency, such as Nanog and Oct-4 (octamer-binding transcription factor 4) (Tsai et al. 2012), should have been used to
confirm the undifferentiated state of these cells. Nucleostemin itself is however used as a marker to validate the proliferative state of stem cells (Zhang & Wang 2013).

4.2.2.1.3 Passaging Human Articular Chondrocyte and Mesenchymal Stem Cell Cultures

At ~80% confluence, cells were washed twice with PBS (1X) (Invitrogen™) and 500µl-1ml (depending on the surface area of the culture dish) of TrypLE™ Express (1X) (Life Technologies™) was added and incubated at 37°C for 5min. Cells were confirmed to be detached under an Olympus® CK2 microscope. The appropriate culture medium (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)), 10 times the volume of TrypLE™ Express (1X) (Life Technologies™), was then added to the cells to inhibit the trypsin activity. This was then transferred to a sterile 50ml polypropylene tube (BD Falcon™) and centrifuged (Sigma® 4K15) at 2000rpm for 5min. The supernatant was then removed and the cell pellet re-suspended in 1ml of the appropriate culture medium. The solution was homogenised using a P1000 pipette (Eppendorf®) and divided between new plates containing the appropriate culture medium. Cells were then returned to the incubator (Sanyo) at 37°C with 5% CO2. The media was changed every 2-3 days until optimal confluence was reached.

4.2.2.1.4 Human Articular Chondrocyte and Mesenchymal Stem Cell Culture in Chondrogenic Media

Upon acquiring a sufficient number of cells for all downstream experiments, cells were passaged as above (Section 4.2.2.1.3) and re-suspended in 1ml StemPro® chondrogenic media (Appendix Solution 7.2.10), which was prepared according to manufacturer’s instructions. Cell density was calculated as described in (Section 3.2.1.3.2). For each time point, cells were plated in 5 wells of a 12 well plate (Corning®) at 4x10^4 cells per well in a final volume of 1ml chondrogenic media and placed at 37°C in the 5% CO2 incubator (Sanyo). The media was changed approximately every 3 days until the desired time point was reached. Culture media was then removed from all wells. One ml of TRI Reagent® (Ambion®) was then divided among three wells and left at room temperature. After 5min the solution in each well was homogenised using a 1ml syringe (BD Plastipak™) with a 21G needle.
investigating the role of GNL3 in osteoarthritis

(BD Microlance™ 3) attached. This was then transferred to a 1.5ml tube (Eppendorf®) and stored at -80°C.

4.2.2.2 Genotyping at rs11177 in Human Articular Chondrocyte and Mesenchymal Stem Cell Cultures

4.2.2.2.1 DNA Extraction
DNA was extracted from all cultures using TRI Reagent® (Ambion®) (Section 3.2.7.1). DNA concentration and quality were determined using the methods described in Section 2.2.1.3. Samples were stored at -20°C.

4.2.2.2.2 PCR
Details regarding the amplification of exon 3 are as described in Section 2.2.2.

4.2.2.2.3 Sequencing and Analysis
Sequencing and analysis of each sample at rs11177 are as described previously in Section 2.2.3.

4.2.2.3 mRNA Expression in Human Articular Chondrocyte and Mesenchymal Stem Cell Cultures

4.2.2.3.1 RNA Extraction
RNA was extracted using TRI Reagent® (Ambion®) as described previously (Section 3.2.8.1)

4.2.2.3.2 Reverse Transcription
RNA was reverse transcribed using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2)

4.2.2.3.3 cDNA Amplification
The primers for the amplification of chondrogenic markers, GNL3 and β-Actin from 200ng cDNA, are listed in Appendix Table 7.11. Cycling conditions are as stated in Section 3.2.10.2.2. Visualisation of the cDNA end-point PCR products are as described in Section 3.2.10.2.3.
4.2.3 Gateway® Cloning

4.2.3.1 Overview

Gateway® technology (Invitrogen™) was used to create several skin melanoma cell lines (A37529) which express every possible combination of wildtype and mutant variants at rs11177 and rs2289247. Protein and RNA from these cells was then used in western blotting and cDNA end-point PCR experiments respectively.

Gateway® technology (Figure 4.2) uses a modified bacteriophage lambda (λ) recombination system. This system, when facilitated by site-specific enzymes, results in conservative recombination between specific attachment (att) sites. During the Gateway® cloning process the recombination system switches between lysogenic (BP) and lytic pathways (LR). The lysogenic pathway, which results in an exchange between attB and attP sites (Figure 4.3), is catalysed by the Escherichia coli integration host factor (IHF) and bacteriophage λ integrase hereafter referred to as the BP Clonase® II Enzyme. The lytic pathway which results in the exchange attL and attR sites (Figure 4.4), requires LR Clonase® II Enzyme. The latter consists of bacteriophage λ excisionase in addition to the two enzymes which make up BP Clonase® II Enzyme (Invitrogen™ 2010a).

Figure 4.2: The lysogenic (BP) and lytic (LR) reactions of the Gateway® Technology cloning system. BP Clonase® II catalyses the reaction between attB (flanking the gene of interest) and attP (donor vector) sites, which gives rise to the attL sites (entry clone). LR Clonase® II catalyses the reaction between the attL (entry clone) and attR (destination vector) sites, which gives rise to attB (expression clone) sites. The ccdB gene inhibits growth in E.coli by interfering with DNA gyrase.

29 The A375 cell line is derived from a 54 year old female human melanoma sample (ATCC 2012)
thereby allowing for negative selection of the untransformed donor and destination vectors and by-products (Invitrogen™ 2010a).

**Figure 4.3:** BP reaction results in recombination between the \( attB-GNL3 \) PCR product and the \( attP \) sites of the donor vector to create an entry clone containing \( attL \) sites and a by-product containing \( attR \) sites (Invitrogen™ 2010a).

**Figure 4.4:** LR reaction results in recombination between \( attL \) sites of the entry clone and \( attR \) sites of the destination vector to create an expression clone containing \( attB \) sites and a by-product containing \( attP \) sites (Invitrogen™ 2010a).
4.2.3.2 **Obtaining the GNL3 transcript**
cDNA from the osteosarcoma cell line, U2OS, was used to obtain a wildtype GNL3 cDNA transcript. Cells were cultured as described in Section 3.2.3.2.

4.2.3.2.1 **RNA Extraction**
RNA was extracted from a 75cm² flask of U2OS cells using the GenElute™ Mammalian Total RNA kit (Sigma®) (Section 3.2.8.2).

4.2.3.2.2 **Measuring RNA Concentration and Quality**
RNA concentration was measured as described in Section 3.2.8.4.

4.2.3.2.3 **Reverse Transcription**
I used 2µg of RNA in the first, reverse transcription, step from the Long-Range 2Step RT-PCR kit (Qiagen®) (Section 3.2.9.1).

4.2.3.3 **Creating the attB flanked GNL3 transcript**
The initial cloning of the GNL3 transcript into the pDONR™221 vector (Invitrogen™) requires attB sites to be attached prior to the BP cloning step (Figure 4.2 and Figure 4.3) of the Gateway® protocol.

4.2.3.3.1 **Primer Design**
Using the H.sapiens (GRCh37/hg19) reference sequence NM_014366.4 obtained from the UCSC Genome Browser (Kent et al. 2002) and manually adding the attB sites, as prescribed in Gateway® Technology (Invitrogen™ 2010a), a 1781bp attB-GNL3 sequence was created. The online tool, Primer3 v0.4.0 (Untergasser et al. 2012) was used to design primers (Invitrogen™).

In an attempt to reduce the melting temperature of the primers, to ensure reproducible amplification, two primers sets were designed. The first primer set contained 20bp of GNL3 on either side of the amplicon with 10bp from the attB site. The stop codon for GNL3 was not included, to allow for the expression of a downstream V5 tag in the pDONR™221 vector (Invitrogen™). The second primer set included the full attB site which attached to the 10bp attB site of the amplicon from first reaction. No nucleotides corresponding to the GNL3 sequence were
included in the second primer set. Please refer to Table 4.1 for primer sequences, annealing temperatures and Figure 4.5 for a description of the attB site composition.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 4: FUNCTIONAL ANALYSIS OF GNL3

### Table 4.1: Primer sequences for attachment of attB sites to GNL3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward [5’-3’ +]</th>
<th>Reverse [5’-3’ -]</th>
<th>Product size (bp)</th>
<th>Anneal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB (10bp) + GNL3 (20bp)</td>
<td>AGATAGAACCATGAAAAGGCCCTAAGTTAAA</td>
<td>GCTGGGTCACATAATCTGTACTGAAGTC</td>
<td>1723</td>
<td>69</td>
</tr>
<tr>
<td>Full attB only</td>
<td>GGGGACAAGTTTGTAACAAAAAAGCAGGCTTGAAGGAGATAGAACC</td>
<td>GGGGACCCTTTGTACAAAGAAGGCTGGTCCAGATAGAACC</td>
<td>1781</td>
<td>72</td>
</tr>
</tbody>
</table>

Underlined bases refer to attB site sequence used in both primer sets. bp, base pairs.

Figure 4.5: Primer design for the attachment of attB sites

Shine-Dalgaro and Kozak sequences allow the native protein to be expressed in both mammalian and E.coli cells. The GNL3 stop site was excluded from the reverse primer sequences. ATG, is the GNL3 start site; ORF, Open Reading Frame (adapted from Invitrogen™ 2010a; GibcoBRL® 2012).
4.2.3.3.2 Amplification

The attB sites were attached by means of two PCR amplifications which used a *Pyrococcus*-like enzyme, Phusion® High Fidelity DNA Polymerase (New England BioLabs®). Master mixes were prepared on ice according to manufacturer guidelines for a 50µl reaction using HF buffer, no dimethyl sulphoxide (DMSO) and the primers described in Table 4.1. The first PCR reaction used ~70ng of cDNA to attach the first primer set. The second PCR reaction used ~800ng of the PCR product generated in the first reaction. All reactions were run on the G-Storm GS1 thermocycler according to the conditions stated in Appendix Table 7.19 and Appendix Table 7.20. This resulted in a complete GNL3 transcript with attB sites attached to the full transcript of GNL3 (without the stop codon).

4.2.3.3.3 Purification

The attB-flanked GNL3 amplicon was purified by combining 100µl of 30% (m/v) polyethylene glycol 8000 (Sigma®) in 30mM magnesium chloride (MgCl₂) (New England BioLabs®) with 150µl tris-EDTA (TE) buffer (1X, pH 8.0) (Invitrogen™) and 50µl PCR product. The mixture was then vortexed and centrifuged (Eppendorf® 5415D) 10000 x g for 15min at room temperature. The supernatant was then removed and the pellet dissolved in 50µl TE buffer (1X, pH 8.0) (Invitrogen™). Sample concentration and purity were then measured using the NanoDrop1000 v3.7.0 (Thermoscientific).

4.2.3.3.4 Visualisation

All amplified products were visualised as described in Section 2.2.2.3 except, a 1kb DNA ladder (New England BioLabs®) was run alongside the PCR products on a 1% agarose (Bioline)–TBE (1X) gel.

4.2.3.4 Entry Clone (BP reaction)

4.2.3.4.1 pDONR™221 Donor Vector

The donor vector (Figure 4.2 and Figure 4.3) used was pDONR™221 (Invitrogen™) (300ng/µl) which has an attP-flanked site (Figure 4.6).
Figure 4.6: pDONR™221 donor vector (Invitrogen™) map (4761bp) T2, rrmB T2 transcription termination sequence (c) (268-295bp) and T1, rrmB T1 transcription termination sequence (c) (427-470bp), prevent the expression of the cloned gene by vector-encoded promoters; M13, sites for the attachment of forward (537-552bp) and reverse (3026-3042bp) sequencing primers; attP1 (570-801bp) and attP2 (c) (2753-2984bp), allow for recombination with attB-GNL3; ccdB gene (c) (1197-1502bp), allows for negative selection of the plasmid; Cm<sup>R</sup>, chloramphenicol resistance gene (c) (1825-2505bp); Kanamycin, kanamycin resistance gene (3155-3964bp); pUC origin (4065-4758bp), allows for replication and maintenance in E.coli; (c), complementary strand (Invitrogen™ 2012).

4.2.3.4.2 BP reaction
The BP reaction will facilitate the transfer of the attB-GNL3 to the attP sites of the pDONR™221 (Invitrogen™). The attP sites will be converted to attL sites when
recombined with attB sites in a reaction catalysed by BP Clonase® II Enzyme (Invitrogen™) (Figure 4.2 and Figure 4.3).

In a 1.5ml microcentrifuge tube (Eppendorf®) I added 1ul (10.05fmol or 11.43ng) (Equation 4.1) of attB-flanked GNL3 amplicon to 1ul (300ng) pDONR™221 (Invitrogen™) and 14µl TE (1X, pH 8.0) (Invitrogen™). To this mixture, 4µl of Gateway® BP Clonase® II Enzyme Mix (Invitrogen™) was added, vortexed and incubated overnight at 25ºC. A negative control, which contained no attB-GNL3, was also incubated overnight. The next day, 2µl of Proteinase K (2µg/µl) (Invitrogen™) was added to the reaction and incubated at 37ºC for 10min.

\[
ng = (fmol)(N) \left( \frac{660fg}{fmol} \right) \left( \frac{1ng}{10^6fg} \right)
\]

Equation 4.1: Converting DNA from nanograms (ng) to femtomoles (fmol) N, number of base pairs in attB-flanked GNL3 amplicon (Invitrogen™ 2010a).

4.2.3.4.3 Transformation of DH5α™ Competent Cells
I then took 1µl of the BP reaction (Section 4.2.3.4.2) and added it to 50µl Subcloning Efficiency™ DH5α™ competent cells (Invitrogen™). The mixture was left on ice for 30min then heat shocked at 42ºC for 45sec and placed back on ice. I then added 450µl lysogeny broth (LB) media and shook for 1hr at 200rpm in a 37 ºC incubator. The transformed cells were grown overnight on 50ug/ml kanamycin (Gibco®) LB agar plates. Individual colonies were then selected and grown overnight in 3ml 50ug/ml kanamycin LB medium at 200rpm in a 37 ºC incubator (INFORS HT EcoTron).

4.2.3.4.4 Plasmid DNA Mini-Prep
Plasmid DNA was prepared using QIAprep® Miniprep (Qiagen®) according to manufacturer’s protocol. Briefly, bacterial cells were centrifuged (Eppendorf® 5810R) at 3000rpm for 15min at room temperature. The pellet was then lysed in an alkaline buffer which results in a neutral and high salt solution, both of which are essential if DNA is to bind to the QIAprep® silica membrane. The lysate is cleared by a series of centrifugation (Eppendorf® 5415R) and wash steps which remove unwanted proteins, RNA, endonucleases and salts. The plasmid DNA was then
eluted from the membrane in a low salt elution buffer (10mM tris-Cl, pH 8.5) (Qiagen® 2012). DNA concentration and quality was then measured as described previously (Section 2.2.1.3).

4.2.3.4.5 Sequencing Entry Clone Plasmid DNA
Plasmid DNA was sequenced using M13 primers (Appendix Table 7.21). These were supplied by HGU services (MRC, Edinburgh) where the sequencing was carried out.

4.2.3.5 Expression Clone (LR Reaction)
4.2.3.5.1 pEF5/FRT/V5-DEST™ Destination Vector
The GNL3 insert was then transferred to the destination vector pEF5/FRT/V5-DEST™ (Invitrogen™) (Figure 4.2 and Figure 4.4) which has an attR-flanked recombination site (Figure 4.7).
Figure 4.7: pEF5/FRT/V5-DEST™ destination vector (Invitrogen™) map (7528bp) EF-1α, human elongation factor 1α promoter which allows for overexpression of GNL3 in mammalian cells (348-1531bp); T7, promoter allows for sense-orientated transcription in vitro (1548-1567bp); attR1 (1654-1769bp) and attR2 (3225-3349bp), allow for recombination with the attL sites of the entry clone; CmR, chloramphenicol resistance (1878-2537bp); ccdB gene, allows for negative selection of the expression clone (2879-3184bp); V5 epitope, can be detected with anti-V5 antibody when expressed with GNL3 (3402-3443bp); BGH pA, bovine growth hormone polyadenylation (3487-3711bp) and SV40 pA, simian virus 40 early polyadenylation signal (5201-5331bp), allow mRNA transcription termination and polyadenylation; FRT, Flp recombinase target site for Flp-Recombinase (3994-4041bp); Hygromycin, hygromycin resistance (no ATG) (4049-5069bp); pUC origin (5714-6387bp), allows for replication and maintenance in E.coli; Ampicillin, ampicillin resistance gene (6532-7392bp); (c), complementary strand (Invitrogen™ 2010b).

4.2.3.5.2 LR reaction
The LR reaction will facilitate the transfer of the attL-flanked GNL3 from the entry clone to the attR site of the pEF5/FRT/V5-DEST™ vector (Invitrogen™). The attR sites will be converted to attB sites when recombined with attL sites in a reaction catalysed by LR Clonase® II Enzyme (Invitrogen™) (Figure 4.2 and Figure 4.4).
In a 1.5ml microcentrifuge tube (Eppendorf®) I added 150ng of the entry clone to 150ng pEF5/FRT/V5-DEST™ vector. This was then brought to a volume of 8µl with TE buffer (1X, pH 8.0) (Invitrogen™). To this mixture, 2µl of Gateway® LR Clonase® II Enzyme Mix (Invitrogen™) was added, vortexed and incubated overnight at 25°C. A negative control, which had no entry clone, was also incubated overnight. The next day, 2µl of Proteinase K (2µg/µl) (Invitrogen™) was added to the reaction and incubated at 37°C for 10min.

4.2.3.5.3 Transformation of DH5α™ Competent Cells
Subcloning Efficiency™ DH5α™ competent cells (Invitrogen™) were transformed as before (Section 4.2.3.4.3), using heat shock methods. Transformed cells were cultured as above (Section 4.2.3.4.3) except 100µg/ml ampicillin (Sigma-Aldrich®) replaced the kanamycin.

4.2.3.5.4 Plasmid DNA Mini-Prep
Plasmid DNA was prepared using QIAprep® Miniprep (Qiagen®) as described in Section 4.2.3.4.4.

4.2.3.5.5 Sequencing Expression Clone
The entire GNL3 insert was sequence by HGU services (MRC, Edinburgh) using the primers described in Appendix Table 7.22.

4.2.3.5.6 Site Directed Mutagenesis
4.2.3.5.6.1 Primer Design
Using NM_014366.4 (GRCh37/hg19) (NCBI 2014c) as a reference, primers were designed to be approximately 30bp in length and end in a guanine or adenine base. The substitute base (minor allele) at rs11177 or rs2289247 was located in the middle of the primer sequence to ensure that the site of interest would be altered. Primer sequences are listed in Appendix Table 7.23.

4.2.3.5.6.2 Amplification
Using 50ng of expression clone plasmid DNA (Section 4.2.3.5.4), site-directed mutagenesis was performed at each variant site using PfuUltra DNA polymerase Master Mix (Agilent Technologies) according to the PCR conditions described in
Appendix Table 7.24 and Appendix Table 7.25. All reactions were run on the G-Storm GS1 thermocycler.

4.2.3.5.6.3 Restriction Enzyme (*DpnI*) Digestion

Prior to digestion, 5µl of the mutagenesis PCR product (Section 4.2.3.5.6.2) was removed and used as a control. The remaining 20µl was then digested with 10U *Diplococcus pneumoniae* (*DpnI*) (New England BioLabs®) at 37°C for 4hrs in a microbiological incubator (Heraeus®, ThermoFisher Scientific), followed by 10min at 65°C on a heat block (UBD, Grant) to inactivate the enzyme. Any plasmids which had not been mutated were destroyed. *DpnI* only digests the DNA when the recognition site is methylated (Figure 4.8), hence only the parental DNA is digested and the mutant DNA remains intact.

![Methylated DpnI recognition site](adapted from (New England BioLabs® 2014)).

Subsequently, undigested and *DpnI*-treated mutant plasmids were transformed into of DH5α™ competent cells (Invitrogen™) (Section 4.2.3.5.3). Plasmid DNA was prepared using QIAprep® Miniprep (Qiagen®) as described in Section 4.2.3.4.4 and sequenced using primers described in Appendix Table 7.22 (Section 4.2.3.5.5).

Site-directed mutagenesis (Section 4.2.3.5.6) was then repeated using the expression clones mutated at a single site with primers for the alternate variant site, to create all four possible combinations of wildtype and mutant variants rs11177 and rs2289247.

4.2.3.6 Transfecting the Melanoma Cell Line (A375)

4.2.3.6.1 Overview

The Flp-In™ system (Invitrogen™) (Figure 4.9) was used to integrate the *GNL3* insert from the expression clone (Figure 4.7 and Section 4.2.3.5) into a specific location in the genome of the A375 cell line. This system requires the host cell line to
possess a Flp recombination target (FRT) site at the location where the gene of interest is to be incorporated. The cells are then co-transfected with pOG44 and the expression clone, which also houses an FRT site. pOG44 constitutively expresses the enzyme Flp-recombinase which is essential for homologous recombination between the FRT sites (Invitrogen™ 2002).

**Figure 4.9: Overview of the Flp-In™ system (Invitrogen™)** Recombination takes places between the FRT sites of the A375 cells and the GNL3 expression vector (Figure 4.7) in the presence of Flp-recombinase which is expressed from pOG44. This results in GNL3 protein expression in hygromycin-resistant A375 cells (Invitrogen™ 2002).
4.2.3.6.2 Introduction of FRT Sites

The FRT site (Figure 4.10) is vital to the Flp-In™ system and must be present in both the host cell and the expression clone. The FRT site originates from Saccharomyces cerevisiae and is well characterised. The site is comprised of an 8bp sequence, which contains the Xba I restriction site, situated between two imperfect inverted repeats, both 13bp in length. (An additional 13bp site may occur although this is not necessary for cleavage.) Upon the binding of Flp-recombinase to the 13bp sequence, cleavage occurs at the boundaries of the 8bp region (Invitrogen™ 2002).

**Figure 4.10: FRT site** The minimal FRT site consists of two imperfect inverted repeats, both 13bp in length (black arrows), situated either side of an 8bp spacer region which also contains a Xba I restriction site. An additional 13bp (grey arrow) is often found but is not essential for cleavage. Blue triangle, FRT cleavage site (adapted from (Invitrogen™ 2002)).

Members of the Hupp lab (Cancer Research United Kingdom (CRUK), Edinburgh) engineered the melanoma cell line to include a single FRT site in the A375 genome by transfecting a pFRT/lacZeo vector. The simian virus 40 (SV40) (NCBI Gene) promoter in this vector controls the expression of the fusion gene lacZ-Zeocin™, which provides the cells with Zeocin™ resistance. Downstream of the lacZ-Zeocin™ gene is the FRT site which is essential if Flp-recombinase is to bind (Invitrogen™ 2002). Southern blotting confirmed only a single copy of the pFRT/lacZeo vector had been integrated into the genome thus preventing undesirable intramolecular recombination (Invitrogen™ 2002; Gray 2012).

The second FRT site is found in the expression clone (Figure 4.7). The gene of interest is under the influence of the human elongation factor 1α promoter. The vector also contains a gene which encodes for hygromycin resistance. This is expressed by the SV40 promoter of the pFRT/lacZeo vector, following successful recombination at the FRT sites.
4.2.3.6.3 POG44 production

pOG44 was prepared by members of the Hupp lab (CRUK, Edinburgh) using Qiagen® Plasmid Maxiprep.

4.2.3.6.4 Transfection

Unless stated otherwise, all tissue culture work involving A375 cells was carried out in a class II microbial safety cabinet (TriMAT). A375 cells were plated in 10cm² dishes (Corning®) in DMEM (Gibco®) (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)). Cells were grown to ~70% confluence in a 10% CO₂ incubator (Heraeus® HERAcell®, ThermoFisher Scientific) at 37°C.

Expression clones (1µg) were co-transfected (1:9) with pOG44 using Attractene transfection reagent (Qiagen®). Mixtures were prepared at room temperature and incubated for 15min before adding the complex in a drop-wise manner to the cells. The cells were placed in an incubator (Galaxy® 170R, New Brunswick (Eppendorf®)) at 32°C with 10% CO₂ for 24hrs.

4.2.3.6.5 Hygromycin Treatment

The next day, cells were incubated (Heraeus® HERAcell®, ThermoFisher Scientific) at 37°C with 10% CO₂ in DMEM (Gibco®) (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)) and challenged with 0.2mg/ml hygromycin-B (Invitrogen™) for approximately two weeks, changing the media every couple of days. A control plate of non-transfected cells was also challenged with 0.2mg/ml hygromycin-B (Invitrogen™).

Cells which had been successfully transfected and correspondingly acquired a resistance to hygromycin formed aggregates. These were collected and grown in new plates (Section 4.2.3.6.6).

Transfected cell were then allowed to proliferate in a hygromycin-free environment. Prior to reaching confluence (~80%) cells were either split (Section 4.2.3.6.6) and/or frozen (Section 4.2.3.6.7) for downstream experiments.

The transfection was carried out twice, resulting in two cell lines for each variant combination. This enabled all downstream experiments to be carried out in duplicate.
4.2.3.6.6 Passaging Transfected A375 Cells

Cells were washed twice with PBS (1X) (Invitrogen™) and 0.05% trypsin-EDTA (1X) (Gibco®), one fifth the volume of the plate, was added and incubated at 37°C for 5min. Cells were confirmed to be detached under a Nikon Eclipse TS100 microscope. DMEM (Gibco®) (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)), three times the volume of trypsin-EDTA, was then added to the cells to inhibit the trypsin activity. This was homogenised using a P1000 pipette (Eppendorf®) and 1ml added to DMEM (Gibco®) (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)) in a new plate. Cells were then returned to the incubator (Heraeus® HERAcell®, ThermoFisher Scientific) at 37°C with 10% CO₂. The media was changed every 2-3 days until optimal confluence was reached.

4.2.3.6.7 Freezing Transfected A375 Cells

Cells were detached as above (Section 4.2.3.6.6) and centrifuged (accuSpin 1, Fisher Scientific) at 1000rpm for 5min at room temperature. The supernatant was removed and the pellet re-suspended in FCS (Sigma®) containing 10% DMSO (Fisher Scientific). The volume added was equivalent to 0.35 times the total surface area of the plate. The mixture was homogenised using a P1000 pipette (Eppendorf®) and divided in 1ml aliquots which were dispensed into CryoTubes® (Nunc®) and placed in an isopropanol freezing container (Nalgene™) at -80°C. This was to ensure the rate of cooling was optimal at -1°C/minute. Cells were then removed from the storage container after a minimum of 24hrs and transferred to liquid nitrogen storage.

4.2.3.7 mRNA expression

4.2.3.7.1 RNA Extraction

Culture media was removed from cells upon reaching ~80% confluence and washed twice with PBS (1X) (Invitrogen™). The RNeasy® (Qiagen®) RNA extraction kit was used with the QIAshredder spin column (Section 3.2.8.3).

4.2.3.7.2 cDNA production

RNA was reverse transcribed using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) as described previously (Section 3.2.9.2).
4.2.3.7.3 cDNA amplification

The transcription of GNL3 in the host cell line was confirmed using primers described in Appendix Table 7.11 and cDNA amplification and visualisation techniques described in Section 3.2.10.2.2 and Section 3.2.10.2.3, respectively.

4.2.3.8 Protein Expression

4.2.3.8.1 Protein Extraction

Protein was extracted from cells which had reached ~80% confluence. The procedure was carried out on ice to reduce the risk of protein degradation. Firstly, all culture media was aspirated and cells washed twice with ice cold PBS (1X) (Invitrogen™). Cells were then scraped in 500µl of PBS (1X), placed in a 1.5ml microcentrifuge tube (Eppendorf®) and spun at 4°C for 5min at 5000rpm in a microcentrifuge (Eppendorf® 5415R). The supernatant was discarded and the cell pellet homogenised in an appropriate volume (typically 40-50µl) of radio immunoprecipitation assay (RIPA) lysis buffer (Appendix Solution 7.2.11) which contained triton X-100 (Sigma®) detergent which had previously been used in other studies (Tsai & McKay 2002) to successfully extract nucleostemin. Lysis was carried out on ice for 20min with periodic vortexing after which, the cells were centrifuged (Eppendorf® 5415R) at maximum speed for 12min at 4°C. The supernatant containing the protein was transferred to a new microcentrifuge tube and stored at -80°C.

4.2.3.8.2 Protein Concentration

Protein concentration was measured using the Pierce protein assay described in Section 3.2.12.2.

4.2.3.8.3 Western Blot

Western blots were carried out to quantify the expression of GNL3, p53, MDM2 and the loading control β-Actin, as described in Section 3.2.13. The antibodies used to detect the aforementioned proteins are listed in Table 4.2. A protocol to detect the V5 protein tag was also optimised however, this was not used in the final analysis and the results are not shown here. Antibodies for the detection of p53, MDM2 and V5 were kindly provided by the Hupp group (CRUK, Edinburgh).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

### Table 4.2: Antibodies used in western blotting to detect the protein expression of MDM2, GNL3 (R&D Systems®), p53 and β-Actin (Sigma®)

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Protein</th>
<th>Host</th>
<th>Clonality</th>
<th>Conc.</th>
<th>Secondary Antibody Host</th>
<th>Band size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A10</td>
<td>MDM2</td>
<td>mouse</td>
<td>monoclonal</td>
<td>1:1000</td>
<td>donkey</td>
<td>75°</td>
</tr>
<tr>
<td>AF1638</td>
<td>GNL3</td>
<td>goat</td>
<td>polyclonal</td>
<td>1:1000</td>
<td>donkey</td>
<td>62</td>
</tr>
<tr>
<td>DO-1</td>
<td>p53</td>
<td>mouse</td>
<td>monoclonal</td>
<td>1:5000</td>
<td>donkey</td>
<td>53</td>
</tr>
<tr>
<td>A5060</td>
<td>β-Actin</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:1000</td>
<td>donkey</td>
<td>42</td>
</tr>
</tbody>
</table>

All antibodies were diluted in 5% (w/v) milk-TBS (1X). All secondary antibodies (Stratech®) were diluted 1:1000. MDM2 resulted in two bands, both of which were quantified together.

#### 4.2.3.9 Cisplatin Treatment

Transfected cells representing each combination of variants were plated at equal density in a six well plate (Corning®). Cells which had not been transfected with the GNL3 insert were also plated at the same density. Two wells were plated per cell type, one to act as an untreated control and the other, to be treated with the DNA cross-linker, cisplatin (Sigma®) (Section 4.1).

Cells were grown for 1-2 days in 2ml DMEM (Gibco®) (with 10% FCS (Sigma®) and 1% penicillin-streptomycin (Gibco®)). Prior to reaching complete confluence, 5µM cisplatin (Sigma®) was added and the cells returned to the incubator (Heraeus® HERAcell®, ThermoFisher Scientific) at 37°C with 10% CO₂ for 16hrs. No cisplatin (Sigma®) was added to the control wells.

Protein was extracted from the cultures and run on western blots as described above (Section 4.2.3.8). The results were analysed using densitometry (Section 3.2.13.6.1). All experiments were carried out in duplicate for each transfection.

#### 4.2.3.10 Hypoxia

The A375 cells, both transfected and those containing no GNL3 insert were placed under hypoxic conditions. This was accomplished by placing cells in a hypoxic incubator (H35 Hypoxystation, Whitley) which provided only 0.5% O₂ to the cells. Cells under control conditions, otherwise referred to as normoxic conditions, were maintained as before and were subjected only to the naturally occurring oxygen levels in the atmosphere.
4.2.3.10.1 AlamarBlue® Cell Survival Assay
To ensure the A375 cells would survive in a hypoxic environment, a survival test was conducted prior to continuing with the experiment. Cells from the first transfection were cultured in triplicate until ~50% confluence and then placed under hypoxic and normoxic conditions for 48hrs.

The number of surviving cells was assayed using alamarBlue® (Invitrogen™). The latter detects the metabolic activity of the cells and relies on a redox indicator which changes colour and fluoresces in response to changes in the oxidative state of the culture. As the metabolic activity of the cells increases, alamarBlue® (Invitrogen™) is altered from a non-fluorescent blue, if the cells are in an oxidative state, to a fluorescent red, indicative of a chemical reduction due to an increase in cell growth (Invitrogen™ 2013).

AlamarBlue® (Invitrogen™) was placed in the hypoxic incubator 2hrs prior to use on the hypoxic cultures, to ensure no oxygen was added to the cells upon the addition of this reagent. AlamarBlue® was added at 10% volume of the culture media per well, 3hrs prior to analysis. The fluorescence level in each well was read at an emission wavelength of 590nm and excitation wavelength of 540nm using a BIO-TEK™ SynergyHT plate reader and Gen5™ software. A blank well, which contained only culture media but no cells, was used as a control to correct for background fluorescence.

4.2.3.10.1.1 Statistical Analysis
All values were corrected for background fluorescence. Any values which exceeded the average ± 3SD were removed. P values were calculated using MiniTab® v12.23 (MiniTab Inc). A general linear model ANOVA was conducted to determine whether the cells differed significantly (p≤0.05) from one another in the ability to survive 48hrs under hypoxic conditions. Any significant results were further investigated using Tukey pairwise comparison test (95% confidence interval) to determine which cells, if any, differed significantly from one another.
4.2.3.10.2 Hypoxia Treatment

Having confirmed cell viability, cultures were subjected to various periods under normoxic and hypoxic conditions. Cells were first subjected to 24hrs hypoxia with corresponding normoxic control conditions for 24hrs. Cells were also placed under oxidative stress during which cultures initially placed under hypoxic conditions for 24hrs were returned to normoxic conditions for a further 24hrs. Controls for this condition include cells subjected to normoxic conditions for 48hrs and a second control during which cells were incubated under hypoxic conditions for 48hrs.

Protein was extracted from the cultures and run on western blots as described above (Section 4.2.3.8). The results were analysed using densitometry (Section 3.2.13.6.1). All experiments were carried out in triplicate for each transfection.

4.2.3.11 Statistical Analysis of Protein Expression in Response to Cisplatin and Hypoxia

All protein expression (Section 4.2.3.9 and Section 4.2.3.10.2) was corrected for β-Actin. Any values which exceeded the average ± 3SD were removed. P values were calculated using MiniTab® v12.23 (MiniTab Inc). A general linear model ANOVA was employed to determine whether the cells differed significantly (p≤0.05) from one another in the expression of proteins, GNL3, p53 and/or MDM2 (corrected for β-Actin), in response to stressful stimuli. This response was first analysed in FRT cells alone. The same statistical analysis was then carried out, independently of the latter, to investigate whether cells containing the GNL3 insert differed significantly from one another in response to the stimulus. Any significant results were further investigated using Tukey pairwise comparison test (95% confidence interval) to determine whether variants under the same conditions, differed in the amount of protein expressed.
4.3 Results

4.3.1 siRNA GNL3 Knockdown

Cultured HACs (taken from femoral head cartilage) (Sections 3.2.1.3) and JJ012 chondrosarcoma cells (Section 3.2.3.1) were successfully transfected with siRNA (Santa Cruz Biotechnology®) as described above (Section 4.2.1). RNA was extracted using TRI Reagent® (Ambion®) (Section 3.2.8.1) and reverse transcribed using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) (Section 3.2.9.2).

4.3.1.1 Confirmation of GNL3 Knockdown by cDNA end-point PCR

cDNA (200ng) was successfully amplified using DNA Polymerase Kit (Qiagen®) as described in Section 4.2.1.4.3. The GNL3 and β-Actin amplicons are shown in Figure 4.11. The expression of GNL3 appears decreased in HACs and JJ012 cells transfected with nucleostemin siRNA (Santa Cruz Biotechnology®). β-Actin expression appears uniform throughout.

Figure 4.11: cDNA end-point PCR results for mRNA expression of GNL3 (208bp) and β-Actin (250bp) in cultured HACs and JJ012 chondrosarcoma cell line cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X). All amplicons from the same cell type were run on the same gel. No bands were seen in the no template PCR control.

4.3.1.2 Quantification of GNL3 Knockdown by qPCR

Samples were run in a qPCR experiment to quantify the level of GNL3 knockdown in nucleostemin siRNA cells using the methods described in Section 4.2.1.4.3 to quantify the level of GNL3 (corrected for 18S) expression.
The results (Figure 4.12) show successful knockdown of GNL3 in both HACs and JJ012 cells. The expression of GNL3 (corrected for 18S) was not significantly different between the controls in JJ012 cells. Nor, was there a statistically significant difference in the expression of GNL3 between any of the controls and nucleostemin knockdown JJ012 cells. The same was true when samples were normalised to the untreated control.

Figure 4.12: qPCR results for mRNA expression of GNL3 in control and GNL3 knockdown HACs and JJ012 chondrocyte cell lines HAC, Cultured HACs. Transfection carried out once (n=1). All samples were run in duplicate. JJ012, JJ012 chondrosarcoma cell line. Transfection carried out in triplicate (n=3). Samples from the first transfection were run in duplicate. Samples from all other transfections were run in triplicate. Values are average GNL3/18S percentage calculated using molecules/µl values. Blank subtracted from all values. Values are normalised to the untreated control. Error bars are ± SEM.

4.3.1.1.3 Effect of GNL3 Knockdown on mRNA Expression of Chondrogenic Markers

cDNA was successfully amplified using DNA Polymerase Kit (Qiagen®) as described in Section 4.2.1.4.4 except varying amounts of cDNA from HACs\textsuperscript{30} and JJ012\textsuperscript{31} cells was amplified.

\textsuperscript{30} MMP3, 200ng; MMP13, 200ng; ACAN, 200ng; SOX9, 200ng; COL2A, 200ng; RUNX2, 2µg; GNL3, 200ng; β-Actin, 200ng

\textsuperscript{31} MMP3, 2µg; MMP13, 5µg; ACAN, 10µg; SOX9, 200ng; COL2A, 5µg; RUNX2, 2µg; GNL3, 200ng; β-Actin, 500ng
4.3.1.1.3.1 Cultured Human Articular Chondrocytes

The cDNA end-point PCR results are shown in Figure 4.13. Amplicons for all chondrogenic markers were seen in all control and nucleostemin knockdown samples.

![Image of PCR gel showing expression of chondrogenic markers](Image)

Figure 4.13: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in control and GNL3 knockdown cultured HACs. cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X) alongside a low molecular weight marker (New England BioLabs®). All genes are represented by a single sample. All samples for the same gene were run on the same gel. No bands were seen in the no template PCR controls.

4.3.1.1.3.2 Chondrosarcoma Cell Lines (JJ012)

The cDNA end-point PCR results are shown in Figure 4.14. Amplicons for all chondrogenic markers were seen in all control and nucleostemin knockdown samples.
Figure 4.14: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in control and GNL3 knockdown JJ012 chondrocyte cell lines. cDNA PCR products were run on a 2% agarose gel at 75V for 45min in TBE buffer (1X) alongside a low molecular weight marker (New England BioLabs®). All genes are represented by a single sample (Results may vary in alternate samples). No bands were seen in the no template PCR control.

4.3.2 Genotyping Cells Cultured in Chondrogenic Media

4.3.2.1 Human Articular Chondrocytes

Genotyping was performed on DNA extracted from primary HAC cultures (Section 4.2.2.1.1) using TRI Reagent® (Ambion®). Genotyping for rs11177 was carried out as described in Section 4.2.2.2. Total RNA was extracted from the same samples following culture in chondrogenic media at various time points (Section 4.2.2.1.4). This was used to compare the expression of chondrogenic markers and GNL3 in patients of different genotypes. In total, RNA was extracted from HAC cultures of all three rs11177 (c.116G>A (p.R39Q)) genotypes (GG, n=6; GA, n=4; AA, n=3) (Section 4.2.2.3). Details regarding age, gender and disease status for each genotype are listed in Table 4.3.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

<table>
<thead>
<tr>
<th>rs11177</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Normal</td>
<td>1 (16.7%)</td>
<td>1 (25.0%)</td>
</tr>
<tr>
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<td>Gender</td>
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<td>2 (50.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>5 (83.3%)</td>
<td>2 (50.0%)</td>
<td>2 (66.7%)</td>
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<tr>
<td>Age</td>
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<td>55.3 ± 11.3</td>
<td>63.7 ± 14.0</td>
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</tbody>
</table>

Table 4.3: Details of the patient samples, from which HAC were cultured in chondrogenic media and used to compare the mRNA expression of chondrogenic markers and GNL3 between different genotypes at rs11177 Age, mean (years) ± 1SD.

4.3.2.2 Mesenchymal Stem Cells

Genotypes at rs11177 were determined as described in Section 4.3.2.1. Total RNA was extracted from MSC cultures following culture in chondrogenic media at various time points (Section 4.2.2.1.4). This was used to compare the expression of chondrogenic markers and GNL3 in patients of different genotypes. In total, RNA was extracted from MSC cultures of all three rs11177 (c.116G>A p.(R39Q)) genotypes (GG, n=5; GA, n=4; AA, n=2) (Section 4.2.2.3). Details regarding age, gender and disease status for each genotype are listed in Table 4.4.

<table>
<thead>
<tr>
<th>rs11177</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Normal</td>
<td>0 (0.0%)</td>
<td>1 (25.0%)</td>
</tr>
<tr>
<td>OA</td>
<td>5 (100.0%)</td>
<td>3 (75.0%)</td>
<td>1 (50.0%)</td>
</tr>
<tr>
<td>Gender</td>
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<td>57.6 ± 8.5</td>
<td>55.3 ± 11.3</td>
<td>64.0 ± 19.8</td>
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</tbody>
</table>

Table 4.4: Details of the patient samples, from which MSCs were cultured in chondrogenic media and used to compare the mRNA expression of chondrogenic markers and GNL3 between different genotypes at rs11177 Age, mean (years) ± 1SD.

4.3.3 Association between rs11177 variants and mRNA Expression of Chondrogenic Markers in Human Articular Chondrocyte and Mesenchymal Stem Cell Cultures
4.3.3.1 Human Articular Chondrocytes

cDNA end-point PCR (Section 4.2.2.3.3) confirmed the expression of all chondrogenic markers, \textit{GNL3} and \textit{\(\beta\)-Actin} in HAC cultures for different rs11177 genotypes during 21 days of culture as shown in Figure 4.15.
Chapter 4: Functional Analysis of GNL3
Investigating the Role of GNL3 in Osteoarthritis

Figure 4.15: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in HACs cultured in StemPro® chondrogenic media for 0, 7, 14 or 21 days. cDNA PCR products were run on a 2% agarose gel at 75V for 1hr in TBE buffer (1X). All genes are represented by a single sample for each genotype (Results may vary in alternate samples). No bands were seen in the no template PCR control. B, No template PCR control; L, Low molecular weight DNA ladder (New England BioLabs®).

4.3.3.2 Mesenchymal Stem Cells

cDNA end-point PCR (Section 4.2.2.3.3) confirmed the expression of all chondrogenic markers (except COL2A), GNL3 and β-Actin in MSC cultures for different rs11177 genotypes during 21 days of culture as shown in Figure 4.16.
CHAPTER 4: FUNCTIONAL ANALYSIS OF GNL3

Investigating the Role of GNL3 in Osteoarthritis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Band Size (bp)</th>
</tr>
</thead>
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<td>MMP3</td>
<td>614</td>
</tr>
<tr>
<td>MMP13</td>
<td>347</td>
</tr>
<tr>
<td>ACAN</td>
<td>346</td>
</tr>
<tr>
<td>SOX9</td>
<td>323</td>
</tr>
<tr>
<td>COL2A</td>
<td>207</td>
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<tr>
<td>RUNX2</td>
<td>177</td>
</tr>
<tr>
<td>GNL3</td>
<td>208</td>
</tr>
<tr>
<td>β-Actin</td>
<td>250</td>
</tr>
</tbody>
</table>

rs11177
Figure 4.16: mRNA expression of MMP3, MMP13, ACAN, SOX9, COL2A, RUNX2, GNL3 and β-Actin in MSCs cultured in StemPro® chondrogenic media for 0, 7, 14 or 21 days cDNA PCR products were run on a 2% agarose gel at 75V for 1hr in TBE buffer (1X). All genes are represented by a single sample for each genotype (Results may vary in alternate samples). No bands were seen in the no template PCR control (Figure 4.15). L, Low molecular weight DNA ladder (New England BioLabs®).

4.3.4 Confirming Site Directed Mutagenesis in Expression Clones

Site directed mutagenesis was carried out as described in Section 4.2.3.5.6 to create all four possible combinations of wildtype and mutant variants at rs11177 and rs2289247. The expression clones were sequenced as described in Section 4.2.3.5.5 to confirm successful mutagenesis had been carried out (Figure 4.17).

Figure 4.17: Site directed mutagenesis of expression clones Chromatograms show expression clones sequences for primers ‘DEST_FRT’ and ‘Insert_2’ (Appendix Table 7.22). Primer sequences for ‘R39Q’ and ‘V367M’ are listed in Appendix Table 7.23. Black bar indicates amino acid codon. All DNA sequences are 5’ to 3’ orientation. Chromatograms from Chromas LITE v2.1.1 (Technelysium 2012); Green, Adenine; Blue, Cytosine; Black, Guanine; Red, Thymine.

4.3.5 Confirming mRNA Expression of GNL3 in Transfected A375 Cells

Initially, cDNA end-point PCR (Section 4.2.3.7.3) was used to examine the expression of GNL3 in A375 cells transfected with the different variants. This confirmed expression of the GNL3 transcript in all A375 cells. Cells not transfected with the GNL3 insert also showed endogenous expression of GNL3 (Figure 4.18).
Investigating the Role of GNL3 in Osteoarthritis

Figure 4.18: mRNA expression of GNL3 confirmed in A375 cells. PCR products for GNL3 and β-Actin run on a 2% agarose gel at 75V for 30min in TBE buffer (1X). Results shown here are from the first transfection; FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247). L, 100bp DNA ladder (New England BioLabs®); B, No template PCR control.

4.3.6 Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to Cisplatin Treatment

Western blots (Section 4.2.3.8.3) confirmed the expression of GNL3, p53, MDM2 and β-Actin proteins in wildtype A375 cells and those transfected with the different variants of GNL3 (Figure 4.19).
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

CHAPTER 4: FUNCTIONAL ANALYSIS OF GNL3
Cells treated with 5µM cisplatin (Sigma®) appear to show a decrease in GNL3 whereas MDM2 and p53 expression increased (Figure 4.19 and Figure 4.20).

Independent analysis (Section 4.2.3.11) of FRT cells showed no significant difference in the expression of any protein between control and cisplatin-treated cells in either transfection, GNL3 (1st, p=0.072; 2nd, p=0.722), MDM2 (1st, p=0.729; 2nd, p=0.753) and p53 (1st, p=0.312 2nd, p=0.560).

Excluding FRT from the calculations (Section 4.2.3.11), the expression of GNL3 (1st, p=0.087; 2nd, p=0.775) and MDM2 (1st, p=0.077; 2nd, p=0.301) were not altered significantly when variants were treated with cisplatin in either transfection. Nor were there any differences in the expression of GNL3 (1st, p=0.111; 2nd, p=0.719) or MDM2 (1st, p=0.791; 2nd, p=0.934) between the variants overall. The difference in p53 between control (1st, 0.52 ± 0.09 SEM; 2nd, 0.20 ± 0.06 SEM) and cisplatin-treated (1st, 1.31 ± 0.19 SEM; 2nd, 0.79 ± 0.21 SEM) variants was significantly different in both transfections (1st, p=0.001; 2nd, p=0.048). However, there were no significant differences in p53 expression between variants overall (1st, p=0.109; 2nd, p=0.786) and, any differences between different variants under the same condition or the same variant under alternate conditions were negligible.
Figure 4.20: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in control and cisplatin-treated (5µM) A375 cells (A) GNL3 expression in first transfection cells; (B) MDM2 expression in first transfection cells; (C) p53 expression in first transfection cells; (D) GNL3 expression in second transfection cells; (E) MDM2 expression in second transfection cells; (F) p53 expression in second transfection cells. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in duplicate for each transfection (n=2). Dark grey, Protein/β-Actin expressed in control cells; Light grey, Protein/β-Actin expressed in cisplatin-treated cells. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).
4.3.7 Influence of Hypoxia on A375 Cell Survival

On average, approximately 88% (± 0.02 SEM) of A375 cells survived when placed under hypoxic conditions for 48hrs (Section 4.2.3.10.1). FRT cells, which contained no GNL3 insert had 85.69% (± 0.03 SEM) cell survival, while those carrying various GNL3 inserts showed cell survival rates of; RV, 82.99% (± 0.05 SEM); QV, 91.70% (± 0.04 SEM); QM, 86.9% (± 0.01 SEM) and RM, 90.66% (± 0.01 SEM). There were no significant differences in cell viability between the samples (Figure 4.21).

![Figure 4.21: A375 cell survival after 48hrs under hypoxic stress](image)

Values are average fluorescence readouts for alamarBlue® redox reaction. All samples were run in triplicate. All values corrected for background. Error bars are ± SEM (n=3 (except QV, n=2)). Dark grey, Control samples under 48hrs normoxic conditions; Light grey, Cells under 48hrs hypoxic conditions. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).

4.3.8 Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to Hypoxia

Western blots (Section 4.2.3.8.3) confirmed the expression of GNL3, p53, MDM2 and β-Actin proteins in wildtype A375 cells and those transfected with the different variants of GNL3 (Figure 4.22 and Figure 4.24).
4.3.8.1 Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to 24hrs Hypoxia

FRT cells containing no GNL3 insert as well as those containing the rs2289247 variant encoding for valine showed increased expression of GNL3 and decreased expression of MDM2 after 24hrs under hypoxic conditions. Conversely the expression of GNL3 appeared unchanged (or only slightly increased) and the expression of MDM2 increased in cells containing the GNL3 insert encoding methionine at rs2289247. All cells had increased levels of p53 expression when placed under hypoxic condition for 24hrs. Only cells from the first transfection were analysed in the experiment (Figure 4.22 and Figure 4.23).

Independent analysis (Section 4.2.3.11) of FRT cells demonstrated no significant differences in the expression of any protein between cells placed under normoxic and hypoxic conditions for 24hrs, GNL3 (p=0.791), MDM2 (p=0.468) and p53 (p=0.190).

Comparing only the cells containing the GNL3 insert (Section 4.2.3.11) showed no significant difference in the GNL3 (p=0.383) or MDM2 (p=0.807) response to hypoxic conditions. Nor were there any significant differences in the expression of either protein between variants, GNL3 (p=0.911) and MDM2 (p=0.422). The expression of p53 increased significantly (p=0.002) in response to decreased oxygen levels (normoxic, 1.97 ± 0.21 SEM; hypoxic, 4.86 ± 0.75 SEM) however there were no significant differences between the variants overall (p=0.601) nor were there any differences between different variants under the same conditions or the same variant under different conditions.
Investigating the Role of GNL3 in Osteoarthritis

Chapter 4: Functional Analysis of GNL3
Figure 4.22: Western blot for GNL3, MDM2, p53 and β-Actin expression in A375 cells placed under normoxic (N) or hypoxic (H) conditions for 24hrs. Both bands were taken into account when quantifying MDM2. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247). L, Magic Mark™ XP (Life Technologies™).
Figure 4.23: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in A375 cells placed under normoxic or hypoxic conditions for 24hrs. (A) GNL3 expression in first transfection cells; (B) MDM2 expression in first transfection cells; (C) p53 expression in first transfection cells. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in triplicate. Error bars are ± SEM (n=3). Dark grey, Protein/β-Actin expressed in cells under normoxic conditions for 24hrs; Light grey, Protein/β-Actin expressed in cells under hypoxic conditions for 24hrs. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247).
4.3.8.2 **Influence of GNL3 Variants on Interactions with the p53 Pathway in Response to 48hrs Hypoxia and Oxidative Stress**

Overall cells appeared to show a decreased expression of GNL3 in response to hypoxia whereas MDM2 and p53 expression increased. This response was observed when cells were placed under hypoxic condition for 48hrs and when returned to normoxic conditions for 24hrs after being incubated under reduced oxygen levels for 24hrs prior. In general, the cell response was greater the longer the period of hypoxia and any results which deviated from this were only observed in a single transfection (Figure 4.24 and Figure 4.25).

Independent analysis (Section 4.2.3.11) of FRT cells showed there were no significant differences in the expression of any protein in response to altered oxygen levels in either transfection, GNL3 (1st, p=0.728; 2nd, p=0.547), MDM2 (1st, p=0.348; 2nd, p=0.905) and p53 (1st, p=0.143 2nd, p=0.310).

Comparing the variants separately from the FRT cells (Section 4.2.3.11) revealed no significant difference in the expression of either protein between the variants, GNL3 (1st, p=0.958; 2nd, p=0.711), MDM2 (1st, p=0.819; 2nd, p=0.730) and p53 (1st, p=0.829 2nd, p=0.603). There were no significant differences in the GNL3 (1st, p=0.129; 2nd, p=0.120) or MDM2 (1st, p=0.192; 2nd, p=0.387) response to the altered oxygen levels. However there was a slight trend for the expression of GNL3 to decrease and MDM2 to increase overall, the longer the cells were exposed to hypoxia. There was a significant difference (1st, p=0.001; 2nd, p=0.009) in the p53 response between cells placed under 48hrs normoxia (1st, 0.08 ± 0.02 SEM; 2nd, 0.11 ± 0.03 SEM) compared to those surrounded by a hypoxic environment for 48hrs (1st, 0.49 ± 0.10 SEM; 2nd, 0.71 ± 0.22 SEM). Similarly, cells which were returned to normal oxygen levels after 24hrs hypoxic incubation (1st, 0.13 ± 0.03; 2nd, 0.17 ± 0.05 SEM), also differed significantly (1st, p=0.002; 2nd, p=0.020) in the amount of p53 expressed when compared to those under hypoxic conditions for 48hrs. The level of p53 expression was not significantly different between cells which were placed under normoxic condition for 48hrs and those which although initially placed under hypoxic conditions, were returned to the normoxic incubator after 24hrs (1st, p=0.854; 2nd, p=0.943). There were no significant observations in the expression of
either protein between variants under the same conditions, nor when the same variant was compared under different conditions.
CHAPTER 4: FUNCTIONAL ANALYSIS OF GNL3
Figure 4.24: Western blot for GNL3, MDM2, p53 and β-Actin expression in A375 cells placed under normoxic (N) or hypoxic (H) conditions for 48hrs or ‘re-oxidated’ (R) from 24hrs hypoxic to 24hrs normoxic. Both bands were taken into account when quantifying MDM2. FRT, A375 cells possessing only a FRT site and not transfected with GNL3; RV, GNL3 insert encoding R (rs11177) and V (rs2289247); QV, GNL3 insert encoding Q (rs11177) and V (rs2289247); QM, GNL3 insert encoding Q (rs11177) and M (rs2289247); RM, GNL3 insert encoding R (rs11177) and M (rs2289247). L, Magic Mark™ XP (Life Technologies™).
Investigating the Role of GNL3 in Osteoarthritis

Chapter 4: Functional Analysis of GNL3

Figure 4.25: Western blot densitometry for protein expression of GNL3, MDM2 and p53 in A375 cells placed under normoxic or hypoxic conditions for 48hrs or ‘re-oxidated’ from 24hrs hypoxic to 24hrs normoxic (A) GNL3 expression in first transfection cells; (B) MDM2 expression in first transfection cells; (C) p53 expression in first transfection cells; (D) GNL3 expression in second transfection cells; (E) MDM2 expression in second transfection cells; (F) p53 expression in second transfection cells. Protein/β-Actin ratios are the average protein expression values (corrected for β-Actin). All experiments were run in triplicate for each transfection. Error bars are ± SEM (n=3). Dark grey, Protein/β-Actin expressed in cells under normoxic conditions for 48hrs; Light grey, Protein/β-Actin expressed in cells under ‘re-oxidative’ conditions; Medium grey, Protein/β-Actin expressed in cells under hypoxic conditions. FRT, A375 cells possessing only a FRT site and not
transfected with *GNL3*; RV, *GNL3* insert encoding R (rs11177) and V (rs2289247); QV, *GNL3* insert encoding Q (rs11177) and V (rs2289247); QM, *GNL3* insert encoding Q (rs11177) and M (rs2289247); RM, *GNL3* insert encoding R (rs11177) and M (rs2289247).
4.4 Discussion

The aims of this chapter were to 1) Examine the effect of GNL3 knockdown on cartilage homeostasis, 2) Investigate the influence of the GNL3 variants at rs11177 on the chondrogenic potential of HACs and MSCs and 3) Determine if the different GNL3 variants (rs11177 and rs2289247), transfected into A375 cells, affected the p53/MDM2 response of these cells to hypoxia and cisplatin.

The expression of GNL3 was successfully knocked down in JJ012 chondrosarcoma cells and HACs. The results were confirmed and quantified using cDNA end-point PCR (Figure 4.11) and qPCR (Figure 4.12) techniques. HAC transfections were not repeated and hence it was not possible to perform statistical analysis on these results. Instead, all calculations were performed using data from the JJ012 transfections which were repeated in triplicate. This eliminated any chance of random factors such as patient age, gender or disease status interfering with the outcome. The HAC experiment serves as an example of the gene response in one patient and should not be considered a representation of all HACs.

In JJ012 cells, GNL3 expression was quantified, using qPCR, in all controls and not found to be statistically different. siRNA-A is theoretically the most accurate control due to the fact that these cells have been treated in precisely the same manner as the GNL3 knockdown cells regarding the addition of media, transfection reagent and siRNA with the only exception being the non-mammalian siRNA-A target. For this reason, the latter is discussed in further detail below.

The cDNA end-point PCR experiments showed (Figure 4.14) GNL3 and the chondrogenic markers were expressed in nucleostemin knockdown and siRNA-A control JJ012 cells. Using qPCR (Figure 4.12), GNL3 expression was calculated to have dropped by 81.16% (± 39.23 SEM) in knockdown JJ012 cells, relative to the siRNA-A control (normalised to the untreated control). However, this difference was not found to be statistically significant. The experiments did not reveal any significant effect of the knockdown of GNL3 on expression of other chondrogenic markers however an effect cannot be excluded, given the limitations of the experiment.
Shortcomings of this experiment are the small sample size and the fact that chondrosarcoma cells were used. While this reduced the number of errors which might occur when using HACs extracted from patients. The fact that these cells are cancerous and should therefore have increased expression of GNL3 could mean that the response seen here is restricted to these cells (and perhaps others with a high level of proliferative ability) and therefore one need be bear this in mind when extrapolating these findings to HACs. Furthermore, the number of cycles carried out during PCR amplification could mean that the results shown here are representative of the plateau phase of an end-point PCR and therefore not reflect the differences in chondrogenic marker expression between samples.

Unfortunately attempts to transfect the cultured HACs (Figure 4.13), was only successful on one occasion. Using qPCR, GNL3 expression was calculated to have dropped by 62.20% in knockdown HACs, relative to the siRNA-A control (normalised to the untreated control). cDNA end-point PCR experiments showed the expression of all chondrogenic markers (and GNL3) in the knockdown cells. These results need be treated with caution and validated in additional samples.

Future work should perhaps utilise qPCR to investigate the expression of chondrogenic markers. OA chondrocytes are reported to show an initial increase in production of anabolic factors at the onset of OA (Section 1.2.4) and therefore investigating the cellular response at a protein level would provide valuable insight into the effect GNL3 knockdown on the chondrogenic process. Therefore, western blotting and perhaps co-localisation or immunoprecipitation experiments should also be carried out to determine whether the results seen at the mRNA level differ in any way from the protein expression and perhaps provide insight into how affected genes might interact. This work should also examine chondrogenic marker expression in more HAC cultures. Overexpression of GNL3 which has been shown to have an adverse effect on the cell (Section 1.4.1.4) (Tsai & McKay 2002), should also be explored to determine whether chondrogenic markers are affected.

Aims to investigate whether the GNL3 variants at rs11177 influenced the chondrogenic potential of HACs and MSCs grown in chondrogenic media over a 21
day period were successfully examined at the mRNA level. HAC and MSC samples showed expression of all chondrogenic markers (except \textit{COL2A} in MSCs) across all variants (Section 4.3.3).

The images could not be quantified due to the fact that the results shown here may not be representative of the exponential amplification phase of PCR. Hence, these results can only determine whether mRNA was present or absent and cannot be used to quantify the initial amount of mRNA present.

It should be noted that any alterations in chondrogenic marker expression could be a consequence of the culture methods used here which maintain the chondrocytes in a 2D environment, unlike the physiological 3D ECM from which these cells were extracted. It is difficult to assess how these alterations in the expression of chondrogenic markers might affect the ECM in a 2D system. While the cells were also cultured as 3D pellets (in StemPro® chondrogenic media (Appendix Solution 7.2.10) at the bottom of a 15ml tube) to conquer this obstacle, unforeseen difficulties in cell culturing and RNA extraction have prevented these results from being included here.

It would also be of interest to compare whether any alterations in one chondrogenic marker such as MMP levels, could influence the expression of others, for example, \textit{ACAN} and \textit{COL2A} (Section 4.1). Given that \textit{COL2A} is an essential structural component of cartilage and the fact that expression in MSCs was poor (despite constant expression of \textit{SOX9}, \textit{RUNX2} and \textit{GNL3}), is reason to suggest that chondrogenesis was not successful in these experiments.

Further studies, with larger numbers should therefore be investigated using a reduced number of PCR cycles or qPCR before any definite conclusions can be made. Western blots might also be carried out in order to investigate the expression of chondrogenic markers at a protein level. Fluorescent co-localisation or immunoprecipitation experiments would provide insight as to how nucleostemin might interact with other proteins in the cell to influence their locality and expression. cDNA end-point PCR was used as a screening tool to determine which, if
any, genes should be investigated further by qPCR. The results overall appear largely varied between samples of the same genotype at the same time. Furthermore, the effect of the variants at rs11177 may not be strong enough to be seen using these methods.

The fact that the cells were passaged several time in order to achieve sufficient cell numbers for culture could also have influenced the expression of chondrogenic markers, for example MSCs may exhibit a loss in proliferative ability or pluripotent potential (Sawada et al. 2007). Although the constant expression of differentiation, SOX9 and RUNX2, and proliferation, GNL3, factors suggests this was not the case. A lack of ECM, in 2D culture, could also have influenced the results.

There does not appear to be a trend in the expression of these chondrogenic markers between different genotypes at the same time point. So, in summary, the transcription of the genes examined here and GNL3 itself, in MSCs and HACs, do not appear to be dependent on the rs11177 genotype when examined using the cDNA end-point PCR methods described above.

Aims to investigate the effect of the GNL3 variants at rs11177 and rs2289247 on the p53 stress response required A375 cells to be transfected with the expression clone containing every possible combination of the variants at the aforementioned sites. This was successfully achieved twice.

The mRNA expression of GNL3 was confirmed in all cells (including those lacking the GNL3 insert) by means of cDNA end-point PCR (Figure 4.18).

Further investigations concerning the influence of the variants on the p53 stress response, were investigated solely at a protein level (Section 4.3.6 and Section 4.3.8). Nucleostemin protein expression was confirmed in all cells using a nucleostemin antibody prior to conducting any further investigation. Cells were then subjected to stimuli intended to induce a p53 stress response. The first being treatment with cisplatin, a DNA cross-linker known to result in increased expression of p53 in cancer cells (Section 4.1) (as reviewed in (Boulikas & Vougiouka 2003; Barabas et al. 2008)). The second being oxidative stress, as discussed later in this
In Investigating the Role of GNL3 in Osteoarthritis

In Section 1.4.1.4 and Section 4.1 (Tsai & McKay 2002; Dai et al. 2008; Meng et al. 2008), GNL3 is reported to interact with p53 and MDM2. Therefore, the aim here was to determine whether the GNL3 variants were able to influence the stress response in the transfected melanoma cells.

The cisplatin results (Section 4.3.6) were first analysed in FRT cells alone to determine whether there were any differences in the protein expression of GNL3, MDM2 or p53 in response to the stimulus. While the expression of GNL3 decreased and the other proteins increased, the FRT response to cisplatin was not statistically significant in either transfection. Furthermore, cells with the GNL3 insert were not significantly different from FRT cells in the GNL3, MDM2 or p53 response to cisplatin treatment in either transfection.

Analysis of the variants alone did not show a significant difference in the amount of GNL3 or MDM2 expression in response to being treated with cisplatin. The p53 response was significant (1st, p=0.001; 2nd, p=0.048) overall however there was no difference between any variants under the same conditions. The expression of GNL3 decreased after cisplatin treatment whereas both p53 and MDM2 increased. The reduced level of nucleostemin could therefore have triggered nucleolar stress, releasing unassembled riboproteins, L5, L11 and 5S into the nucleoplasm. This could subsequently inhibit MDM2-mediated ubiquitination and proteasomal degradation of p53, resulting in increased p53 activity (Dai et al. 2008; Donati et al. 2013). This increase in p53 expression could increase the level of MDM2 via the auto-regulatory feedback loop (Barak et al. 1993; Wu et al. 1993) and this in turn could have led to an increase in the MDM2-mediated proteasomal degradation of GNL3 itself (Huang et al. 2009) (Section 1.4.1.4 and Section 4.1). It should also be noted that although the experiment here investigated the influence of GNL3 variants on the p53 stress response, alternative p53-independent regulatory pathways may have been influenced. Therefore, it may be worth considering how the decrease in GNL3 expression could alter the ability of these cisplatin-treated cells to repair DNA damage via the recruitment of RAD51 (Section 1.4.1.5) (Hsu et al. 2012; Meng et al. 2013).
As discussed (Section 4.1), chondrocytes in OA cartilage are subject to oxidative stress when cartilage is degraded (as reviewed in (Pfander & Gelse 2007)). Hence a second, more physiologically relevant, stimulus was employed in an attempt to induce a p53 response in the A375 cells. Cells were placed in a normoxic or hypoxic incubator for 24hrs or 48hrs and cells initially placed under hypoxic conditions for 24hrs were then also returned to normal oxygen conditions for a further 24hrs. Protein was extracted after 24hrs and 48hrs.

Prior to carrying out stress response experiments, cell survival (Section 4.3.7) was measured after 48hrs hypoxia. Results showed on average ~88% (± 0.02 SEM) cell survival. This suggests an apoptotic response to hypoxia in these cells which might be confirmed in future experiments with annexin V staining. Apoptosis is most likely due to the increase in p53 expression as demonstrated in the stress response experiments (see below).

It should be noted that only cells from the first transfection (Section 4.3.8.1) were successful in the 24hrs experiment. Protein was successfully extracted from cells in repeat experiments however, unforeseen errors at the western blot stage resulted in only the first experiment being completed.

The hypoxia results were first analysed in FRT cells alone to determine whether there were any differences in the expression of GNL3, MDM2 or p53 in response to the stimulus. After 24hrs hypoxia, the expression GNL3 and p53 increased and MDM2 decreased. After 48hrs hypoxia (Section 4.3.8.2) the expression of GNL3 decreased and the other proteins increased in both transfections. In response to ‘re-oxidation’, cells from the first transfection showed an increase in expression of p53 and MDM2 (relative to 48hrs normoxia). Although this was only marginal in p53 and in MDM2 this was not as high as the response to 48hrs hypoxia. Cells from the second transfection showed a slight decrease in the expression of p53 and MDM2 (relative to 48hrs normoxia). Both transfections showed a decrease in GNL3 in response to ‘re-oxidation’ (relative to 48hrs normoxia). None of these changes were statistically significant in either transfection. Furthermore, cells with the GNL3 insert
were not significantly different from FRT cells in the GNL3, MDM2 or p53 response at either time point or in either transfection.

Comparing the variants independently from the FRT cells proved the expression of GNL3 and MDM2 overall to be insignificant in response to alterations in oxygen levels in both transfections. On the contrary, p53 expression increased significantly in response to reduced oxygen levels in both transfections. Even though this was observed between 24hrs normoxic and 24hrs hypoxic (p=0.002), 48hrs normoxic and 48hrs hypoxic (1st, p=0.001; 2nd, p=0.009) and when ‘re-oxidated cells were compared to the latter (1st, p=0.002; 2nd, p=0.020), there were no significant differences between variants under the same conditions nor the same variants under different conditions at the same time point.

The results showed varied expression of GNL3 and MDM2 at 24hrs in cells containing the GNL3 insert. Although there was an overall increase in the expression of GNL3 and a slight decrease in MDM2, these alterations were not significant. There did appear to be a trend in the expression of cells which contained the rs2289247 variant encoding methionine. The expression of GNL3 appeared unchanged in these cells compared to the cells containing a wildtype valine residue which showed an increase in GNL3. Furthermore, cells containing the methionine variant showed an increase in the expression of MDM2 whereas valine cells exhibited a decrease in MDM2. These changes were not significant between variants but do suggest that perhaps the variants at rs2289247 could affect the shuttling and nuclear location of GNL3 and thereby influence its interaction with MDM2. For example, the mutant methionine residue may inhibit the nucleolar translocation of GNL3 in response to stress due to the fact that GTP-binding is unable to release a switch in the intermediate residue of GNL3 which anchors the protein in the nucleoplasm (Section 1.4.1.2) (Tsai & McKay 2005). This could trigger an increase in the ubiquitination and degradation of GNL3 by MDM2 in the nucleoplasm (Huang et al. 2009) resulting in an apparent lack of response by GNL3 to the stimulus in methionine cells. Or, an increase in the nucleoplasmic accumulation of GNL3 could bind MDM2 and inhibit the degradation of p53 (Section 1.4.1.4) (Dai et al. 2008). An increase in p53 could increase the expression of MDM2 via the auto-regulatory
feedback loop (Section 4.1) (Barak et al. 1993; Wu et al. 1993). MDM2, in turn, could then degrade GNL3 in the nucleoplasm. These suggestions are of course highly speculative and the fact that the observed differences in protein expression between the variants are not significant and data presented with large error bars, are reason to suggest that these may be due to experimental error and need be repeated.

At 24hrs the results did show a significant increase in the expression of p53 across all samples (containing the GNL3 insert) when placed under reduced oxygen conditions. However, there did not appear to be any differences in this expression, significant or otherwise, between the variants, suggesting that differences between variants in the expression of GNL3 and MDM2 do not influence the expression of p53.

At 48hrs, hypoxic cells containing the GNL3 insert appeared to show a decrease in the expression of GNL3 and an increase in the expression of MDM2 and p53 when compared to normoxic cells which had not encountered hypoxic conditions at any point. Cells which had been exposed to both hypoxic and normoxic conditions at 24hrs intervals showed a similar trend, of reduced magnitude, in MDM2 and GNL3 expression. Whereas p53 expression, was similar to that seen at 48hrs normoxia. Although some results did stray, this seemed to occur at random, was not repeated in cells from the alternate transfection and did not appear to alter the response of the other proteins. Hence, this can most likely be attributed to experimental error (as discussed below).

It is interesting to note however that although not significant in either case, the expression of GNL3 did increase overall after 24hrs hypoxia whereas it decreased after 48hrs. Exposure to normal oxygen levels after 24hrs hypoxia also appeared to be able to rescue cells from a decrease in GNL3 expression, as was observed at 48hrs hypoxia. Unfortunately, no comparison can be made between the 24hrs normoxia or hypoxia and the ‘re-oxidated’ cells as these were not run on the same blot (hence, this is suggested for future experiments). It does however suggest that the GNL3 response may initially increase in an attempt to rescue damaged cells however after prolonged exposure to hypoxia, the expression of GNL3 decreases. Perhaps, as Huang et al. (2011) observed in U2OS cells, the initial response to ROS (H\textsubscript{2}O\textsubscript{2})
results in nucleostemin being protected from degradation but after prolonged exposure this could result in an increase in insoluble nucleolar aggregates and ultimately trigger an apoptotic response via an increase in p53. It is possible that the altered GNL3 protein structure may have influenced the antibody binding affinity and/or efficiency of protein extraction or membrane transfer which could have reduced the level of nucleostemin detected in these cells, although there were no unexpected bands in the western blot. However, it is unlikely that the detergent used to lyse these cells would solubilise these nucleolar aggregates. Future methods should include a sonication step (Huang et al. 2011). Alternatively, the decrease in GNL3 might result in nucleolar disruption and release unassembled riboproteins such as L5, L11 and 5S which could target MDM2 and prevent it from degrading p53 (Section 1.4.1.4) (Dai et al. 2008; Donati et al. 2013). An increase in the expression of p53 could then activate the transcription of MDM2 via the auto-regulatory feedback loop (Section 4.1) (Barak et al. 1993; Wu et al. 1993). The altered levels of GNL3 expression could of course act independently of MDM2 and p53. Furthermore, the fact that wildtype rs2289247 cells appear to be responsible for this increase in GNL3 expression at 24hrs and the methionine cells, exhibit a delayed response could suggest that the delayed response to hypoxia by mutant cells could influence the onset of OA. This is once again highly speculative and the fact that ultimately there appears to be no difference between the variants after prolonged exposure to hypoxia or in the cells ability to rescue protein expression levels when oxygen levels increased, suggest that this could have little influence on the progression of OA and perhaps the observed trends between the variants at 24hrs are merely due to experimental error.

The results clearly show that although unaffected by GNL3 genotype, the expression of p53 is increased significantly when exposed to reduced oxygen levels for 48hrs. Furthermore, the increase in p53 after exposure to hypoxia can be rescued by increasing oxygen supply. The expression of GNL3 and MDM2 was not significantly altered in response to 48hrs hypoxia. While the purpose of this study was to determine the influence which GNL3 variants might have on the stress response to hypoxia, it may be worth speculating on the pathways which may have contributed to
the alterations in protein expression which were observed in the results. It is well established that HIF1α is increased in response to hypoxia (as reviewed in (Hammond & Giaccia 2005)) (Section 4.1). This in turn can induce apoptosis via p53 up-regulation ((Chiarugi et al. 1999) and as reviewed in (Hammond & Giaccia 2005)). Consequently, an increase in the transcription of MDM2 (Barak et al. 1993; Wu et al. 1993) and subsequent increase in GNL3 degradation (Huang et al. 2009) could trigger nucleolar stress and sequester the inhibitory action of MDM2 on p53 activity (Dai et al. 2008; Donati et al. 2013). HIF1α may also up-regulate the expression of iNOS (Chiarugi et al. 1999) which in the presence of GTP, can increase the level of NO, a ROS ((Senda et al. 1995) and as reviewed in (Nordberg & Arner 2001)). Initially exposure to ROS can trigger an increase in proliferation (Huang et al. 2011), which may explain the increase in GNL3 expression observed at 24hrs. This in turn could sequester MDM2-mediated p53 degradation (Huang et al. 2009). Prolonged exposure to ROS however can result in the formation of insoluble nucleolar GNL3 aggregates and prompt an apoptotic response (Huang et al. 2011).

The increase in p53 can also up-regulate the expression of miR-34 (Section 3.4) which consequently inhibits guanine synthesis by IMPDH (Kim et al. 2012) and results in a reduction of GTP. Huang et al. (2009) reported that in the absence of GTP, the MDM2-mediated degradation of GNL3 is more pronounced and could ultimately elicit a nucleolar stress response, sequester MDM2 and activate p53 (Dai et al. 2008; Huang et al. 2008; Donati et al. 2013).

It is also interesting that in fact the expression of GNL3 increased in response to hypoxia in human tendon stem cells (Zhang & Wang 2013) and suggests that perhaps these observations may be tumour cell line specific and respond differently in cells which might otherwise be better adapted to a hypoxic environment. Although the expression of GNL3 did decrease significantly in OA HACs (Figure 3.8), this was an *in vitro* study carried out in a normoxic environment and could involve numerous other pathological factors. Therefore it cannot be compared here however, the effect of oxidative stress on GNL3 expression in HACs does warrant further investigation.

A shortcoming of these experiments is clearly the fact that the cells used here are melanoma cells and not chondrocytes. The reason the A375 cell line was chosen was
not only because it was readily available from our collaborators with the FRT site but also being a cancer cell line, the p53 response is well characterised and known to respond to cisplatin. I do however acknowledge that the results shown here may be tumour specific. Although a more relevant physiological stimulus for OA was investigated by placing the cells under oxidative stress, the results showed no significant differences between variants in the response, regardless of the stimulus used. Had the results been significant between GNL3 variants, the stress response would have been investigated further in a more relevant chondrocyte cell line.

Although it may be argued that experimental errors could have reduced the significance of the findings here. While no blots were over-exposed and every effort was made to reduce non-specific background, there were differences in antibody binding affinity between the blots. However, all values were corrected for β-Actin and these values were then assessed for outliers prior to calculating average values. The MDM2 antibody used here is particularly difficult to achieve reproducible results. However, the p53 antibody did bind efficiently and showed less inter-blots errors, which does contribute to the trustworthiness of the results shown here. It is also acknowledged that the GNL3 antibody would detect both endogenous GNL3 and that which had been transfected into the cells. One would therefore expect the transfected cells to have higher expression of the GNL3 protein however this difference was not found to be significantly different between the parental cells and those which contained the GNL3 insert. Future work should consider using the V5 antibody for the detection of GNL3 protein abundance in these cells.

In addition, these experiments were carried out at a protein level and perhaps this is not sensitive enough to detect any differences which might be present between the variants investigated here or perhaps additional variants are required in order to achieve a significant result. Moreover, the small sample number used here may not be powerful enough and could therefore also have contributed to the outcome. Had the results been significant these would also have been investigated at the mRNA level which, as observed in comparison of GNL3 expression between normal and OA HACs (Figure 3.4 and Figure 3.8) and a study by Lo et al. (2012), may elicit an alternate response to that seen at the level of protein expression.
So in conclusion, exposure to stressful stimuli appears to alter the expression of all proteins investigated here however, only p53 was significantly different in stressed cells. There were no significant differences between the different variants under the same condition or between alternate conditions for the same variant. The results of this experiment demonstrate that, regardless of whether the minor alleles at rs11177 and rs2289247 occur together or independently of one another, there is no difference in the p53 stress response at a protein level. Hence, the overall significance in the altered p53 expression does not appear to be influenced by GNL3 genotype in these cells.

Future experiments might include the use of co-localisation experiments to determine whether these alterations in expression are due alterations in the location of GNL3 and its ability to interact with MDM2 and p53. Protein extracted should be subject to sonication and run on western blots under non-reducing conditions in order to ensure that all forms of GNL3 are quantified. The affect of other stimuli such as mechanical stress or mycophenolic acid (i.e. a reduction in GTP levels) might also be assessed. The influence which other polymorphisms, such as the novel variant (Table 2.2) identified in the mutation screen which lies upstream of GNL3 in the AHR/ARNT/ARNT2 transcription factor binding site for HIF1-α, have on the expression of GNL3 and other proteins in response to hypoxia could also be investigated. The affect which GNL3 variants have on the cells ability to proliferate might be investigated using a MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assay.

In summary of this chapter, the initial aim of this project to examine the effect of GNL3 knockdown on cartilage homeostasis in J012 cells and HACs did not eliminate the expression of chondrogenic markers. The aims of this project to investigate the role of the alleles at rs11177 in chondrogenesis showed that, despite the fact that rs11177 was identified as the tagging SNP in the arcOGEN GWAS, it does not appear to influence the chondrogenic potential of HACs or MSCs in these experiments. Further investigation is needed in order to quantify the difference at both the RNA and protein level. The variants at both rs11177 and rs2289247 did not have an effect on the protein expression of p53 or MDM2 in A375 melanoma cells in
response to cisplatin treatment or oxidative stress. As discussed, the effect of the variants may not be strong enough to be seen using the methods described here and may require additional variants to be investigated in conjunction with rs11177 and rs2289247 if a significant effect is to be seen.
Chapter 5: Role of gnl3 in Zebrafish Cartilage Development

5.1 Introduction

The skeletal structure of zebrafish (*Danio rerio*) starts to develop at approximately 2-3 days post fertilisation (dpf) (Schilling et al. 1996). The trunk is divided into motor neurons and somites (Piotrowski & Nusslein-Volhard 2000). The chondrocranium (*Figure 5.1*) is partitioned along the anterior-posterior axis and consists of a dorsal neurocranium and seven pharyngeal arches. The anterior neurocranium consists of the ethmoid plate, trabeculae crania, anterior basicranial commissure and orbital cartilage. The posterior neurocranium consists of the posterior basicranial commissure, basal plate, parachordal cartilage, otic capsule and occipital arch. The first mandibular pharyngeal arch comprises of anterior Meckel’s cartilages and posterior palatoquadrates which form the jaw. The second hyoid pharyngeal arch includes the hyosymplectics, basihyal, ceratohyals and interhyals which support the jaw. The remaining five posterior pharyngeal arches are the branchial arches which form the gills. Branchial arches have ceratobranchials that extend dorsoventrally and are connected along a midline via the basibranchials. Ceratobranchials of pharyngeal arches three to six are connected to the basibranchials via hypobranchials. The most posterior arch has three teeth on either side (Schilling et al. 1996; Schilling & Kimmel 1997).
Figure 5.1: Wildtype zebrafish cartilaginous pharyngeal skeleton (A-C) Whole mount alcian blue staining and (D-F) schematic representations of pharyngeal skeleton. (A, D) Lateral view, (B, E) Ventral view, (C, F) Dorsal view. (D, E) Pharyngeal arches: 1, blue; 2, yellow; 3, pink; 4, orange; 5, green; 6, purple; 7, black. (F) Neurocranium, grey. abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; c, cleithrum; cb, ceratobranchial; ch, ceratohyal; e, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; m, Meckel’s cartilage; n, notochord; ot, otic capsule; pc, parachordal; pq, palatoquadrate; t, trabeculae cranii. (Adapted from (Schilling et al. 1996)).

Embryonic development of the muscles and cartilaginous pharyngeal skeleton is synchronised and plays an important role in feeding and respiratory processes ((Schilling & Kimmel 1997) and as reviewed in (Graham 2003)). Each pharyngeal segment consists of a central mesoderm surrounded by neural crest cells (NCCs) and has an exterior epidermal ectoderm covering and interior epithelial endoderm lining (as reviewed in (Graham 2001; Graham 2003)). Pharyngeal muscles and endothelia are derived from the mesoderm ((Noden 1983; Schilling and Kimmel 1994; Piotrowski & Nusslein-Volhard 2000) and as reviewed in (Graham 2001; Graham 2003)). The endoderm gives rise to pharyngeal pouches which are necessary in order to segment the pharyngeal arches ((Piotrowski & Nusslein-Volhard 2000) and as reviewed in (Graham 2001; Graham 2003)). Segregation has also been shown to be partly controlled by localised NCC apoptosis in chick embryos (as reviewed in (Graham 2003)). The NCC migrate from the mid- and hindbrain and colonise arches to form connective tissue, cartilage, pigmentation and neurons by interacting with the
endodermal tissue and responding to signalling molecules ((Noden 1983; Schilling & Kimmel 1994; Veitch et al. 1999; Piotrowski & Nusslein-Volhard 2000) and as reviewed in (Graham 2001; Graham 2003)). The role of the mesoderm and ectoderm in signalling NCC fate is unclear (as reviewed in (Graham 2001)).

The NCCs produce clones that are restricted to a single segment (Schilling & Kimmel 1994). These cells condense and progressively subdivide to form dorsal and ventral structures which are later chondrofied (Schilling & Kimmel 1997). Cells initially undergo a period of morphogenesis and differentiation during which the chondrocytes deposit an ECM and are rearranged and assembled into stacks, which are vital in determining cartilage shape. This is then followed by a period of isometric growth (Kimmel et al. 1998).

The bone of teleosts, such as zebrafish, lacks haemopoietic bone marrow (as reviewed in (Spoorendonk et al. 2010)) (which is instead filled with adipose tissue) and teleost chondrocytes rarely undergo endochondral ossification. Instead osteoblast-like cells from the perichondrium are positioned at the cartilage barrier and secrete bone matrix or a mixture of both bone and cartilage matrices (as reviewed in (Witten and Huysseune 2009; Apschner et al. 2011)).

Regulators of skeletal development, such as sox9 (Yan et al. 2002; Yan et al. 2005) and runx2 (Flores et al. 2004; Li et al. 2009) are highly conserved in both humans and teleost fish ((Mitchell et al. 2013) and as reviewed in (Spoorendonk et al. 2010)). In addition, indian hedgehog (ihh) (Hammond & Schulte-Merker 2009) which signals chondrocyte proliferation and differentiation and osteoblast differentiation is reported to contribute to skeletal development in the zebrafish. Genes such as exostosin (ext2) and solute carrier family 35 member B2 (slc35b2/papst1), which are involved in glycosaminoglycan synthesis and are associated with osteochondroma in humans, can also influence the cartilage phenotype in zebrafish ((Clement et al. 2008; Wiweger et al. 2011) and as reviewed in (Apschner et al. 2011)). Furthermore, homologues of several OA-associated genes (Section 1.3) namely mcf2l, gdf5, col1a1, col2a1, col9a2, col10a1, pthlh are expressed during the skeletal development of zebrafish (Mitchell et al. 2013).
Mutating zebrafish provides insight into chondrogenesis. Phenotype characteristics in the fish such as ECM staining, chondrocyte stacking, individuation and shaping are assessed to determine how the process of development, differentiation and growth might be affected. For example, fish which have mutations that disrupt chondrocyte differentiation such as *hammerheads*, exhibit a reduced pectoral skeleton and abnormal cell morphologies which manifests much later than fish which have developmental mutations such as *flatheads* which have small eyes, brain necrosis, flat compressed heads and either absent or a reduced number of branchial arches (Schilling et al. 1996). *Jellyfish* which occurs as a result of mutant alleles in *sox9*, not only display poor cartilage differentiation but also irregular morphologies in chondrocyte stacking, arch individuation and cartilage shaping (Yan et al. 2002). Abnormal stacking often results in rounded chondrocytes and disorganised clustering (Wiweger et al. 2011). In *van gogh* mutants, the endoderm does not segment and fish exhibit fused pharyngeal arches (Piotrowski & Nusslein-Volhard 2000).

The use of *in vivo* models in cartilage studies is advantageous when comparing the protocols executed during *in vitro* experiments due to the fact that the latter, not only remove the chondrocytes from the surrounding matrix but also subject the cells to physiological stresses (as reviewed in (van der Kraan 2013)). The use of zebrafish in the study of OA may seem unrealistic due to a lack of synovial joints and alternate mechanical stress, due to their aqueous habitat ((Mitchell et al. 2013) and as reviewed in (van der Kraan 2013)). However, the skeletal development pathways in both humans and teleost fish are highly conserved ((Mitchell et al. 2013) and as reviewed in (Spoorendonk et al. 2010)). Furthermore, large numbers of offspring, ease of genetic manipulation and simple maintenance, are all advantageous when considering zebrafish as an *in vivo* model. Embryos are also translucent and develop rapidly which allows for the generation of organs to be observed while the fish are still alive ((Mitchell et al. 2013) and as reviewed in (Spoorendonk et al. 2010; van der Kraan 2013)). Despite the advantages listed here, the fact remains that these zebrafish are intended to act as a model for the human disease and hence cannot be wholly representative of the OA process in humans but do provide insight into the chondrogenic development of vertebrates (as reviewed in (van der Kraan 2013)).
Zebrafish which acquire lethal loss of function mutations in skeletal development are able to survive much longer than other in vivo models such as mice (Hammond & Schulte-Merker 2009) and as reviewed in (Spoorenndonk et al. 2010)). It has been suggested that this may be because there is a lack of respiratory stress due to the aquatic environment of the zebrafish (as reviewed in (Spoorenndonk et al. 2010)). This is advantageous to this study as the lifespan extends at least to the point of chondrogenesis. Whereas homozygous Gnl3 knockout mice died at embryonic day 4 (Beekman et al. 2006; Zhu et al. 2006), the homozygous gnl3 zebrafish were able to survive up to 5dpf (Essers et al. 2014). Bearing in mind that chondrogenesis occurs at 2dpf (Schilling & Kimmel 1997) in the zebrafish and the fact that embryogenesis is ex utero and embryos are translucent, this provides a valuable model with which the role of gnl3 in cartilage might be determined (Mitchell et al. 2013) and as reviewed in (Spoorenndonk et al. 2010; van der Kraan 2013)).

The ns$^{hu3259}$ (nucleostemin$^{hu3259}$) is a loss of function gnl3 mutant (Paridaen et al. 2011). In previous studies which examined retinal neurogenesis, the ns$^{hu3259}$ mutant zebrafish displayed a phenotype typical of ribosomal biogenesis mutants (Paridaen et al. 2011; Essers et al. 2014). Compared to the wildtype fish, ns$^{-/-}$ were smaller in size and had smaller eyes and heads. The forebrain and mid-hindbrain were reduced, hindbrain hyperinflated and epiphysis expanded (Paridaen et al. 2011). Mutant fish died at 5pdf (Essers et al. 2014). Heterozygous fish presented a normal phenotype. mRNA expression of gnl3 was concentrated at sites of proliferation, namely the retina, hindbrain and optic tectum. As the fish developed, gnl3 expression levels decreased which correlated with a reduction in proliferation. The authors suggested that gnl3 was important in maintaining progenitor cell populations. Retinal cells displayed a delay in cell cycle exit whereas brain cell cycle exit was premature. These alterations in cell cycle exit and differentiation were not dependent on p53 and were suggested to be due to abnormal expression of cell cycle regulators, cyclinD1 which promotes proliferation and p57kip2, an inhibitor of G1-S transition (Paridaen et al. 2011). This was in concordance with the knockout mice studies which showed a decrease in cell proliferation and G1-S arrest (Beekman et al. 2006) (Section 1.4.1.4). In ns$^{-/-}$ fish, there was an increase in the number of p53-mediated apoptotic
cells (Paridaen et al. 2011). P53 mutants could not rescue the \( n s^{-/-} \) embryo from lethality but could partially rescue the morphological phenotypes (Paridaen et al. 2011; Essers et al. 2014). Further investigation into ribosomal biogenesis in these fish revealed that pre-rRNA processing was considerably impaired. In \( n s^{-/-} \) fish this resulted in improper cleavage of the pre-rRNA transcript, affecting predominantly 5.8S and 28S (but also 18S to a far lesser extent), and retention of intermediate cleavage products. This primarily had an impact on the formation of the large 60S ribosomal subunit, however 80S was also affected and ultimately resulted in a significant reduction in protein synthesis (Essers et al. 2014). Disruption in the 60S subunit could release the L5/L11/5S complex and inhibit MDM2 (Donati et al. 2013; Essers et al. 2014). P53 was able to rescue the 60S subunit however this increase was also observed in wildtype fish (Essers et al. 2014).

In previous studies, other mutant fish have exhibited simultaneous alterations in retinotectal, pharyngeal arches and pectoral fin development. Factors which regulate limb development, such as BMPs and hedgehog signalling, have also been observed in the pharyngeal arches and brain and may have similar functions (Schilling et al. 1996). Accordingly, perhaps the effect of \textit{gnl3} depletion in retina and brain in zebrafish could have a similar effect in cartilage, which I have shown to express \textit{GNL3} in humans (Section 3.3).

The aim of this project was to investigate whether \textit{gnl3} had an effect on the cartilage phenotype \textit{in vivo}. To achieve this, zebrafish were used as a model to compare the differences, between wildtype and \( n s^{ha3259} \) mutant fish. I not only examined the amount of cartilage present but also its structure, by quantifying the number of chondrocytes and ECM content.
5.2 Materials and Methods

5.2.1 Zebrafish

In a reverse genetic screen which specifically targeted gnl3 (NM_001002297) in the zebrafish genome, the \( n^+_{\text{hu}3259} \) mutant fish was generated by our collaborators at the Hubrecht Institute (Utrecht, The Netherlands). Briefly, chemical mutagenesis was performed on wildtype male fish using \( N \)-ethyl-\( N \)-nitrosourea (ENU) to create random mutations throughout the genome. These fish were then crossed with wildtype females. DNA was then extracted from the offspring and the TILLING (target induced local lesions in genomes) method used to identify mutations of interest. In short, TILLING involves amplifying the gene of interest using nested PCR, followed by CEL-1 enzyme mediated cleavage of heteroduplexes and electrophoresis. Mutations are then confirmed by re-sequencing and fish selected for breeding (Wienholds & Plasterk 2004; Paridaen et al. 2011).

The \( n^+_{\text{hu}3259} \) allele is a non-sense mutation in exon 5 of gnl3 of the zebrafish genome which converts the glutamic acid at position 117 (NP_001002297.1) (NCBI 2012) to a stop codon (Figure 5.2) (Paridaen et al. 2011). This corresponds to exon 5 of the human transcript (NP_055181.3) (NCBI 2014c) (Tsai & McKay 2002; EMBL-EBI 2012).

![Figure 5.2: The \( n^+_{\text{hu}3259} \) allele is a nonsense mutation in exon 5 of gnl3 in the zebrafish genome.](image)

This results in the alteration of a glutamic acid at position 117 (GAA) being converted to a premature stop codon (TAA) (Paridaen et al. 2011).
5.2.2 Histology

5.2.2.1 Paraffin-Embedding
Paraffin-embedded wildtype and ns<sup>hua3259</sup> mutant zebrafish at both 3 and 5dpf were kindly prepared and donated by Paul Essers and Alyson MacInnes (Hubrecht Institute, Utrecht).

5.2.2.2 Sectioning
Sections were cut at 5µm by Helen Caldwell and Susan Harvey (Breakthrough Unit, Edinburgh) using a Leica RM2235 microtome. In total, five sections taken at every 4th or 5<sup>th</sup> interval were attached to glass slides (VWR International™ SuperFrost® Plus). Fish were sectioned in either the coronal or sagittal plane.

5.2.2.3 Alcian Blue and Safranin-O Staining

5.2.2.3.1 Staining
Images of whole mount alcian blue stained fish are courtesy of Paul Essers and Alyson MacInnes (Hubrecht Institute, Utrecht).

Paraffin section staining was carried out at the Breakthrough Unit Edinburgh with the help of Susan Harvey and Helen Caldwell. Slides were de-waxed in xylene (Genta Medical), rinsed in running water, then immersed in a filtered 1% alcian blue solution (in 3% acetic acid, pH 2.5) for 30min and rinsed in water again. Alcian blue is a cationic stain which, under acidic conditions, binds to the acidic glycosaminoglycans present in the cartilage ECM (Section 1.2.2.2) (Society for Developmental Biology 2008). Fish were then counterstained by briefly submerging the slides in a 0.1% safranin-O solution and rinsed in water. (All stains were kindly prepared by staff at the Royal Infirmary Edinburgh.) Slides were dehydrated through increasing concentrations of industrial methylated spirits (Genta Medical) and finally submerged in xylene (Genta Medical) before mounting a glass coverslip using DPX mounting medium (Fisher Scientific) and left to air-dry.

5.2.2.3.2 Visualisation
Sections were visualised at 40X magnification on a Zeiss Axio Imager A1 microscope. Paraffin sections 1, 5 10, 14 and 18 were used in analysis. A series of consecutive images were captured along the body of each fish for every section.
5.2.2.3.3 Analysis

5.2.2.3.3.1 Quantification

The amount cartilage, other tissue, chondrocytes and ECM was determined by overlaying a 361 point graticule (Figure 5.3) over each image. The cell or tissue type which lay in the centre of each graticule cross-point was counted. Graticule points which did not cover the fish were not counted.

Figure 5.3: Graticule used to quantify cartilage, other tissue, chondrocytes and ECM in zebrafish

Quantification was carried out blind. The graticule was placed over every image along the body of the fish and through the alternate paraffin-sections of the same fish. The average of these values was then used to calculate the cartilage, other tissue, chondrocytes and ECM. Fish were then grouped by genotype, dpf and histological plane. When possible, five fish were analysed in each category.

5.2.2.3.3.2 Statistical Analysis

The average percentage cartilage (vs. other tissue) or chondrocytes (vs. cartilage ECM) was then calculated for all the wildtype or mutant fish at each time point and any values which exceeded the group average ± 3SD, were removed. P values were calculated using MiniTab® v12.23 (MiniTab Inc). A general linear model ANOVA was employed to determine whether groups differed significantly (p ≤ 0.05) from one another. A Tukey comparison test (95% confidence interval) was used to assess individual differences between the groups.
5.3 Results

5.3.1 The Effect of Nonsense Mutation $ns^\text{hu3259}$ on Cartilage in Zebrafish

The results (Figure 5.4) show a general dysmorphia in mutant fish with a reduction in pharyngeal cartilage becoming more apparent, particularly in older mutant fish. The mutant fish display shortened Meckel’s, palatoquadrate and ceratohyal cartilage and the ceratobranchial arches did not develop. Furthermore, the wildtype fish not only have more cartilage but the chondrocytes display a greater pericellular matrix and the cartilage structure is more ordered than that of the mutant counterparts (Figure 5.5) (ZF Atlas 2014).

![Figure 5.4: Whole mount alcian blue staining of wildtype and $ns^\text{hu3259}$ mutant gnl3 zebrafish at both 3 and 5dpf](image)

Mutant fish show a general dysmorphia with shortened anterior cartilage and no ceratobranchials. These images were kindly provided by Alyson MacInnes and Paul Essers (Hubrecht Institute, Utrecht). m, Meckel’s cartilage; pq, palatoquadrate; cb, ceratobranchial arches (1-5); ch, ceratohyal; pf, pectoral fin.
5.3.1.1 **Mutation $ns^{hu3259}$ Results in a Reduction of Cartilage**

Quantification of histological sections (Section 5.2.2 and Figure 5.5) confirmed a significant reduction in the amount of cartilage relative to other tissue present in the mutant zebrafish. This was found to be significantly different between mutant and wildtype fish at 3dpf ($p=0.007$) and 5dpf ($p<0.001$). There was a significant increase ($p<0.001$) in the percentage cartilage from 3 to 5dpf in wildtype fish. The percentage cartilage also increased from 3 to 5dpf in mutant fish, although not significantly so (Table 5.1 and Figure 5.6).
5.3.1.2 Mutation ns<sup>hu3259</sup> Results in a Disruption of Cartilage Structure

Quantification of histological sections (Section 5.2.2 and Figure 5.5) confirmed a significant reduction in the number of chondrocytes relative to the cartilage ECM present, in the cartilage of mutant zebrafish. This was found to be significantly different between mutant and wildtype fish at 3dpf (p<0.001) and 5dpf (p<0.001). There was a significant increase (p=0.001) in the percentage chondrocytes from 3 to 5dpf in wildtype fish. This appeared to occur as a result of a disproportionately larger increase in chondrocyte number compared to cartilage ECM. The percentage chondrocytes in 5dpf mutant fish decreased slightly however this difference was not significantly different from that of the 3dpf mutant fish and was most likely due to a

Table 5.1: Data from histological quantification of cartilage and other tissue in wildtype and ns<sup>hu3259</sup> mutant gnl3 zebrafish at both 3 and 5dpf. Values are average ± SEM (n=10 (except ns<sup>hu3259</sup> mutant zebrafish at 5dpf, n=6)).

<table>
<thead>
<tr>
<th>dpf</th>
<th>Fish</th>
<th>Cartilage</th>
<th>Other Tissue</th>
<th>Cartilage/Other Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>wildtype</td>
<td>146.40 ± 9.80</td>
<td>3415.30 ± 378.69</td>
<td>4.76% ± 0.68</td>
</tr>
<tr>
<td></td>
<td>ns&lt;sup&gt;hu3259&lt;/sup&gt;</td>
<td>80.90 ± 9.89</td>
<td>3037.00 ± 159.96</td>
<td>2.64% ± 0.28</td>
</tr>
<tr>
<td>5</td>
<td>wildtype</td>
<td>182.80 ± 13.84</td>
<td>2123.50 ± 114.17</td>
<td>8.68% ± 0.61</td>
</tr>
<tr>
<td></td>
<td>ns&lt;sup&gt;hu3259&lt;/sup&gt;</td>
<td>121.00 ±17.73</td>
<td>3685.83 ± 314.48</td>
<td>3.45% ± 0.64</td>
</tr>
</tbody>
</table>

Figure 5.6: Percentage cartilage in wildtype and ns<sup>hu3259</sup> mutant gnl3 zebrafish at both 3 and 5dpf. Values are average percentage cartilage/other tissue. Error bars are ± SEM (n=10 (except ns<sup>hu3259</sup> mutant zebrafish at 5dpf, n=6)). Dark grey, wildtype; Light grey, ns<sup>hu3259</sup> zebrafish.

5.3.1.2 Mutation ns<sup>hu3259</sup> Results in a Disruption of Cartilage Structure

Quantification of histological sections (Section 5.2.2 and Figure 5.5) confirmed a significant reduction in the number of chondrocytes relative to the cartilage ECM present, in the cartilage of mutant zebrafish. This was found to be significantly different between mutant and wildtype fish at 3dpf (p<0.001) and 5dpf (p<0.001). There was a significant increase (p=0.001) in the percentage chondrocytes from 3 to 5dpf in wildtype fish. This appeared to occur as a result of a disproportionately larger increase in chondrocyte number compared to cartilage ECM. The percentage chondrocytes in 5dpf mutant fish decreased slightly however this difference was not significantly different from that of the 3dpf mutant fish and was most likely due to a
disproportionately larger increase in cartilage ECM compared to chondrocyte number (Table 5.2 and Figure 5.7).

<table>
<thead>
<tr>
<th>dpf</th>
<th>Fish</th>
<th>Chondrocytes</th>
<th>Cartilage ECM</th>
<th>Chondrocytes/Cartilage ECM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>wildtype</td>
<td>60.20 ± 5.42</td>
<td>87.16 ± 16.60</td>
<td>67.09% ± 5.82</td>
</tr>
<tr>
<td></td>
<td>ns\textsuperscript{hu3259}</td>
<td>15.20 ± 1.92</td>
<td>99.80 ± 26.46</td>
<td>19.97% ± 3.11</td>
</tr>
<tr>
<td>5</td>
<td>wildtype</td>
<td>118.70 ± 8.26</td>
<td>102.90 ± 25.05</td>
<td>154.93% ± 23.27</td>
</tr>
<tr>
<td></td>
<td>ns\textsuperscript{hu3259}</td>
<td>25.67 ± 7.41</td>
<td>171.67 ± 57.64</td>
<td>16.39% ± 2.21</td>
</tr>
</tbody>
</table>

Table 5.2: Data from histological quantification of chondrocytes and cartilage ECM in wildtype and ns\textsuperscript{hu3259} mutant gnl3 zebrafish at both 3 and 5dpf. Values are average ± SEM (n=10 (except ns\textsuperscript{hu3259} mutant zebrafish at 5dpf, n=6)).

Figure 5.7: Percentage chondrocytes in wildtype and ns\textsuperscript{hu3259} mutant gnl3 zebrafish at both 3 and 5dpf. Values are average percentage chondrocytes/ECM in cartilage tissue. Error bars are ± SEM (n=10 (except ns\textsuperscript{hu3259} mutant zebrafish at 5dpf, n=6)). Dark grey, wildtype; Light grey, ns\textsuperscript{hu3259} zebrafish.
5.4 Discussion

The use of zebrafish as a model to investigate the influence which gnl3 has on cartilage development, showed that this gene plays a vital role in the chondrogenic process.

Assessment of the whole fish revealed an overall reduction in cartilage in the mutants. This was the result of a shortening of the first two pharyngeal arches and an absence of arches 3 through to 7. The first arch includes the Meckel’s cartilage and palatoquadrate which are present but appear compressed. The second arch includes the ceratohyal cartilage which extends postero-medially in the mutant fish. Arches 3 to 7 are the branchial arches which do not develop in the ns^hu3259 mutants. This is a comment on the whole mount images however these characteristics were also noted when performing more in depth histological analysis of the cartilage.

Quantification of the histology results revealed there was significantly (3dpf, p=0.007; 5dpf, p<0.001) less cartilage in the mutants when compared to the wildtype fish. This was observed as early as 3dpf and failed to develop at the same rate as in the wildtype fish. Furthermore, comparison of the cartilage structure between mutant and wildtype fish showed, not only was there a significantly (3dpf, p<0.001; 5dpf, p<0.001) higher percentage of chondrocytes present in the cartilage of wildtype fish but these chondrocytes appeared larger, due mostly to a greater surrounding pericellular matrix, and the layout was more structured. These differences were observed as early as 3dpf. Chondrocyte shape and organisation failed to improve as the mutant fish matured. There was also more ECM surrounding the chondrocytes in mutant fish as early as 3dpf, which also did not differ much from the cartilage structure at 5dpf in these fish. It interesting to note, that while the percentage cartilage showed a marginal increase in mutant fish between 3 and 5dpf, the amount of cartilage ECM also increased however, the number of chondrocytes did not increase in proportion with cartilage growth. This is in contrast to the wildtype fish which showed an increase in cartilage and chondrocyte number and relatively less of an increase in cartilage ECM between 3 and 5dpf. This suggests that not only the amount of cartilage but also its structure is affected by a loss of gnl3 function.
Other studies on pharyngeal arch development in zebrafish have noted that in general fish tend to fail in either the cartilage differentiation, or overall developmental morphology (Schilling et al. 1996). As discussed previously (Section 5.1), the jellyfish mutation in sox9 affects cartilage differentiation which results in improper chondrocyte stacking, arch individuation and cartilage shaping (Yan et al. 2002). Developmental delays such as those exhibited in flatheads typically manifest symptoms earlier and exhibit a reduction of entire cartilages, possibly due to aberrant NCC positioning (Schilling et al. 1996).

The ns\textsuperscript{hu3259} mutants were observed in previous studies to have aberrant cell cycle exit and differentiation, incomplete development of retina and brain, as well as an overall decrease in body size, small eyes and reduction in brain ventricles (Paridaen et al. 2011). The cartilage phenotype in mutant fish exhibited a reduction in cartilage, particularly the posterior branchial arches. This is similar to the phenotype of the developmental mutant group (Schilling et al. 1996). Schilling et al. (1996) suggested this could be due to a loss of NCCs in the brain which migrate to populate the posterior arches. The ns\textsuperscript{hu3259} mutants also exhibited some characteristics similar to the phenotype of the differentiation impaired mutants (Schilling et al. 1996; Yan et al. 2002). While there was an overall reduction in development, the chondrocytes which did populate the pharyngeal arches were successful in depositing the ECM, however the chondrocytes themselves had a reduced pericellular matrix and stacking was disorganised. This suggests there is a delay in differentiation in the mutant chondrocytes which could be due to aberrant expression of cell cycle regulators, as was reported in retinal neurogenesis studies. This may also be due to apoptosis, as previous studies observed high number of apoptotic cells in other tissues (Paridaen et al. 2011). A disruption in chondrocyte stacking and ECM structure could subsequently affect the cartilage morphology and ultimately the joint shape (Kimmel et al. 1998). Previous ns\textsuperscript{hu3259} mutant studies (Paridaen et al. 2011) also noted a reduction in cell proliferation which could contribute not only to a reduction in chondrocyte numbers but also inhibit cartilage growth (Kimmel et al. 1998). In wildtype chondrocytes, only a thin layer of ECM surrounds the chondrocytes (Kimmel et al. 1998). The fact that the mutant fish in this study had a large ECM and
minimal pericellular matrix, could suggest that the chondrocytes which are present may not be able to achieve efficient protein synthesis due to a disruption of the 60S subunit (Essers et al. 2014). Further studies are needed to investigate the effect of p53 and ribosome biogenesis on the cartilage phenotype. Perhaps the expression of cell cycle regulators such as cyclinD1 and p57kip2 should be investigated in cartilage. These might be responsible for the reduction in cartilage development and hence IHC for col2a, sox9 and aggrecan should also be carried out. In addition, it would be of interest to conduct IHC for col6 given the enlarged pericellular matrix in mutant fish.

These results clearly show a striking difference in the cartilage of wildtype and \textit{ns}^{hu3259} mutant fish. Bearing in mind the lack of diarthrodial joint and mechanical stresses when using fish as a model for OA ((Mitchell et al. 2013) and as reviewed in (van der Kraan 2013)), these results undoubtedly confirm \textit{gnl3} as a viable candidate in the study of cartilage development and warrants further investigation as to how this might contribute to the pathogenesis of OA in humans. Particularly as the skeletal development pathways are highly conserved between these species ((Mitchell et al. 2013) and as reviewed in (Spoorendonk et al. 2010)) and therefore the loss of cartilage integrity, delayed or absent development of cartilage structure and the influence this could have on joint shape, are all plausible explanations as to how deficiencies in \textit{GNL3} might affect skeletal development and predispose individuals to the onset of OA. To this end studies which employ Cre-Lox recombinase technology to generate conditional knockout mice with joint-specific \textit{Gnl3} mutations might also be considered. This would provide valuable information from an \textit{in vivo} model which shares greater sequence homology with humans which would otherwise be embryonic lethal.
Chapter 6: Discussion

OA is a complex disease and is influenced by a number of environmental and genetic factors all contributing varying individual effects (Loughlin 2011a). The most significantly associated signal from the recent arcOGEN GWAS was found in locus 3p21.1 and tagged by two SNPs, rs11177 and rs6976. Rs11177 lies within the exon 3 of GNL3 which encodes nucleostemin (Zeggini et al. 2012).

Nucleostemin is a nucleolar protein which was originally identified in cancer cells and stem cells (Tsai & McKay 2002). It is involved in a number of different biological processes, namely embryogenesis (Beekman et al. 2006; Zhu et al. 2006), cell cycle progression, proliferation (Tsai & McKay 2002), DNA damage repair (Hsu et al. 2012) and rRNA processing (Romanova et al. 2009). The GTP-mediated shuttling of nucleostemin between the nucleolus and nucleoplasm are thought to be vital in enabling it to interact with other proteins to carry out these functions as well as mediating its own stability. Nucleostemin is predominantly located in the nucleolus and exported to the nucleoplasm during mitosis or stressful conditions. Nucleostemin is directed to the nucleolus by its N-terminal upon GTP binding which releases the nucleoplasmic anchor in its intermediate domain (Tsai & McKay 2005). A loss of GTP results in a less stable nucleoplasmic nucleostemin which is subject to ubiquitination and proteasomal degradation (Huang et al. 2009; Lo et al. 2012).

Nucleostemin null mice are embryonic lethal and fail to progress beyond the blastocyst stage due to G1-S cell cycle arrest. However these mice were not able to be rescued by p53 null mice (Beekman et al. 2006; Zhu et al. 2006). It is thought that nucleostemin is able to mediate the recruitment of RAD51 to DNA repair sites via sumoylated-TRF1 and PMLIV. Nucleostemin may also directly inhibit telomere shortening by binding to TRF1. It has been suggested that these mechanisms of DNA repair failure are responsible for the death of the nucleostemin null mice (Hsu et al. 2012).

Nucleolar stress has been shown to lead to cell cycle arrest. A number of studies have suggested that this could be carried out via interactions with members of the
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

p53 pathway (Tsai & McKay 2002; Ma & Pederson 2007). In particular, nucleoplasmic nucleostemin has been shown to bind to MDM2, inhibiting the ubiquitination and proteasomal degradation of p53 leading to cell cycle arrest (Dai et al. 2008; Meng et al. 2008). Other cell cycle regulators such as ARF (Ma & Pederson 2007), cyclinD1 and p57kip2 (Paridaen et al. 2011) have also exhibited alterations in response to changes in nucleostemin levels.

There are conflicting studies regarding the involvement of nucleostemin in rRNA processing. Some have noted that nucleostemin is found in nonribosomal subnucleolar regions (Politz et al. 2005). Whereas others have shown it to be part of a large ∼700kDa ribosomal protein and be involved in processing 32S, part of the large 60S subunit (Romanova et al 2009). Furthermore, a decrease in nucleostemin can result in nucleolar stress and subsequent release of ribosomal proteins resulting in the inhibition of MDM2 by L5/L11/5S (Donati et al. 2013).

Nucleostemin levels rapidly decrease in response to a reduction in GTP levels, thought to be due to an increase in the less stable nucleoplasmic nucleostemin which may be subject to MDM2-mediated ubiquitination (Huang et al. 2009). However, some studies have suggested alternate factors may be involved in targeting nucleostemin for proteasomal degradation (Lo et al. 2012).

An increase in ROS has also been shown to result in the formation of thiol-reversible bonds and increase in nucleolar nucleostemin. Prolonged exposure to ROS can result in detergent-insoluble nucleolar aggregates (Huang et al. 2011).

Nucleostemin levels have also been shown to increase in limb regeneration in the newt (Maki et al. 2007), in response to injury of the heart (Siddiqi et al. 2008) and liver (Shugo et al. 2012) as well as increases in FGF2 (Kafienah et al. 2006; Siddiqi et al. 2008) and oestrogen (Charpentier et al. 2000).

Nucleostemin was chosen due to the fact that there is significant evidence in the literature regarding its functional role in cell proliferation and DNA repair to suggest that, were it present in joint tissues, it may contribute to joint maintenance and ultimately the onset of OA.
This thesis has shown mRNA expression of \textit{GNL3} in several human joint tissues (Section 3.3.3) and protein expression in HACs (Section 3.3.1). It has also shown dysregulation in this expression at both the mRNA (Section 3.3.4.2.1) and protein (Section 3.3.5.1) levels which increased and decreased respectively, in HACs extracted from OA patients relative to the controls. Other studies which measured nucleostemin levels in response to GTP-depletion have noted that mRNA levels were not affected whereas protein expression decreased (Huang et al. 2009; Lo et al. 2012). With regard to the OA relevant mechanisms which may influence the expression of nucleostemin, the influence of cytokines (IL1β, IL13 and TNFα) and growth factor (FGF2) were investigated at the mRNA and protein level (Section 3.3.6) however these did not influence nucleostemin expression. Furthermore, knockdown of \textit{GNL3} did not influence the mRNA expression of chondrogenic markers (Section 4.3.1.1.3). GTP-signalling is known to play a role in mechanotransduction (Jutila et al. 2014). Nucleostemin could therefore be vital in determining the joint response to mechanical stresses and influence the onset and progression of OA. Experiments which treat chondrocytes with an IMPDH inhibitor, such as mycophenolic acid, should be carried out in order to determine whether there is any difference in the mRNA and protein expression of \textit{GNL3} in response to GTP depletion.

The experiments carried out in this thesis aimed to identify the causal variant(s) in \textit{GNL3}. The functional effect of two non-synonymous coding variants was investigated further in this thesis. All variants (Table 2.2) could be investigated in order to determine whether there is an AEI. It would also be interesting to determine whether the promoter variants identified are able to influence \textit{GNL3} expression in a luciferase assay or affect the binding of transcription factors such as AHR/ARNT/ARNT2 and ZFP161 by means of an electrophoretic mobility shift assay.

While the effect of nucleostemin rs11177 variants on the chondrogenic potential of MSCs and HACs was investigated here at an mRNA level (Section 4.3.3), the effect should be further quantified at both an mRNA and protein level using qPCR and western blotting respectively. The effect of nucleostemin knockdown in these cells
should also be investigated using these techniques to quantify the expression of chondrogenic markers and GNL3 itself. Additional tests which use cell markers to confirm the extraction of MSCs should also be performed prior to conducting these experiments.

The influence which the variants at rs11177 and rs2289247 have on the p53/MDM2 response was investigated at a protein level (Section 4.3.6 and Section 4.3.8). While no significant results were found, the location of nucleostemin should be investigated further using confocal microscopy to determine whether there is a difference in the distribution of nucleostemin variants in the nucleolus that might ultimately affect its abundance and interactions with other proteins. This technique might also be used to determine whether there are any differences in the nucleolar expression pattern of nucleostemin between GNL3 variants, and/or OA and normal HACs.

The p53/MDM2 response was investigated after stimulation with cisplatin and oxidative stress in a melanoma cell line. The western blot experiments in this thesis were carried out under reducing conditions, which would reverse the thiol bonds reported to mediate the nucleolar aggregation of nucleostemin. It would be interesting to determine the effect of hypoxia on oligomerisation in chondrocytes which are found at reduced oxygen levels under physiological conditions. Hence a western blot should perhaps be performed under non-reducing conditions (Huang et al. 2011).

The use of ns\textsuperscript{hu3259} mutant zebrafish presented a dysmorphic cartilage phenotype which not only resulted in a loss of cartilage but also a decrease in chondrocyte numbers, dysmorphic chondrocyte shape and an increase in ECM in the mutant fish (Section 5.3). Further IHC should be conducted on these fish to determine the location of nucleostemin expression (which was found at the sites of proliferation in neurogenesis studies (Paridaen et al. 2011)). Considering, the large pericellular matrix in the wildtype fish and the increase in ECM in mutant fish, IHC which investigates the level of collagens II and VI would be of interest, as well as perhaps staining for aggrecan and sox9. Previous retinal neurogenesis (Paridaen et al. 2011) studies reported an aberrant level of cyclinD1 and p57kip2 expression in ns\textsuperscript{hu3259}
mutant zebrafish and hence perhaps the role of these cell cycle regulators should be investigated in relation to cartilage development.

Nucleostemin null mice die at embryonic day 4 (Beekman et al. 2006; Zhu et al. 2006). The Gnl3 homolog shares 71% protein identity with that of human GNL3 (compared to 44% homology in zebrafish) (as reviewed in (Tsai 2014)). Henceforth it would be of interest to investigate the effect of removing Gnl3 by means of Cre-Lox conditional knockout in mouse joint tissue.

This research has confirmed the mRNA expression of GNL3 in several human joint tissues and shown evidence of dysregulation of GNL3 expression at both an mRNA and protein level in OA HACs. Furthermore, the zebrafish studies illustrate that nucleostemin plays a vital role in normal cartilage development in zebrafish.

The mechanisms by which GNL3 expression, itself is regulated might be explored in future studies. The extent to which the promoter variants identified in this study or other variants at locus 3p21.1 could influence GNL3 expression should be investigated further. The location of nucleostemin may be responsible for the differences in expression seen between normal and OA cases. This may be mediated by alteration in GTP levels in response to mechanotransduction signalling. This is likely to be deficient in OA cartilage tissue as it is degraded and lacking in hydrophilic proteoglycans and glycosaminoglycans which would otherwise provide efficient shock absorption. The nuclear location of nucleostemin may also be effected by oxidative stress. This could occur as a result of a further reduction in oxygen levels and subsequent increase in ROS in OA chondrocytes which might otherwise be found in a less hypoxic environment in intact cartilage.

The mechanisms by which these alterations in GNL3 expression influence the development and repair of joint tissues should also be considered. The experiments described above would aid in defining these. While it is recognised that the chondrocytes in healthy cartilage are in a dormant state, they do undergo proliferation, clustering and ultimately apoptosis in OA cartilage. Cell cycle regulators, such as p53, MDM2, ARF, cyclinD1 and p57kip2 have been suggested to
play a role in previous experiments. The functioning of these and others in cartilage in response to nucleostemin levels would be of interest. With regard to the development and maintenance of joint tissues, deficiencies in DNA repair mechanisms, increased telomere shortening and aging of tissues are all viable mechanisms by which nucleostemin could influence the development and maintenance of joint tissues. Errors in processing of ribosomal subunits in response to aberrant nucleostemin expression could affect the transcription and translation of ECM proteins.

How these processes affect the chondrogenic potential of MSCs would also be of interest. Although present in low numbers, MSCs are found in the synovium and hence it is plausible that they could contribute to the repair of cartilage. The extent to which nucleostemin maintains this function might be investigated further.

The work carried out in this thesis is the first to confirm nucleostemin as a viable candidate in the study of OA due to the differential expression identified in normal and OA chondrocytes and its clear role in cartilage development. Hence, further research into mechanisms by which this contributes to the pathogenesis of OA would be of great value to the field.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

References


Albagha, O. M. E. Chi-squared analysis of allele frequencies. 2012. Ref Type: Personal Communication

INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Ref Type: Pamphlet

http://www.arthritisresearchuk.org. 2013
Ref Type: Electronic Citation

Ref Type: Electronic Citation

Ref Type: Electronic Citation

REFERENCES

211
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

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INVESTIGATING THE ROLE OF GNL3 IN OSTEARTHRITIS


REFERENCES 213
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Boulidakas, T. & Vougiouka, M. 2003, Cisplatin and platinum drugs at the molecular level (review), *Oncology Reports*, vol. 10, no. 6, pp. 1663-1682.


REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


REFERENCES


INVESTIGATING THE ROLE OF GNL3 INOSTEOARTHRITIS


Ref Type: Electronic Citation


Ref Type: Abstract


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Duval, E., Leclercq, S., Elissalde, J. M., Demoor, M., Galera, P., & Boumediene, K. 2009. Hypoxia-inducible factor 1 alpha inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen in the hypoxia-inducible factor 1 alpha-dependent redifferentiation of chondrocytes, *Arthritis and Rheumatism*, vol. 60, no. 10, pp. 3038-3048.


REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEARTHRITIS


Ref Type: Abstract


GibcoBRL®. GATEWAY™ Cloning Technology. 2012. Ref Type: Catalog

INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Gray, T. A. FRT Site. 2012.
Ref Type: Personal Communication


REFERENCES 221


data from the Baltimore Longitudinal Study of Aging (BLSA), *Osteoarthritis and Cartilage*, vol. 6, no. 4, pp. 245-251.

Hsu, J. K., Lin, T., & Tsai, R. Y. L. 2012, Nucleostemin prevents telomere damage by promoting PML-IV recruitment to SUMOylated TRF1, *Journal of Cell Biology*, vol. 197, no. 5, pp. 613-624.


Ref Type: Abstract

INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Invitrogen™. Gateway® pDONR™ vectors. 2012. Ref Type: Catalog

Invitrogen™. alamarBlue® assay. 2013. Ref Type: Catalog


J. Craig Venter Institute. SIFT v4.0.3 (Sorting Intolerant From Tolerant). http://sift.jcvi.org/. 2011. Ref Type: Electronic Citation

REFERENCES 224
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Kafienah, W., Mistry, S., Williams, C., & Hollander, A. P. 2006, Nucleostemin is a marker of proliferating stromal stem cells in adult human bone marrow, *Stem Cells*, vol. 24, no. 4, pp. 1113-1120.


REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Kent, J. UCSC In-Silico PCR. http://genome.ucsc.edu/cgi-bin/hgPcr?command=start. 2011.

Ref Type: Electronic Citation


chondrocyte development and growth factor signaling during cartilage morphogenesis, *Development*, vol. 132, no. 17, pp. 3989-4003.


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Lefebvre, V., Huang, W. D., Harley, V. R., Goodfellow, P. N., & de Crombrugghe, B. 1997, SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha 1(II) collagen gene, Molecular and Cellular Biology, vol. 17, no. 4, pp. 2336-2346.


Ref Type: Abstract


REFERENCES 228


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Moos, V., Menard, J., Sieper, J., Sparmann, M., & Muller, B. 2002, Association of HLA-DRB1*02 with osteoarthritis in a cohort of 106 patients, *Rheumatology*, vol. 41, no. 6, pp. 666-669.


Moxley, G., Han, J., Stern, A. G., & Riley, B. P. 2007, Potential influence of IL1B haplotype and IL1A-IL1B-IL1RN extended haplotype on hand osteoarthritis risk, *Osteoarthritis and Cartilage*, vol. 15, no. 10, pp. 1106-1112.


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


NCBI. GLT8D1 glycosyltransferase 8 domain containing 1 [Homo sapiens (human)]. http://www.ncbi.nlm.nih.gov/gene/55830. 2014b. Ref Type: Electronic Citation

NCBI. Homo sapiens guanine nucleotide binding protein-like 3 (nucleolar) (GNL3), transcript variant 1, mRNA. http://www.ncbi.nlm.nih.gov/nuccore/NM_014366.4. 2014c. Ref Type: Electronic Citation

NCBI. Homo sapiens guanine nucleotide binding protein-like 3 (nucleolar) (GNL3), transcript variant 2, mRNA. http://www.ncbi.nlm.nih.gov/nuccore/NM_206825.1. 2014d. Ref Type: Electronic Citation

NCBI. Homo sapiens guanine nucleotide binding protein-like 3 (nucleolar) (GNL3), transcript variant 3, mRNA. http://www.ncbi.nlm.nih.gov/nuccore/NM_206826.1. 2014e. Ref Type: Electronic Citation


NCBI. MUSTN1 musculoskeletal, embryonic nuclear protein 1 [Homo sapiens (human)]. http://www.ncbi.nlm.nih.gov/gene/389125. 2014i. Ref Type: Electronic Citation

REFERENCES
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


NCBI. NT5DC2 5'-nucleotidase domain containing 2 [Homo sapiens (human)]. http://www.ncbi.nlm.nih.gov/gene/64943. 2014k. Ref Type: Electronic Citation


NCBI. PubMed. www.ncbi.nlm.nih.gov/pubmed. 2014m. Ref Type: Electronic Citation


NCBI. SPCS1 signal peptidase complex subunit 1 homolog (S. cerevisiae) [Homo sapiens (human)]. http://www.ncbi.nlm.nih.gov/gene/28972. 2014s. Ref Type: Electronic Citation


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Ref Type: Electronic Citation


Ref Type: Abstract


Ref Type: Electronic Citation

REFERENCES 237
INVESTIGATING THE ROLE OF GNL3 IN OSTEARTHRITIS


Ref Type: Abstract


Pelosi, M., Lazzarano, S., Thoms, B. L., & Murphy, C. L. 2013, Parathyroid hormone-related protein is induced by hypoxia and promotes expression of the differentiated phenotype of human articular chondrocytes, Clinical Science, vol. 125, no. 9-10, pp. 461-470.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Poole, A. R. Chapter 3: The normal synovial joint. OARSI primer. 2011.


REFERENCES

239
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Ref Type: Catalog


Investigating the Role of GNL3 in Osteoarthritis


Salter, D. M., Buckwalter, J. A., & Sandell, L. J. Chapter 4: Pathology of OA. OARSI Primer. 2011. Ref Type: Electronic Citation


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Senda, M., Delustro, B., Eugui, E., & Natsumeda, Y. 1995, Mycophenolic-acid, an inhibitor of IMP dehydrogenase that is also an immunosuppressive agent, suppresses the cytokine-induced nitric-oxide production in mouse and rat vascular endothelial-cells, Transplantation, vol. 60, no. 10, pp. 1143-1148.


Ref Type: Abstract


Sigma-Aldrich Co. 293 Cell Line human.
Ref Type: Electronic Citation

Sigma-Aldrich Co. CHO Cell Line from Chinese hamster ovary.
Ref Type: Electronic Citation

Sigma-Aldrich Co. SAOS-2 Cell Line human.
Ref Type: Electronic Citation
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Sigma-Aldrich Co. U-2 OS Cell Line human.
Ref Type: Electronic Citation

Sigma. qPCR Technical Guide. 2008.
Ref Type: Electronic Citation

Smith, A. J. P., Gidley, J., Sandy, J. R., Perry, M. J., Elson, C. J., Kirwan, J. R.,
low-density lipoprotein receptor-related protein 5 (LRP5) gene: Are they a risk factor

Smith, A. J. P., Keen, L. J., Billingham, M. J., Perry, M. J., Elson, C. J., Kirwan, J.
haplotypes and linkage disequilibrium in the IL1R1-IL1A-IL1B-IL1RN gene cluster:
association with knee osteoarthritis, Genes and Immunity, vol. 5, no. 6, pp. 451-460.

Society for Developmental Biology. Staining and observation of cartilaginous
structures in zebrafish complete larvae.
Ref Type: Electronic Citation

Solovieva, S., Hirvonen, A., Siivola, P., Vehmas, T., Luoma, K., Riihimaki, H., &
Leino-Arjas, P. 2006, Vitamin D receptor gene polymorphisms and susceptibility of
hand osteoarthritis in Finnish women, Arthritis Research and Therapy, vol. 8, no. 1,
pp. R20

Song, R. H., Tortorella, M. D., Malfait, A. M., Alston, J. T., Yang, Z. Y., Arner, E.
C., & Griggs, D. W. 2007, Aggrecan degradation in human articular cartilage
explants is mediated by both ADAMTS-4 and ADAMTS-5, Arthritis

Sophocleous, A. 2009, The role of type 2 cannabinoid receptor in bone metabolism.
Ref Type: Thesis

Sorge, R. E., Trang, T., Dorfman, R., Smith, S. B., Beggs, S., Ritchie, J., Austin, J.
S., Zaykin, D. V., Vander Meulen, H., Costigan, M., Herbert, T. A., Yarkoni-
Abitbul, M., Tichauer, D., Livneh, J., Gershon, E., Zheng, M., Tan, K., John, S. L.,
Slade, G. D., Jordan, J., Woolf, C. J., Peltz, G., Maixner, W., Diatchenko, L., Seltzer,
Z., Salter, M. W., & Mogil, J. S. 2012, Genetically determined P2X7 receptor pore
formation regulates variability in chronic pain sensitivity, Nature Medicine, vol. 18,
no. 4, pp. 595-599.

Southam, L., Dowling, B., Ferreira, A., Marcelline, L., Mustafa, Z., Chapman, K.,
Bentham, G., Carr, A., & Loughlin, J. 2004, Microsatellite association mapping of a
primary osteoarthritis susceptibility locus on chromosome 6p12.3-q13, Arthritis
and Rheumatism, vol. 50, no. 12, pp. 3910-3914.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Ref Type: Electronic Citation

Swiss Institute of Bioinformatics. SwissRegulon Portal. PrFont34Bin0BinSub0Frac0Def1Margin0Margin0Jc1Indent1440Lim0Lim1http://swissregulon.unibas.ch/gbrowse2/fcgi/gbrowse/hg19/?name=chr3%3A52719976.52719
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

Ref Type: Electronic Citation

Ref Type: Electronic Citation


Ref Type: Electronic Citation


Tsai, C.C., Su, P-F., Huang, Y-F., Yew, T-L., Hung, S-C. 2012, Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells, Molecular Cell, vol. 47, no. 2, pp. 169 – 182.


Tsai, R. Y. L. & McKay, R. D. G. 2002, A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells, Genes and Development, vol. 16, no. 23, pp. 2991-3003.


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


REFERENCES 246
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Wakitani, S., Imoto, K., Mazuka, T., Kim, S., Murata, N., & Yoneda, M. 2001, Japanese generalised osteoarthritis was associated with HLA class I - A study of HLA-A, B, Cw, DQ, DR in 72 patients, *Clinical Rheumatology*, vol. 20, no. 6, pp. 417-419.


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Ref Type: Abstract


INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS


Ref Type: Unpublished Work


Ref Type: Electronic Citation

REFERENCES 250


Zhang, J. Y. & Wang, J. H. C. 2013, Human tendon stem cells better maintain their stemness in hypoxic culture conditions, *Public Library of Science One*, vol. 8, no. 4, pp. e61424.


### Chapter 7: Appendix

#### 7.1 Supplementary Tables

<table>
<thead>
<tr>
<th>GNL3 Region</th>
<th>Forward [5'-3' +]</th>
<th>Reverse [5'-3' -]</th>
<th>Product size (bp)</th>
<th>Anneal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter &amp; Exon 1</td>
<td>GTGCGACAGGGCTACTGG</td>
<td>TACCAGGAAATAGGGCCAAG</td>
<td>695</td>
<td>51</td>
</tr>
<tr>
<td>Exon 2</td>
<td>TGTTCGCATAGGCATTTCG</td>
<td>TTGTGCCCATGGCTAGTCTA</td>
<td>331</td>
<td>61</td>
</tr>
<tr>
<td>Exon 3</td>
<td>GCCCTTCTAGTTACAATTTGCTTG</td>
<td>TGCCCTGAGTTTCTGCTG</td>
<td>485</td>
<td>61</td>
</tr>
<tr>
<td>Exon 4</td>
<td>GGGGAAGCTGAAGCTAAGGAAA</td>
<td>GACTGAGGTGTAAGACTGCTTG</td>
<td>396</td>
<td>61</td>
</tr>
<tr>
<td>Exon 5</td>
<td>CACAGGCTGCTGAGCTTCTT</td>
<td>CGTCACATGCCACTTCTCA</td>
<td>341</td>
<td>61</td>
</tr>
<tr>
<td>Exon 6</td>
<td>TGAGCTCAACCTGGCTTAA</td>
<td>GTGCACGTACTGCTGGCTT</td>
<td>382</td>
<td>61</td>
</tr>
<tr>
<td>Exon 7</td>
<td>ACTCGTGATCCGCCTGTCT</td>
<td>AAATCAAGATTTGTACACTCATCATA</td>
<td>288</td>
<td>50</td>
</tr>
<tr>
<td>Exon 8</td>
<td>TTCAGCCAGAGATCATCTGAAA</td>
<td>ACGTGATCTGGCAGAGTAT</td>
<td>466</td>
<td>61</td>
</tr>
<tr>
<td>Exon 9</td>
<td>TTTCCCTTGCTTTGTACATTCT</td>
<td>CTCCAGCCTGGGACAAG</td>
<td>402</td>
<td>51</td>
</tr>
<tr>
<td>Exon 10</td>
<td>AGAACAGTCGGTATGCTTG</td>
<td>ACCAAACAAAGGACTGCTCC</td>
<td>473</td>
<td>61</td>
</tr>
<tr>
<td>Exon 11</td>
<td>AGGCTGATGCTGAGCTGGAAGA</td>
<td>GTCCAGATGTAGGGGATG</td>
<td>420</td>
<td>61</td>
</tr>
<tr>
<td>Exon 12</td>
<td>CAAAAGGGTGGAATCCCAAA</td>
<td>CAGACGGGAGACTGGGAAAA</td>
<td>417</td>
<td>61</td>
</tr>
<tr>
<td>Exon 13 &amp; Exon 14</td>
<td>GCTTCAATCTGGAGAGACTGG</td>
<td>GCATCATCCTCTCTATGATTT</td>
<td>751</td>
<td>61</td>
</tr>
<tr>
<td>Exon 15</td>
<td>TTTGCTGCAGAGAGAGACAGG</td>
<td>GATTCCAATGGCCTGGTTT</td>
<td>359</td>
<td>61</td>
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</tbody>
</table>

Table 7.1: Primer sequences for GNL3 amplification bp, base pairs.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

<table>
<thead>
<tr>
<th>GNL3 Region</th>
<th>Reverse [5'-3' -]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 5</td>
<td>TGACTTGAGTCTTTTTAAACCTCATTAC</td>
</tr>
<tr>
<td>Exon 9</td>
<td>TCACCTTCCCTGAGATAACAAA</td>
</tr>
<tr>
<td>Exon 13</td>
<td>TGACTTGAGTCTTTTTAAACCTCATTAC</td>
</tr>
</tbody>
</table>

Table 7.2: Primer sequences for GNL3 sequencing

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200μM each dNTP</td>
</tr>
<tr>
<td>Q solution</td>
<td>1X</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.625U/reaction</td>
</tr>
<tr>
<td>DNA</td>
<td>25ng</td>
</tr>
</tbody>
</table>

Table 7.3: PCR reagent concentrations for Qiagen® Taq DNA polymerase kit with a final reaction volume of 25μl dH₂O, distilled water; dNTP, deoxyribonucleotide triphosphate.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>4min</td>
</tr>
<tr>
<td>94</td>
<td>30sec</td>
</tr>
<tr>
<td>50-61³</td>
<td>1min</td>
</tr>
<tr>
<td>72</td>
<td>1min</td>
</tr>
<tr>
<td>72</td>
<td>8min</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

X 35 cycles

Table 7.4: Thermocycling conditions for amplification of DNA using Qiagen® Taq DNA polymerase kit with heated lid at 100°C. Refer to Appendix Table 7.1 for annealing temperatures.
### Table 7.5: Reagent Concentrations for LongRange 2Step RT-PCR kit (Qiagen®) cDNA synthesis, with a final reaction volume of 20µl

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>LongRange Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1mM each dNTP</td>
</tr>
<tr>
<td>Oligo(dT)₂₁</td>
<td>1µM</td>
</tr>
<tr>
<td>LongRange RNase Inhibitor</td>
<td>0.04U/µl</td>
</tr>
<tr>
<td>LongRange Reverse Transcriptase</td>
<td>1X</td>
</tr>
<tr>
<td>RNA</td>
<td>2-4µg</td>
</tr>
</tbody>
</table>

### Table 7.6: Reagent Concentrations for MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) kit (Mix A) with a final reaction volume of 10µl

<table>
<thead>
<tr>
<th>Reagents (Mix A)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>20ng</td>
</tr>
<tr>
<td>Oligo(dT)₂₁</td>
<td>1.5µM</td>
</tr>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Table 7.7: Reagent Concentrations for MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre®) kit (Mix B) with a final reaction volume of 20µl (including 10µl Mix A). DTT, dithiothreitol.

<table>
<thead>
<tr>
<th>Reagents (Mix B)</th>
<th>Final Concentration</th>
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</thead>
<tbody>
<tr>
<td>MMLV Reaction Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>DTT</td>
<td>10mM</td>
</tr>
<tr>
<td>dNTPs</td>
<td>100µM each dNTP</td>
</tr>
<tr>
<td>RiboGuard RNase Inhibitor</td>
<td>1U/µl</td>
</tr>
<tr>
<td>MMLV Reverse Transcriptase</td>
<td>1.25U/µl</td>
</tr>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
</tbody>
</table>
### Table 7.8: Primer sequences for amplification of cDNA using Qiagen® LongRange2Step RT-PCR Kit

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward [5'-3' +]</th>
<th>Reverse [5'-3' -]</th>
<th>Product size (bp)</th>
<th>Anneal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNL3</td>
<td>ATGACCTGCCATAAGCGGTA</td>
<td>AGCATCATCCTCTTCAATGATTT</td>
<td>1587</td>
<td>53</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACCACAGTCCATGCCATCAC</td>
<td>TCCACCACCCTGTGTGCTGTA</td>
<td>451</td>
<td>53</td>
</tr>
</tbody>
</table>

### Table 7.9: PCR reagent concentrations for amplification of cDNA, using LongRange 2Step RT-PCR kit (Qiagen®), with a final reaction volume of 25μl MgCl₂ magnesium chloride.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>LongRange Buffer (with MgCl₂)</td>
<td>1X, 2.5mM MgCl₂</td>
</tr>
<tr>
<td>dNTPs</td>
<td>500μM each dNTP</td>
</tr>
<tr>
<td>Q solution</td>
<td>1X</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.4μM</td>
</tr>
<tr>
<td>LongRange Enzyme Mix</td>
<td>1U/reaction</td>
</tr>
<tr>
<td>cDNA</td>
<td>500ng</td>
</tr>
</tbody>
</table>

### Table 7.10: Thermocycling conditions for amplification of cDNA, using LongRange 2Step RT-PCR kit (Qiagen®), with a heated lid at 111°C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
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<tbody>
<tr>
<td>93</td>
<td>3min</td>
</tr>
<tr>
<td>93</td>
<td>15sec</td>
</tr>
<tr>
<td>53</td>
<td>30sec</td>
</tr>
<tr>
<td>68</td>
<td>1min 30sec</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

X 35 cycles
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward [5'-3' +]</th>
<th>Reverse [5'-3' -]</th>
<th>Product Size (bp)</th>
<th>Anneal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GNL3</strong></td>
<td>GCATGACCTGCCATAAGCGG</td>
<td>GCCTGTCAAGTTTCTGCTGC</td>
<td>208</td>
<td>50</td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td>CATGTACGTTGCTATCCAGGC</td>
<td>CTCCTTAATGTCACGCACGAT</td>
<td>250</td>
<td>60</td>
</tr>
<tr>
<td><strong>COLII</strong></td>
<td>AGGAGGCTGGCAGCTGTGTGC</td>
<td>CACTGGCAGTGCGAGGTCAG</td>
<td>207</td>
<td>60</td>
</tr>
<tr>
<td><strong>ACAN</strong></td>
<td>TGAGGAGGGCTGGAACAAGTACC</td>
<td>GGAGGTGGAATTTGCAAGGAACA</td>
<td>346</td>
<td>60</td>
</tr>
<tr>
<td><strong>RUNX2</strong></td>
<td>CAGACCAGCAGCACTCCATA</td>
<td>CAGCGTCAACACCATCATCCTC</td>
<td>177</td>
<td>60</td>
</tr>
<tr>
<td><strong>SOX9</strong></td>
<td>GCGGAGGAAATGTCGGTAAGA</td>
<td>TTGAGATGACGTGCTGCT</td>
<td>323</td>
<td>60</td>
</tr>
<tr>
<td><strong>MMP3</strong></td>
<td>AGATGATATAATAAGGACATTCAG</td>
<td>CTCCAACGTGGAAGATCCAG</td>
<td>61</td>
<td>60</td>
</tr>
<tr>
<td><strong>MMP13</strong></td>
<td>GGCGCGGAAATGCGTCTTCTT</td>
<td>ATCAAAATGGGTAGAAAGTCGCCAATGC</td>
<td>347</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 7.11: Primer sequences used to assess the expression of GNL3, β-Actin and chondrogenic markers in human cDNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward [5'-3' +]</th>
<th>Reverse [5'-3' -]</th>
<th>Product Size (bp)</th>
<th>Anneal Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gnl3</strong></td>
<td>Murine</td>
<td>TCGTGGTGATCCCGAGACC</td>
<td>GGTCATACGTCTCTCCTTCTT</td>
<td>91</td>
<td>54.7</td>
</tr>
<tr>
<td><strong>Gapdh</strong></td>
<td></td>
<td>ACGACCCCTTCATTGACC</td>
<td>TAGACTCCACGACATACCTGACGA</td>
<td>195</td>
<td>54.7</td>
</tr>
<tr>
<td><strong>GNL3</strong></td>
<td>Canine</td>
<td>GGAAGAAGGCTTGGGAGAAG</td>
<td>TTGGTGGTGCAGGGTGACA</td>
<td>195</td>
<td>54.7</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td></td>
<td>GCCATCAATGACCCCTTCAT</td>
<td>GCATCAGGAGGAGCAGA</td>
<td>290</td>
<td>54.7</td>
</tr>
<tr>
<td><strong>GNL3</strong></td>
<td>Bovine</td>
<td>TGAAGGCCTCAGATGGTGTGC</td>
<td>GACATGCTTCCCTCCACAT</td>
<td>388</td>
<td>54.7</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td></td>
<td>CATGACCACCTTGGCATCGT</td>
<td>GCCAGGGTGATCCACAAACA</td>
<td>250</td>
<td>54.7</td>
</tr>
</tbody>
</table>

Table 7.12: Primer sequences used to assess the expression of GNL3 and GAPDH in cDNA from murine, canine and bovine cartilage
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>Buffer</td>
<td>1x</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200μM each</td>
</tr>
<tr>
<td>Q solution</td>
<td>1x</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Taq</td>
<td>2.5U</td>
</tr>
<tr>
<td>cDNA</td>
<td>40ng-10μg</td>
</tr>
</tbody>
</table>

Table 7.13: PCR reagent concentrations for amplification of cDNA, using Taq DNA Polymerase kit (Qiagen®), with a final reaction volume of 25μl

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>3min</td>
</tr>
<tr>
<td>94</td>
<td>50sec</td>
</tr>
<tr>
<td>50-60°</td>
<td>45sec</td>
</tr>
<tr>
<td>72</td>
<td>1min</td>
</tr>
<tr>
<td>72</td>
<td>10min</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

X 35 cycles

Table 7.14: Thermocycling conditions for amplification of cDNA, using Taq DNA Polymerase Kit (Qiagen®), with a heated lid at 100°C. Refer to Appendix Table 7.11 and Appendix Table 7.12 for the annealing temperatures.
INVESTIGATING THE ROLE OF GNL3 IN OSTEOARTHRITIS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward [5'-3' +]</th>
<th>Reverse [5'-3' -]</th>
<th>Product size (bp)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNL3</td>
<td>GTGGTCTGAGTGGACAGGTG</td>
<td>AGCCGCTTTTTCATGTCTACC</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.15: Primers and probe (Universal ProbeLibrary (Roche Diagnostics)) for GNL3 qPCR

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>SensiFAST™</td>
<td>1x</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.4μM</td>
</tr>
<tr>
<td>Probe 63</td>
<td>0.1μM</td>
</tr>
<tr>
<td>cDNA</td>
<td>900ng</td>
</tr>
</tbody>
</table>

Table 7.16: qPCR reagent concentrations for amplification of GNL3, with a final reaction volume of 20μl

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>10min</td>
</tr>
<tr>
<td>95</td>
<td>15sec</td>
</tr>
<tr>
<td>55 (GNL3)/60 (18S)</td>
<td>30sec</td>
</tr>
<tr>
<td>72</td>
<td>15sec</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

X 40 cycles

Table 7.18: Thermocycling conditions for qPCR with 100°C heated lid

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-Free H₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>SensiFAST™</td>
<td>1x</td>
</tr>
<tr>
<td>Probe-Primer Mix</td>
<td>1x</td>
</tr>
<tr>
<td>cDNA</td>
<td>900ng</td>
</tr>
</tbody>
</table>

Table 7.17: qPCR reagent concentrations for amplification of 18S, with a final reaction volume of 20μl
### Reagents and Final Concentration

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>N/A</td>
</tr>
<tr>
<td>HF Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200μM each dNTP</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Phusion® DNA</td>
<td>1U</td>
</tr>
<tr>
<td>DNA</td>
<td>see text</td>
</tr>
</tbody>
</table>

Table 7.19: PCR reagent concentrations for Phusion® High Fidelity DNA Polymerase (New England BioLabs®) with a final reaction volume of 50μl. Please refer to Section 4.2.3.3.2 for DNA concentration.

### Thermocycling Conditions

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>30sec</td>
</tr>
<tr>
<td>98</td>
<td>10sec</td>
</tr>
<tr>
<td>69$^a$</td>
<td>30sec</td>
</tr>
<tr>
<td>72</td>
<td>1min</td>
</tr>
<tr>
<td>72</td>
<td>10min</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

X 35 cycles

Table 7.20: Thermocycling conditions for attachment of attB sites using Phusion® High Fidelity DNA Polymerase (New England BioLabs®) with heated lid at 100°C PCR machine preheated to 98°C. $^a$ No annealing step for second set of attB primers.
**Investigating the Role of GNL3 in Osteoarthritis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward [5’-3’+]</th>
<th>Reverse [5’-3’-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>GTAAACGACGGCCAG</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
</tbody>
</table>

Table 7.21: Primers for sequencing the entry clone

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward [5’-3’+]</th>
<th>Reverse [5’-3’-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEST_F</td>
<td>AATACGACTCATAGGAG</td>
<td>-</td>
</tr>
<tr>
<td>Insert_1</td>
<td>GTGATGGAAGGCCTCCGATGT</td>
<td>-</td>
</tr>
<tr>
<td>Insert_2</td>
<td>GAAAGAGGCTTTGGAAAC</td>
<td>-</td>
</tr>
<tr>
<td>Insert_3</td>
<td>GAGTGGACAGGTCCTCATT</td>
<td>-</td>
</tr>
<tr>
<td>DEST_R</td>
<td>-</td>
<td>AATCTCGCCGATCTAAT</td>
</tr>
</tbody>
</table>

Table 7.22: Primers for sequencing the expression clone

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward [5’-3’+]</th>
<th>Reverse [5’-3’-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>V367M</td>
<td>CTGAATTTTTTACT[AG]GTCTTCAGAGAAGG</td>
<td>CCTCTTATCTGACGAGCAAGC[AG]GTTAAAAATCCAG</td>
</tr>
</tbody>
</table>

Table 7.23: Primers for site directed mutagenesis of the GNL3 insert Altered allele in []

**Appendix**

260
## Investigating the Role of GNL3 in Osteoarthritis

### Reagents and Conditions

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>N/A</td>
<td>95</td>
<td>1min</td>
</tr>
<tr>
<td><em>Pfu</em> Master Mix</td>
<td>1x</td>
<td>95</td>
<td>50sec</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.52μM</td>
<td>55</td>
<td>1min</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.52μM</td>
<td>68</td>
<td>18min</td>
</tr>
<tr>
<td>DMSO</td>
<td>2.5%</td>
<td>68</td>
<td>30min</td>
</tr>
<tr>
<td>cDNA</td>
<td>50ng</td>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Table 7.24: Site-directed mutagenesis PCR reagent concentrations for a 25μl reaction

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>X 15 cycles</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>1min</td>
</tr>
<tr>
<td>95</td>
<td>50sec</td>
</tr>
<tr>
<td>55</td>
<td>1min</td>
</tr>
<tr>
<td>68</td>
<td>18min</td>
</tr>
<tr>
<td>68</td>
<td>30min</td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

Table 7.25: Thermocycling conditions for site-directed mutagenesis with 100°C heated lid. Block preheated to 98°C.
7.2 Solutions

7.2.1 Orange-G Loading Dye

7.2.1.1 10X
0.2g Orange-G (Sigma®) + 7ml dH₂O + 3ml 100% glycerol (BDH)

7.2.1.2 1X
2ml Orange-G stock (10X) (Sigma®) (Appendix Solution 7.2.1.1) in 30% glycerol (BDH)

7.2.2 DNA Wash Solution
0.1 M trisodium citrate (BDH) in 10% ethanol (Sigma®) in sterile water (Gibco®)

7.2.3 NaOH
8mM NaOH (Fisher Scientific) in sterile water (Gibco®)

7.2.4 Hepes
1M hepes (Sigma®) in sterile water (Gibco®)

7.2.5 EDTA
0.5M EDTA (Sigma®) in sterile water (Gibco®)

7.2.6 Loading Buffer

7.2.6.1 5X
5.2ml 1M tris-hydrogen chloride (HCl) (pH 6.8) (Sigma®) + 1g DL-DTT (Sigma®) + 1.3g sodium dodecyl sulphate (SDS) (Sigma®) + 6.5ml glycerol (BDH) + 130μl of 10% (w/v) bromophenol blue (Sigma®)

7.2.7 Transfer Buffer

7.2.7.1 1X
25mM tris-base (Sigma®) + 192mM glycine (Sigma®) + 20% (v/v) methanol (Sigma®) + 0.037% (w/v) SDS (Sigma®) in dH₂O
7.2.8 TBS

7.2.8.1 **20X**
1M of tris-base (Sigma®) + 1M tris-HCl (Sigma®) (adjust pH to 7.9) then add 3M sodium chloride (NaCl) (Sigma®)

7.2.8.2 **1X**
50ml TBS stock (20X) (Sigma®) (**Appendix Solution 7.2.8.1**) + 950ml dH$_2$O

7.2.8.3 **TBS-T**
0.1% (v/v) tween 20 (Sigma®) in TBS (1X) (Sigma®) (**Appendix Solution 7.2.8.2**)

7.2.9 Stripping Buffer
1mM DTT (Sigma®) + 2% (w/v) SDS (Sigma®) + 62.5mM tris-HCl (Sigma®) (pH 6.7)

7.2.10 **StemPro® Chondrogenic Media**
90ml StemPro® osteocyte/chondrocyte differentiation basal medium (Gibco®) + 10ml StemPro® chondrogenesis supplement (Gibco®) + 10000U penicillin-streptomycin (Gibco®)

7.2.11 **RIPA Lysis Buffer**
1% triton X-100 (Sigma®) + 1% (w/v) sodium deoxycholate (Sigma®) + 0.1% (w/v) SDS (Sigma®) + 0.01M sodium phosphate (pH 7.2) + 0.15 M NaCl (Sigma®) + 1 protease inhibitor tablet (Roche) in dH$_2$O