# CHAPTER 2

# Materials and Methods

## 2.1 Materials

### 2.1.1 Cells and cell culture

Human osteosarcoma cell lines MG63 and Saos-2 (derived osteosarcomas of a 14yr old male and 11 yr old female respectively, characterised in Pautke *et al.,* 2004) and the mouse pre-osteoblastic cell line MC3T3-E1 were purchased from the European Collection of Cell Cultures (ECACC), Wiltshire, UK. Human foetal osteoblast (hFOB) cell line was purchased from the American Tissue Culture Collection (USA). Human skin fibroblast cell line 149BR was a kind gift from Dr. Eustace Johnson, Oswestry, UK. Human bone marrow stromal cells (hBMSC) were obtained from bone samples taken at surgery from consenting patients with appropriate ethical approval. Sterile IWAKI tissue culture flasks (25cm$^2$), 96- and 24-well plates were purchased from Bibby Sterilin, Staffordshire, UK. Alpha minimum essential medium (αMEM) supplemented with ribonucleotides and deoxyribonucleotides, Dulbecco's modified Eagle medium: F-12 Nutrient Mixture (DMEM/F12) and L-glutamine solution (x100) were purchased from Invitrogen, Paisley, UK. Antibiotic antimycotic solution containing Penicillin, Streptomycin and Amphotericin B, Trypsin-EDTA (0.1% porcine trypsin, 0.04% EDTA), propidium iodide powder, Alizarin Red-S and bovine serum albumin (BSA) were purchased from Sigma Diagnostics, Dorset, UK. Heat inactivated bovine foetal calf serum (FCS) was purchased from PAA Laboratories, Somerset, UK. All other reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

### 2.1.2 Reagents

BMP-2, BMP-7, activin A, IL-6, insulin, serotonin, NPS2390, Triton-X 100, bradykinin and the pathway inhibitors SP600125, SB202190, U0126, LY294002 and BAY 11-7082 were purchased from Sigma Diagnostics, Dorset, UK. Recombinant human proteins PDGF-AA, PDGF-AB, PDGF-BB, VEGF, noggin, TNFα, TGFβ, IGFII, FGF1, HGF, SCF and sFRP1 were purchased from Peprotech (London, UK). PDGFR inhibitor AG1296, calcium ionophore ionomycin, L-type voltage gated calcium channel inhibitor nifedipine, glycogen synthase 3-kinase inhibitor BIO and the PKC inhibitors bisindolylmaleamide (BIM) and staurosporine (STS) were purchased from Merck Biosciences (Nottingham, UK).

### 2.1.3 OPG and DKK-1 enzyme-linked immunosorbant assay (ELISA)

IWAKI 96 well ELISA plates were purchased from Bibby Sterilin, Staffordshire, UK. Starstedt 96 well plates, untreated, were purchased from Starstedt, Leicester, UK. Skimmed milk, obtained from J.Sainsbury's Ltd., UK. Goat anti-human OPG antibody (Cat no. AF805), biotinylated goat anti-human OPG antibody (Cat no. BAF805), recombinant human OPG-Fc, mouse anti-human DKK-1 antibody (Cat no. MAB10962), biotinylated goat anti-human DKK-1 antibody (Cat no. BAF1096) and recombinant human DKK-1 were purchased from R&D Systems, Oxon, UK. Extravidin peroxidise conjugate and Tween 20 were purchased from Sigma Diagnostics, Dorset, UK. SureBlue TMB 1 component micro well substrate was purchased from Insight Biotechnology Ltd., London, UK.

### 2.1.4 RNA extraction and real-time polymerase chain reaction (RT-PCR)

RNeasy® Mini kit, and SYBR green primers for human *Wnt1, Wnt2a, Wnt3a, Wnt4, Wnt5a, Wnt7b, Wnt10a, Wnt11, Wnt16, DKK-1* and *GAPDH* were purchased from Qiagen, Sussex, UK. High capacity cDNA archive kit, Taqman® Universal PCR mastermix, SYBR green PCR mastermix, Assays on Demand© Taqman® primers for human *OPG, RANKL* and *GAPDH* genes, 96 well optiMAX plate and adhesive lid sealers were from Applied Biosystems, Warrington, UK.

### 2.1.5 Immunolocalisation of DKK-1 and β-catenin

Rabbit anti-human DKK-1 antibody (Cat no. Ab61034) and rabbit anti-human β-catenin antibody (Cat no. Ab6302) were purchased from Abcam, Cambridge, UK. Universal LSAB™+ Kit/HRP, Rabbit/Mouse/Goat detection kit (Cat no. K0690) was purchased from Dako, Cambridge, UK.

### 2.1.6 Equipment

Bio-Tek Synergy HT plate reader obtained from Bio-Tek® Instruments, Inc., Vermont, USA. Harrier 18/80 refrigerated centrifuge obtained from Sanyo Biomedical Division, Leicestershire, UK. Real-Time PCR machine, 7500 obtained from Applied Biosystems, Warrington, UK.

## 2.2 Methods

### 2.2.1 Cell culture

Harvested bone marrow tissue was washed in sterile phosphate buffered saline (PBS) and any periosteum removed. The sample was then enzymatically digested using 0.8mg/ml Collagenase XI in serum free medium for 6 hours then filtered through a 70µm nylon filter. The filtrate obtained was centrifuged at 150g, 22˚C for 10 minutes, the supernatant discarded and the bone marrow stromal cell pellet re-suspended in fresh DMEM/F12 supplemented with10% FCS, streptomycin (100µg/ml), penicillin (100U/ml), amphotericin B (250ng/ml). Cells were seeded into tissue culture flasks and maintained at 37˚C in a humidified atmosphere with 5% carbon dioxide in air. Culture medium was changed every 3 to 4 days and any non-adherent cells were removed. Adherant hMSC were passaged on reaching 70% confluence. Cells were detached from the flask using Trypsin-EDTA solution for 5 minutes. Once detached, the cells were transferred to a centrifuge tube containing 1ml full media and spun for 5 minutes at 150g. The supernatant was removed and the cell pellet re-suspended in 2ml full media. 20µl of cell suspension transferred to a 2ml tube containing 20µl of 4% trypan blue in PBS. Trypan blue will enter cells with a damaged membrane and therefore stains them blue. Blue cells indicate non-viable cells and are excluded from the cell count. 10µl of cells in trypan blue mixture was placed in a haemocytometer and the viable (unstained) cells were counted. Cells were counted 4 times and an average taken. hMSC were then seeded into a 96 well plate at a density of $1x10^4$ cells/well in 200µl DMEM/F12 medium with above additions and maintained as above until confluent ready for experiments to commence.

Both MG63 and Saos-2 cells were routinely passaged once weekly and cultured in sterile plastic IWAKI tissue culture flasks ($25cm^2$) with filtered caps in αMEM supplemented with 10% FCS, streptomycin (100µg/ml), penicillin (100U/ml), amphotericin B (250ng/ml) and L-glutamine (2mM), termed complete medium from here on. Cells were maintained at 37˚C in a humidified environment containing 5% carbon dioxide in air until confluent, usually after 3 or 4 days. Once confluent, cells were passaged as above and reseeded into sterile IWAKI 96 well tissue culture plates at a density of $7x10^3$ cells/well in 200µl αMEM complete medium and maintained as above until confluent. For experiments requiring sub-confluent cells, cells were reseeded at a density of $5x10^3$ cells/well in 200µl αMEM complete medium and maintained as above until cells had adhered. Culture medium was changed prior to all experiments. For experiments requiring FCS-free medium,

αMEM was supplemented as above, but 10% FCS was omitted and substituted for 0.2% BSA to maintain a high protein environment within the culture medium. For differentiation assays, culture medium was supplemented with ascorbic acid (50µg/ml), β-glycerophosphate (10mM) and dexamethasone (10nM).

### 2.2.2 Cell number – propidium iodide assay

The propidium iodide (PI) assay was used to determine both necrosis and cell number. PI enters cells with a damaged membrane and becomes incorporated into the cell's DNA where it can be detected by fluorescence at 530/645nm. Culture medium was then removed from each well and stored at -20˚C for future analysis. Cells were washed with 100µl/well sterile PBS prior to addition of 100µl/well of 5µg/ml PI solution and incubated at room temperature in the dark for 20 minutes. To assess apoptosis and/or necrosis after treatment, cells were first observed under fluorescent microscopy. Fluorescence intensity was then determined (excitation 530nm, emission 645nm) on a Bio-Tek Synergy HT plate reader in order to measure necrosis. The PI solution was discarded prior to fixing with 100µl/well of 70% ethanol for 5 minutes. The ethanol was discarded and 100µl/well of 5µg/ml PI solution was added and incubated at room temperature in the dark for a further 20 minutes. Fluorescence intensity was determined as before as a measure of cell number. Fluorescence intensity was converted to cell number using a calibration curve and necrosis expressed as a percentage of total cell number.

### 2.2.3 Alizarin red staining

Mineralised calcium deposits were stained using Alizarin Red-S. Cells were washed 3 times in 100µl/well cold sterile PBS then fixed in 100µl/well 70% ethanol for 10 minutes. Alizarin Red-S solution (pH 4.2) was added to wells (100µl/well) and incubated for 1 hour. Cells were then rinsed extensively with tap water to remove excess dye. Mineralised nodules stained dark red and were photographed using a Nikon Diaphot inverted microscope at a magnification of x200.

### 2.2.4 Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured in cells to assess osteoblastic differentiation. Culture medium was removed and Triton X-100 (50µl/well of 0.1%) was added to cells and incubated at 37˚C for 15 minutes.

Alkaline phosphatase reagent was added (150µl/well of 250mM sodium bicarbonate pH10, 2mM magnesium chloride and 20mM para-nitrophenol phosphate) and incubated at 37˚C for 1 hour. Optical density was determined by reading the plate at 405nm using 650nm as a reference wavelength on a Bio-Tek Synergy HT plate reader.

### 2.2.5 RNA extraction and RT-PCR

#### 2.2.5.1 RNA extraction

MG63 cells were cultured as above but in 24-well plates. Effectors were added or removed depending on experimental design and cells were incubated for 24 hours. Medium was removed from the wells and stored at -20˚C for future analysis. The wells were washed 3 times in 500µl cold sterile PBS. Total RNA was then extracted using the Quiagen RNeasy® kit according to the manufacturer's instructions. In brief, a buffer containing guanidine isothiocyanate was added to each well to lyse the cells and release the RNA whilst protecting it from and RNAses (RNA specific enzymes that will cleave RNA). Ethanol was then added to this buffer and the mixture agitated to dissolve up the RNA. 700µl of this mixture was then transferred to the supplied Quiagen spin column. The presence of the ethanol will promote the binding of the RNA in the sample to the silica gel membrane inside the column. The RNA and membrane was then washed by centrifugation with a series of buffers to leave purified RNA on the membrane. The RNA was then eluted from the spin column using 30µl RNAse free water and a final spin in the centrifuge.

#### 2.2.5.2 Reverse transcription

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe the sample RNA to cDNA according to the manufacturer's instructions. In brief, 5µl of reverse transcriptase buffer, 2µl of dNTP mix, 5µl of random primers, 2.5µl of multiscribe reverse transcriptase and 10.5µl of nuclease free water was added to 25µl of RNA sample and incubated at 25˚C for 10 minutes followed by 37˚C for 2 hours.

### 2.2.5.3 Quantitative / real-time PCR

Quantitative PCR was performed using pre-designed primers as described above. Unlike standard PCR, the Taqman primers used for quantitative or real-time PCR, are oligonucleotides that have a fluorescent probe at the 5' end and a quencher at the 3' end. Unhybridised, the fluorescent molecules are too close to the quencher molecules to be detected. Once the primer has bound to its complimentary cDNA and DNA-dependent DNA polymerase begins the polymerisation, the fluorescent probe is cleaved from the quencher and allows the probe to fluoresce. This fluorescence can be measured each PCR cycle. If the amplification is proceeding at about 100% efficiency which is a requirement for quantitation, then fluorescent intensity will double each cycle. The more target DNA is present, the fewer PCR cycles are needed for the fluorescence to be significantly increased above a specific threshold value. The fractional number of cycles is termed the cycle threshold (CT) value.

As an alternative to Taqman probe based RT-PCR assays, SYBR green based assays were also used. Forward and reverse primers are used which do not have fluorescent probes or quenchers. SYBR green dye intercalates non-specifically with the bases of double stranded DNA and in this state its fluorescence is increased dramatically. However, because the fluorescence is not specific for the nucleotide sequence as are the Taqman probes, it is important that when using SYBR green based assays the homogeneity of the product is verified. The advantage of SYBR green based assays is its cheapness compared to Taqman probe assays. In order to verify the homogeneity and melting point of the product using SYBR Green, at the end of the RT-PCR run, PCR products are heated until they dissociate and the fluorescence is monitored during this process. A homogenious DNA product will show a sharp drop in fluorescence at a temperature characteristic of the number of bases in the product but also the content of the bases. This is shown by a single sharp peak when the first derivative of fluorescence with temperature is plotted.

In brief, 2µl of cDNA from the reverse transcription reaction, 12.5µl SYBR green master mix / Taqman® universal PCR mastermix (depending on primer used), 1.25µl specific primer and 10.25µl nuclease free water were added to each well of an optiMAX plate. The plate was sealed using a plate sealer and run as a quantitative reaction on an Applied Biosystems 7500 real-time thermal cycler. Target gene expression was compared to the housekeeper gene *GAPDH* and

expressed as a ratio using the CT equation, known as the comparative threshold method (Livak and Schmittgen, 2001):

1) Calculate delta CT (δCT)

   δCT = Target CT – Housekeeper control CT

2) Calculate delta delta CT (δδCT)

   δδCT = Target δCT – (δCT not treated/vehicle control)

3) Calculate gene expression

   Gene expression = $2^{-\delta\delta CT}$

### 2.2.6 Human OPG ELISA

This type of ELISA is known as a sandwich ELISA as the protein of interest is "sandwiched" between a primary capture antibody and a secondary detection antibody. An IWAKI 96 well ELISA plate was coated overnight at 4˚C with 100µl/well of 200ng/ml goat anti-human OPG antibody diluted in 0.1M sodium bicarbonate pH 8.6. Non-specific binding was blocked with 200µl/well 8% milk (8% v/v liquid skimmed milk in 0.1M Tris HCl, pH 7.4) for 1 hour at 4˚C. Standard concentrations of recombinant OPG-Fc (100µl of 0, 1, 2, and 4ng/ml in 8% milk) were added to duplicate wells. Samples were also diluted in 8% milk until they were within the linear range of the standards. After blocking, the plate was washed 3 times for 2 minutes with 0.2% Tween 20 in 0.1M Tris HCl buffer pH 7.4. Samples and standards were incubated for 1 hour at 4˚C. The ELISA plate was washed again as above before the addition of 100µl/well of 200ng/ml biotinylated goat anti-human OPG antibody diluted in 8% milk and the plate incubated for 1 hour at 4˚C. The plate was washed as before prior to the addition of 100µl/well of 2µg/ml Extravidin peroxidase (diluted in 8% milk) and incubated for 1 hour at 4˚C. The plate was then washed as before prior to the addition of 100µl SureBlue TMB 1 component micro well substrate (3,3'5,5' tetramethylbenzidine) and incubated for 30 minutes at room temperature to allow the blue colour to develop. The reaction was stopped by the addition of 100µl/well of 10% sulphuric acid. Optical densities of each well were determined at 450nm using 570nm as a reference wavelength on a Bio-Tek Synergy HT plate reader. OPG concentration was calculated by linear interpolation from a standard curve and corrected for dilution and cell number.

### 2.2.7 Human DKK-1 ELISA

An IWAKI 96 well ELISA plate was coated overnight at 4˚C with 100µl/well of 1.8µg/ml mouse anti-human DKK-1 antibody diluted in PBS. Non specific binding was blocked by incubating the plate with 200µl/well 8% by volume of liquid skimmed milk (diluted in 0.1M Tris HCl buffer pH 7.4, described as 8% milk from here on) for 2 hours at 4˚C. Standard concentrations of recombinant DKK-1 (0-, 1-, 2-, and 4ng/ml diluted in 8% milk) were added to duplicate wells. Samples were also diluted in 8% milk until they were within range of the standards. After blocking, the plate was washed 3 times for 2 minutes each time with 0.05% Tween 20 in 0.1M Tris HCl buffer pH 7.4 (described as washing from now on). Samples and standards were then added to the plate and incubated for 2 hours at 4˚C. The ELISA plate was washed again (x3) before the addition of 100µl/well of 25ng/ml biotinylated goat anti-human DKK-1 antibody (diluted in 0.2% BSA in PBS) and the plate incubated for one hour at 4˚C. The plate was washed again (x3) and 100µl/well of 2µg/ml extravidin peroxidase (diluted in 0.2% BSA in PBS) and incubated for 1 hour at 4˚C. The plate was then washed for the final time (x3) before the addition of 100µl/well SureBlue TMB 1 component micro well substrate (3,3'5,5' tetramethylbenzidine) and incubated for 30 minutes at room temperature to allow the blue colour to develop. The TMB reaction was then stopped by the addition of 100µl/well of 10% sulphuric acid. Optical densities of each well were then determined by reading the plate at 450nm using 570 nm as a reference wavelength on a Bio-Tek Synergy HT plate reader.

### 2.2.8 Serum alkaline phosphatase

This assay was performed by laboratory staff at RJAH Orthopaedic Hospital Pathology Department. Total serum alkaline phosphatase (tsALP) was measured on a sample of the serum as part of the routine biochemistry by enzymatic assay (Kodak Ektachem analyzer). Laboratory reference ranges used were females >50years 36-112IU/L and males >50years 43-114IU/L.

### 2.2.9 Formalin-fixed paraffin-embedded (FFPE) tissue samples and immunohistochemistry

#### 2.2.9.1 Preparation of FFPE tissue samples

Preparation performed by laboratory staff at RJAH Orthopaedic Hospital Histopathology Department. In brief, fracture callus and pagetic lesion tissue samples were collected at autopsy or biopsy from consented patients with appropriate ethical approval. The tissue samples were preserved by fixation in 10% neutral buffered formalin (4% formaldehyde in PBS). Tissue samples were then dehydrated by transferring through baths of progressively more concentrated ethanol with a final bath of xylene to remove the alcohol. Dehydrated sections were immersed in a bath of molten paraffin wax and cooled to embed the sample. Embedded samples were stored at room temperature until required.

#### 2.2.9.2 Immunohistochemical localisation of DKK-1 and β-catenin

Assay performed by laboratory staff at RJAH Orthopaedic Hospital Histopathology Department. In brief, 5µM thick sections were cut from each FFPE block using a microtome, floated on water and mounted onto poly-L-lysine-coated glass slides. Slides were then warmed to 60˚C to melt the wax, immersed in xylene to wash off the wax and rehydrated though baths of progressively less concentrated ethanol until the final bath is completely aqueous. Antigen retrieval of the sections was achieved by steaming the slides in 0.01 M sodium citrate buffer, pH 6.0 at 99-100°C for 20 minutes, cooling at room temperature and rinsing with tris-buffered saline (TBS) containing 0.2% Tween. Slides were washed again with TBS containing 0.025% Triton X-100 prior to blocking endogenous binding with 1% BSA in TBS for 2 hours at room temperature. Primary antibody diluted in 1% BSA in TBS (DKK-1 antibody 10µg/ml, β-catenin antibody 1/200) was added and slides incubated overnight at 4˚C. Slides were washed twice prior to addition of biotinylated anti-rabbit immunoglobulins for 15 minutes. After a further two washes, streptavidin peroxidase was added for 15 minutes. The slides were washed again and 3,3’ diaminobenzidine (DAB) chromogen solution added for 15 minutes. Excess DAB-chromogen solution was washed off with distilled water. Slides were then counterstained with Haemotoxylin to aid visualisation of tissue sections and mounted with a glass cover-slip. Slides were then examined under transmitted light using a Leitz Dialux microscope at x200 and x400 magnification.

### 2.2.10 Statistical Analysis

Results presented in this thesis are representative of at least 3 separate experiments unless otherwise stated in the legend. All data are represented as the mean ± the standard deviation (SD) of at least 3 replicates within one experiment unless otherwise stated in the legend. All data were analysed using GraphPad Prism® Version 5.01 and Microsoft Excel. Statistical significance of results in chapters 3-6 was determined using the Student's two-tailed unpaired t-test and one-way analysis of variance (ANOVA) using Dunnett's multiple comparison test where appropriate unless otherwise stated. A probability (P) of less than 0.05 was considered statistically significant. In chapter 7, the data was assessed for normality using the Kolmogorov–Smirnov test and outliers were tested for with Grubb's test. Statistical significance of results was determined using ANOVA with Tukey's multiple comparison test. $P<0.05$ was considered statistically significant.