9 Appendix

APPENDIX A

MATERIALS AND METHODS

A1: ROUTINE CELL CULTURE

A1.1: Human bone-derived cell culture

Human bone-derived cells (HBDC) were obtained using a modified enzymatic digestion technique (Gehron-Robey 1985; Bellows et al. 1986). Small pieces of bone were taken from young teenage patients during surgical operations. The selected patients did not suffer from any conditions known to affect the healing or metabolism of bone. As-received bone was trimmed of fat and soft connective tissue and then cut into small chips approximately 1 mm^3 in size and washed several times in 0.02% (w/v) trypsin (Sigma Bio-Scientific, # T-8253, USA) in calcium-free and magnesium-free PBS (Appendix B1) and then allowed to digest in this solution for 90 minutes at 37°C. Digested bone chips were washed several times in α-Minimum Essential Medium (α-MEM) (Gibco, # 12000-022, USA). HBDC obtained were osteoblast-like cells grown from explanted bone chips and then placed in 25 cm^2 tissue culture flasks (Nunc, # 3013, Denmark) containing α-MEM, 10 % (v/v) foetal bovine serum (FBS) (Gibco BRL, # 10099-141, USA), 2 mM L-glutamine (Gibco Laboratories, # 17605c, USA), 25 mM Hepes Buffer (Gibco Laboratories, USA, # 15630-080), 30 µg/ml penicillin (100 µg/ml streptomycin (Gibco Laboratories, # A393, USA) and 0.1 M L-ascorbic acid phosphate (L-ascorbic acid phosphate magnesium salt: $C_6H_6O_9 \cdot PMg_{3/2}$, Wako Pure Chemical Industries, # 013-12061, OSAKA, Japan) (Asc-2-P) in which the phosphate compound is more stable than the natural form (Hata & Seno 1989).

A1.2: Maintenance of culture

All cultures were incubated at 37°C without CO_2 and the medium was changed three times a week. HBDC were harvested at confluency and passaged using 0.1% (w/v) trypsin (Sigma Bio-Scientific, # T-8253, USA) with 0.2 mM EDTA (Boehringer-Mannheim, # 200-0081, Germany) in calcium-free and magnesium-free PBS (Appendix B1). To harvest cultures, spent culture medium was removed, and flasks rinsed with a small quantity of harvesting solution. Approximately 2 ml of fresh harvesting solution was added to each 25 cm^2 culture flask (Nunc, # 3013, Denmark), and flasks incubated at 37°C for 2 minutes when most of the cells could be freed from the culture surface by gentle tapping of the flask. The cell suspension was collected into a test-tube and immediately centrifuged at 3,000 rpm for 3 minutes at room temperature. The resulting pellet of cells was resuspended and washed in serum free medium. For sub-culturing, the cell suspension was centrifuged again as before, and the cell pellet resuspended in 1 ml of culture medium and seeded into fresh 75 cm^2 culture flask (Falcon, # 3024, USA). For experimental assays 50 μl of this cell suspension were diluted 1/10 in 0.5% (w/v) trypan blue (50 μl of cell suspension and 450 μl of trypan blue) and counted using a hemacytometer. For cell seeding the cell concentration was then adjusted to 5×10^4 cells per ml.

A2: CULTURE OF HBDC ON DIFFERENT SUBSTRATA

HBDC isolated as previously described (Appendix A1) were seeded onto the test substrata. Test materials with a diameter of 10 mm were placed into 48-well tissue culture polystyrene plates (Nunc, # 150687, Denmark) and preincubated in

500 µl of culture medium for 24 hours without cells, and the 24-h wash was then discarded. HBDC were seeded in the culture medium described above in aliquots of 445 µl/well at a concentration of $2.83x10^4$ cells/cm^2 on the different substrates. On every occasion 6 wells were seeded for each substratum at each time point. Plates were incubated at 37°C for the pre-determined time period, after which time cells were washed twice with warm α-MEM (Gibco BRL, # 1200-022, USA) to remove any serum. The final α-MEM wash was aspirated and HBDC were harvested from the surface of the substratum by adding 1 ml of 0.1% (w/v) trypsin (Sigma Bio-Scientific, # T-8253, USA) with 0.2 mM EDTA (Boehringer-Mannheim, # 200-0081, Germany) in 1 X PBS (Appendix B1) to each well, and incubating at 37°C for 2 minutes to allow the attached cells to detach from the culture surface. Disks were agitated to yield a free cellular suspension, in this way 99% of cells from the different biomaterial substrata were isolated.

A3: COMPLIMENTARY DEOXYRIBONUCLEIC ACID (cDNA) PROBES

Samples of cDNA probes were obtained from generous researchers and grown up by standard means to make sufficient quantities for experimentation. cDNA probes arrived in the host bacterium as stab cultures. The bacteria containing the plasmids are then selected, usually by an antibiotic resistance gene that the plasmid carries. The bacterial culture is grown and the plasmid isolated and purified. A summary of the cDNA probes used in this study is listed in Table A1. For a more detailed description and plasmid maps of the probes the reader needs to look up the appropriate references which have been quoted. The following cDNA probes were used in studies performed in this thesis: alkaline phosphatase (ALP), procollagen Iα1 (Col Iα1), procollagen Iα2 (Col Iα2) osteonectin (ON), osteocalcin (OC), and osteopontin (OP). β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

were a generous gift from Dr. P. Hogg, UNSW, Australia. Hybridization solution without probe was used to ensure that the endogenous biotin was completely blocked and produced no more signal than the background.

All the cDNA probes were checked against the Genebank Database (Los Alamos, NM) to ascertain that sequences were as unique as possible to the target of interest, using the Blast-Align program (Altschul et al. 1990) located at the internet site http://www.ncbi.nlm.nih.gov/BLAST/ and highest homology was found to be only 44%. All the cDNA probes used in this study (unless otherwise stated) were generously provided by Dr. W. R. Walsh, Orthopaedic Research Laboratory, University of New South Wales, Sydney, Australia.

A4: ISOLATION OF DEOXYRIBONUCLEIC ACID (cDNA) PROBES

The cDNA probes, as whole plasmids from section A3, were isolated using the Wizard™ Maxipreps DNA Purification System (Promega # A7270, Australia). Large-scale plasmid preparations, such as caesium chloride purification, can be both laborious and time consuming, often requiring an overnight centrifugation, Wizard™ Maxipreps DNA Purification System (Promega # A7270, Australia) is simple and rapid, and requires only a centrifuge and a vacuum source. This system can yield up to one milligram of high copy number plasmid DNA (200-20,000 bp) from 500 ml culture in less than three hours of isolation.

Table A1: List of cDNA probes obtained from generous researchers

cDNA probe	Name	Target mRNA	Insert size	Restriction site for insert	Vector	References
Alkaline Phosphatase	ALP	cDNA to human ALP	0.7 kb	*Eco*RI/*Bam*HI	pUC18	Dracopoli et al. 1991
Osteonectin	ON	cDNA to human ON	1.2 kb	*Eco*RI	pBSK	Villarreal et al. 1989
Osteocalcin	OC	cDNA to Human OC	1.2 kb	*Sac*I	pSP65	
Osteopontin	OP	cDNA to Human OP	1.4 kb	*Pst*I/X*ba*I	pBSK	Kiefer et al. 1989
Collagen Iα1	Col Iα1	cDNA to Human Col Iα1	1.8 kb	*Eco*RI	pBr322	
Collagen Iα2	Col Iα2	cDNA to Human Col Iα2	3.2 kb	*Eco*RI	pBr322	Myers et al. 1981
β-actin	β-actin			*Ecor*V/*Pst*I	pH34	Erba et al. 1986
glyceraldehyde -3-phosphate dehydrogenase	GAPDH	cDNA to Human GAPDH	1.1 kb	*Hin*d 3	pBSK	

A4.1: Cell culture and harvesting

For a 10-500 ml overnight culture of transformed *E. coli* the DNA plasmid yields may vary between 200 μg and 1 mg, depending on the density of the bacterial culture, the plasmid copy number and the bacterial strain. The procedure for growing up plasmids is as follows:

1. Using a heat-sterilized loop, take a sample from the stab culture containing the *E. coli* carrying the cDNA plasmids, and inoculate in a sterilized 5 ml Luria Broth (LB) (Appendix B3) suspension with appropriate antibiotics, incubate with shaking at 37°C for 4-5 hours, cloudy solution indicates the successful growth of bacteria. A sample of the *E. coli* carrying the cDNA plasmid can be stored for long-term by adding 0.5 ml glycerol to 0.5 ml sample from the above mini-culture placed at -20°C, as a stock solution.
2. Plate out a sample from the culture in step 1 containing the *E. coli* carrying the cDNA plasmids, on agar plates (Appendix B3) using a heat-sterilized loop.
3. Incubate agar plates overnight at 37°C, to allow colonies of bacteria to grow.
4. Transfer the contents from the mini-culture (step 1) to a sterilized 400 ml LB broth (Sigma, # L3022) with antibiotic (Appendix B3), and incubate overnight with shaking at 37°C.
5. Transfer suspension to a 500 ml centrifuge bottle and cool on ice for 30 minutes.
6. Centrifuge at 5,000 rpm for 10 minutes at room temperature. Decant supernatant and resuspend cell pellet in 15 ml of cell resuspension solution. This solution has RNase (ribonuclease) which destroys RNA and inhibits the metabolic activity of the cells.
7. Add 15 ml of cell lysis solution (NaOH/SDS) (Appendix B3) and mix gently, but thoroughly by stirring or inverting. Vortexing is not recommended. Cell lysis is

complete when the solution becomes clear and viscous, this could take up to 20 minutes. This solution breaks down the cell membrane to facilitate release of DNA into solution so it can be isolated and purified.

8. Add 15 ml of neutralization solution potassium-acetate (K_2Acetate) (Appendix B3) and immediately mix by gently inverting the centrifuge bottle several times.
9. Centrifuge at 5,000 rpm for 15 minutes at room temperature (Phoenix Clements 900 Orbital, Phoenix, Australia).
10. Transfer the clear supernatant, avoiding any precipitates, into a 50 ml sterile centrifuge tube.
11. Add 0.5 volumes of room temperature isopropanol and mix by inversion, the isopropanol will precipitate the DNA.
12. Centrifuge at 5,000 rpm for 15 minutes at room temperature (Phoenix Clements 900 Orbital, Phoenix, Australia). Discard the supernatant and resuspend the DNA pellet (which may not be visible) in 2 ml Tris-EDTA buffer (Appendix B3). Avoid harsh conditions that might shear some of the supercoiled plasmid as this might affect future transformations. At this stage the plasmid is of main interest as it contains the specific DNA that can be used as a probe.

A4.2: Plasmid purification

A vacuum source is required for this procedure:

1. Add 10 ml of the Wizard™ DNA Purification Resin to the DNA solution from step 12 of Section A4.1. Swirl to mix.

   NOTE: Thoroughly mix the Wizard™ Maxipreps DNA Purification Resin before

removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 25-27°C for 10 minutes. The resin itself is insoluble. It is not recommended to use the resin above 30°C.

2. For each Maxiprep use one Wizard™ Maxicolumn. Insert the Maxicolumn tip into the vacuum source.
3. Transfer the resin/DNA mix into the Maxicolumn, add 13 ml of Column Wash Solution to the bottle that had contained the DNA/resin mix. Swirl and immediately pour into the Maxicolumn.

   NOTE: Prior to using the column wash solution, it is diluted by adding 170 ml of 95% ethanol for a final volume of 295 ml. This will give a final concentration of approximately 55%.
4. Apply a vacuum to draw the wash solution through the Maxicolumn.
5. Add another 12 ml of Column Wash Solution to the Maxicolumn and apply a vacuum to draw the solution through the Maxicolumn.
6. To rinse the resin, add 5 ml of 80% ethanol to the Maxicolumn and apply a vacuum to draw the ethanol through the Maxicolumn. Allow the vacuum to draw for an additional 1 minute. Dry the resin to completion by drawing a vacuum for an additional 5 minutes. Remove the Maxicolumn from the vacuum source. Place the Maxicolumn in the provided Reservoir (50 ml screw cap tube).
7. Apply 1.5 ml of preheated (65-70°C) water to the Maxicolumn. Elute the DNA by centrifuging the Maxicolumn/reservoir at 2,500 rpm for 5 minutes in a table top centrifuge bucket rotor (Phoenix Clements 900 Orbital, Australia).
8. Remove and discard the Maxicolumn. The plasmid DNA may be stored in the screw cap tube at 4°C or -20°C.

9. Determine DNA concentration by Abs_{260} of a 5 or 10 µl sample in 1 ml water. 1 Abs_{260} = 50 µg cDNA. Adjust concentration to 1 µg/µl.

The presence of cDNA insert in plasmid was verified in all the probes by enzyme digestion of the plasmid with restriction endonucleases and agarose gel electrophoresis.

A5: PHOTOBIOTIN^{TM} LABELING OF PROBES

Photobiotin^{TM} (Bresatec, # PLK-1, K053, Australia) can be used for rapid and reliable preparation of stable, non-radioactive, biotin-labeled DNA, RNA and proteins. The photobiotin method of labeling cDNA was originally developed by Forster and colleagues (1985) at the University of Adelaide, South Australia. It was commercialized by Bresatec, Adelaide, South Australia and has been described in a textbook by Symons (1989). The photobiotin reagent (Figure A1) consists of a biotin molecule attached by a linker arm to a photoactivatable (azido) group. When a mixture of nucleic acid and photobiotin is exposed to strong visible light for 20 minutes under defined conditions, the azido group is converted to an extremely reactive nitrene which allows the formation of stable linages, to the nucleic acid. Excess photolysed photobiotin is removed by butan-1-ol extraction. An outline of the labeling procedure is given in Figure A2. The photobiotin method has been found useful in a number of applications, such as single copy detection (McInnes & Symon 1989), mRNA detection (McInnes et al. 1987) and *in situ* hybridization (ISH) (Chantratita et al. 1989). Photobiotin has also been used in protein labeling (Lacey & Grant 1987).

The procedure for Photobiotin labeling is as follows:

1. In a sterile Eppendorf tube (Beckmann, # 616-201, Austria), equal volumes of probe (20 µl, 1 µg/µl) and Photobiotin^{TM} Acetate (Bresatec, # PB31, Australia) (20 µl, 1 µg/µl) both dissolved in sterile distilled water, are combined.
2. The solution is then placed in an ice-water bath with lid open and irradiated for 20 minutes at a distance of 10 cm beneath a 250 wattlamp (Mercury vapour, Philips BHRF).
3. 60 µl of 100 mM Tris-HCL, 1.0 mM EDTA, pH 9.0 is added and mixed well.
4. 10 µl butan-2-ol is added and mixed well, centrifuged at a high speed for 1 minute, and the upper butan-2-ol phase then carefully removed and discarded.
5. The butan-2-ol extraction is repeated, the aqueous phase (colourless) is now concentrated to about 30-40 µl and unreacted photobiotin removed during the extraction.
6. 360 µl of sterile water is added to the mixture to bring the volume to 400 µl.

Figure A1: The chemical structure of Photobiotin®

(Taken from McInnes & Symons 1989)

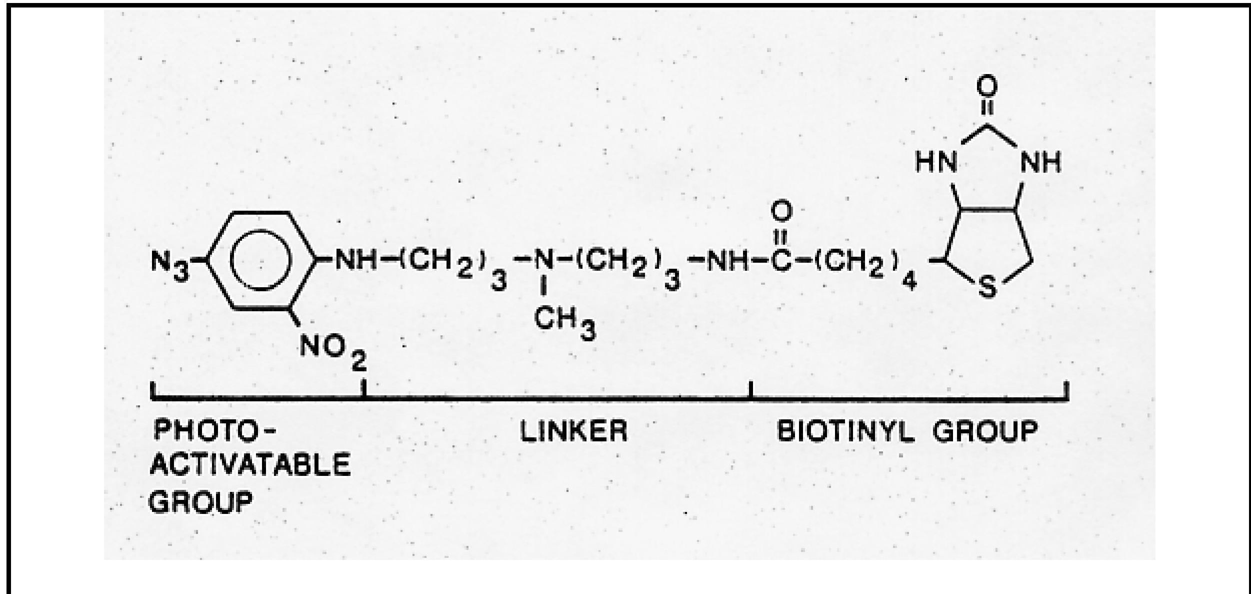
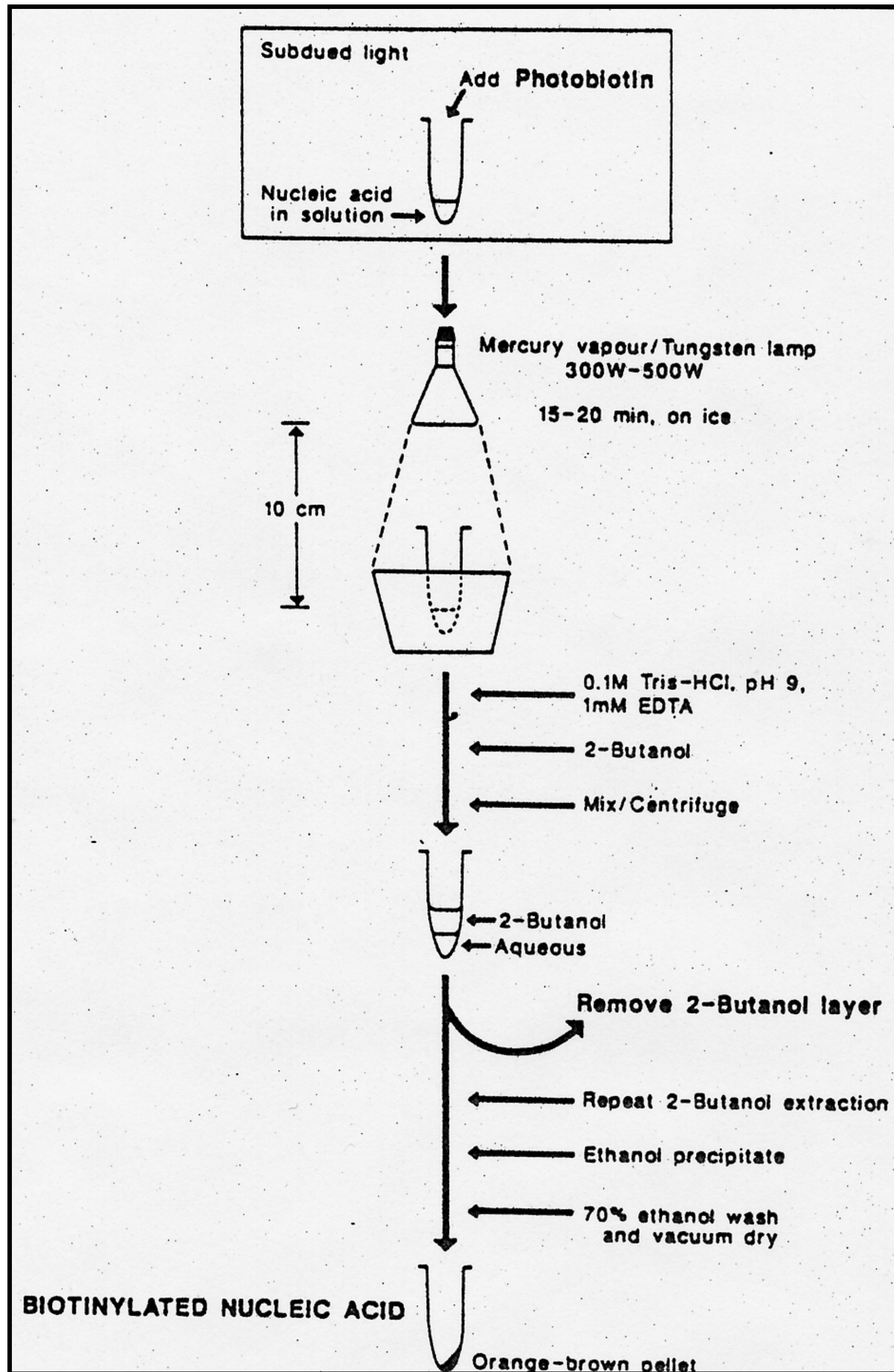


Figure A2: Schematic diagram of the chemical biotinylation procedure for labeling nucleic acids with Photobiotin®



A6: DNA *IN SITU* HYBRIDIZATION (ISH) PROCEDURE

Numerous protocols for DNA ISH utilizing both radioactive and non-radioactive labeling methods have been advised. A method of ISH was adapted and modified (Zreiqat et al. 1996) using photobiotin as the labeling procedure for cDNA probes.

400 µl of the photobiotin labeled probe (20 µg) (Appendix A5) and 50 µl of 10X SSC (Appendix B5) is placed in a sterile Eppendorf tube and heated at 90°C for 15 minutes to dissociate the double stranded DNA into single strands. The labeled probe is then mixed well by high speed vortexing for 20 seconds, chilled in ice, and then 3.6 ml of the hybridization buffer (Appendix B4) is added to each 0.4 ml of the photobiotin probe (20 µg) and mixed well, the preparation is then stored at -20°C until required. The final concentration of the hybridization mixture is 5 µg/ml probe in 5X SSC, 50% formamide (Promega, # 200-0100), 10% dextran SO_4, 10 mM EDTA (pH 7), 250 µg/µl Herring sperm DNA. 50 µl (250 ng/well) of the labeled probe in the hybridization buffer is added for each well.

A6.1: Preparation of cells for hybridization and/or immunocytochemistry

Harvested cells from Appendix A2 were washed once in full culture medium by centrifugation for 3 minutes at 4,000 rpm and resuspended in warm PBS. This was followed by two additional washes in PBS. Cells were again resuspended in PBS and cellular viability was determined using the trypan blue exclusion method as described above (Appendix A1.2). Cellular viability was found to be not less than 98%, and cells were counted with a hemocytometer. Subsequently the cellular concentration was adjusted to $1x10^5$ cells per ml PBS and 100 µl of this suspension was pipetted into each well of tissue culture polystyrene 96-well microtiter plates (Costar #-3596, USA). Plates were centrifuged at 1,200 rpm for 10 minutes in a Clements Orbital 900 Centrifuge (Phoenix Clements 900 Orbital, Phoenix, Australia). The supernatant from

each well was vacuum aspirated using a fine needle, and cells were dried to the plates for 30 minutes in a fan-forced incubator at 37° C. Plates thus dried were wrapped in plastic film and stored at room temperature ready to be used for quantitative *in situ* hybridization (QISH) and/or immunocytochemistry detection. Previous research has shown that the mRNA was preserved for several months without any major decreases in absorbance values obtained using the QISH assay (Markovic et al. 1994, Zreiqat et al. 1996). In practice, it is possible to store away plates for considerable times as internal and quality standards for the assay.

A6.2: Fixation and pre-hybridization of cells

Cell preparations had to be fixed and processed in a manner that would allow the cDNA probes to penetrate through the cell membrane and into the cytoplasm and bind to the mRNA. Cells were fixed in 10% (v/v) formalin analytical grade (Ajax-Chemicals) (Lewis et al. 1987), buffered in 1X PBS for 30 minutes.

Air-dried cells were:

1. Rinsed in two changes of 1X PBS.
2. Rinsed in two changes of 0.25% (v/v) Triton X-100 (Sigma, # T-9284, USA), 0.25% Nonidet P40 (ICN, # 155942-49, USA) for 5 minutes each to extract lipids and perforate the cell membranes for probe penetration.
3. Rinsed in two changes of 1X PBS.
4. Dipped in 20% acetic acid (in water) for 30 seconds.
5. Rinsed in Milli-Q water.
6. Immersed in 100% ethanol for 5 minutes.
7. Cells were air-dried in an incubator at 42°C.

(ii) Hybridization condition

To the air-dried cells, the appropriate 50 µl of the DNA probe in hybridization buffer was added to fill the well completely. Plates were covered with their lids and sealed using polyethylene clear plastic food wrap (Cling Wrap). The plates were placed in a humidified chamber for 22 hours at 42°C. During this process the single stranded DNA probe was able to hybridize to appropriate matching mRNA and DNA sequences in the cell.

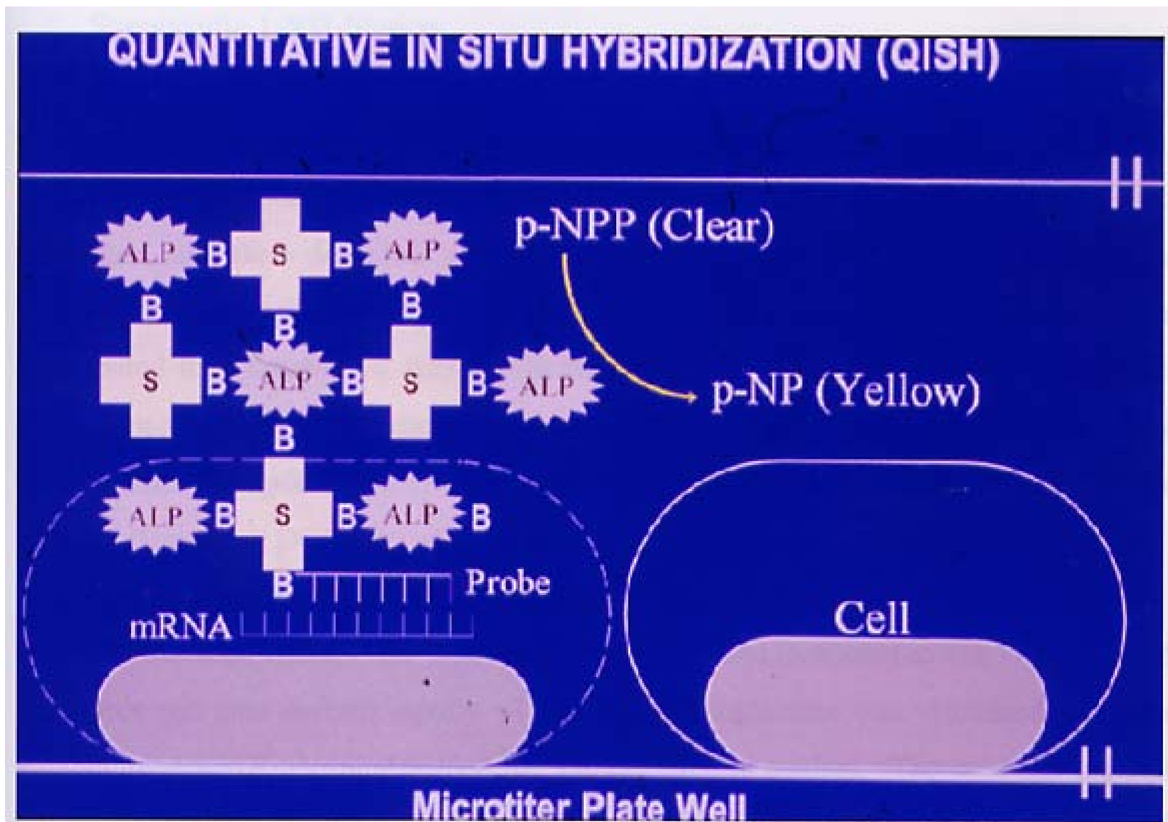
A6.3: Post-hybridization and detection

Post-hybridization involves the stringency washing of the hybridized cells. In this system it was found that washing with 2X SSC-50% formamide at 42°C for 15 minutes followed by two series of washes with 2X SSC and 0.4X SSC at the temperature of hybridization were sufficient to remove all non-specifically bound or trapped sequences. The protocol utilizes avidin-alkaline phosphatase in an indirect affinity detection procedure (Figure A3). The alkaline phosphatase was visualized using the p-NPP substrate (refer to Appendix A6.3, Section ii).

(i) Post-hybridization washes

The plates from Section A6.2 were decanted of probes and hybridization buffer. Excess unbound probe and mismatched bound probe were removed by copious *post-hybridization washings* initially with 2X sodium citrate chloride (SSC) and sodium chloride solution (Appendix B5) in 50% (v/v) formamide for 15 minutes at 42°C; then 2X SSC-0.1% (w/v) sodium dedocyl sulphate (SDS) and 0.4X SSC-0.1% SDS at 42°C, for 15 minutes each. The plates were then washed four times with 0.4X SSC - Milli-Q water at room temperature to remove the SDS, and then placed in 2X PBS and then Tris Buffer 1 (Appendix B5) for 5 minutes each.

Figure A3: Diagrammatical representation of the *in situ* hybridization reaction.



Diagrammatic representation of the *in situ* hybridization reaction in which biotin (B) labeled cDNA probes (probe) hybridize to target mRNA within the cells. Detection is via a streptavidin (S) and biotinylated alkaline phosphatase (BALP) amplification with colourimetric conversion of p-nitrophenyl phosphate (p-NPP) to p-nitrophenyl (p-NP).

(ii) Post-hybridization detection

Plates were decanted. The biotin on the hybridized probe was detected using an *in situ* hybridization detection kit (Dako, Denmark, # K0600). The following were applied sequentially for 10 minutes incubation period each, with four times washing in Tris Buffer 1 between each application:

1. Streptavidin 1:100 dilution
2. Biotinylated alkaline phosphatase 1:100 dilution
3. Streptavidin 1:500 dilution
4. Biotinylated alkaline phosphatase 1:100 dilution

5. <u>Detection method for cells in 96-well tissue culture polystyrene plates:</u>

Plates were then washed four times in Tris Buffer 1 (pH 7.5), and incubated in Tris Buffer 2 (pH 9.5) for 5 minutes and then decanted. Quickly, 100 µl of the p-NPP substrate in 5 ml diethanolamine, preheated to 37°C, was added to each well. The plates were then incubated in a humidified chamber in the dark at 37°C for 20 minutes. The colour developed was yellow and the reaction was stopped by adding 10 µl of 0.5 M NaOH to each well. The optical density of the yellow colour (p-NP) was read by a TIM-200 plate reader (InterMed, Denmark) at 405 nm.

A7: INDIRECT IMMUNOCYTOCHEMISTRY

An indirect immunocytochemical method was used utilising rabbit anti-mouse antibody labeled with biotin, enabling detection with avidin-alkaline phosphatase and visualization using the detection method previously discussed (Appendix A6.3, Section [ii]). The specificity of all the primary antibodies used in studies performed in this thesis was checked by the providers of these antibodies.

A7.1: Cellular quantitative immunocytochemistry assay

The air-dried cells (Appendix A6.1) were fixed for one minute in methanol:acetone (9:1).

The use of streptavidin detection reaction has been reported to cause some non-specific binding (Wood & Warnke 1981); this is probably due to protein binding effects or chemically reactive surfaces, such as glass. The non-specific binding of avidin is removed by applying a blocking solution which comprises a protein mixture (such as bovine serum albumin) which saturates the protein binding sites prior to the addition of the avidin-alkaline phosphatase complex. Endogenous biotin in certain tissues has been thought to be important in non-specific binding. However, all control studies carried out in this system indicate that this does not occur in this test system. Many tissues including kidney and liver contain endogenous biotin, which can be blocked using a biotin blocking kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions.

Cells were:

1. Air-dried and then washed in two changes of 1X PBS.
2. Incubated in 0.25% Triton 100-X, 0.25% Nonidet NP40, for 5 minutes to permeabilize the cell membrane.
3. Washed in two changes of 1X PBS in 0.05% (v/v) Triton 100-X.
4. 50 µl of the blocking solution consisting of 2% (w/v) bovine serum albumin (BSA), heat inactivated (Sigma Aldrich, # A-7030, USA) in HBSS (Sigma Aldrich, # H 9269, USA) were added to the cells to reduce non-specific binding, and incubated for 20 minutes at room temperature in a humidified chamber.

5. The blocking solution was decanted from the wells drained before the appropriate monoclonal (mAbs) or polyclonal antibodies (pAbs) (Table A2) were added. mAbs were used at a 1/100 dilution, and pAbs at a 1/200 dilution. Primary antibodies were diluted in HBSS containing 1% (w/v) BSA and added to cells and incubated at room temperature for 30 minutes in a humidified chamber. Negative controls are routinely conducted with every experiment and involved antibody detection to check for non-specific binding included replacing the primary antibody with 1% (w/v) BSA in HBSS only, as well as irrelevant primary antibodies (e. g. mAbs for HLA-DR and CD45RO, T-cell) (Appendix 7, Table A2).
6. After the primary incubation, plates were washed in four changes of 1X PBS in 0.05% (v/v) Triton 100 X.
7. mAbs were detected with a biotin labeled $F(ab)_2$ fragment of rabbit anti-mouse antiserum (Dako, Denmark), while the pAbs were detected using a Multi-link antibody which is labeled with biotin (Dako, Denmark). The secondary antibodies (Table A3) were diluted 1:200 with HBSS-1% BSA and 50 µl applied to each well. The plates were again placed in a humidified chamber and incubated at room temperature for 30 minutes.
8. Step 6 was repeated.
9. Plates were placed in two changes of Tris Buffer 1 for 5 minutes each. The following were applied sequentially for 10 minutes incubation period each, with Tris Buffer 1 washing four times between each application:
10. 100 µl of streptavidin 1:100 dilution.
11. 100 µl of biotinylated alkaline phosphatase 1:100 dilution.
12. The plates were rinsed in Tris Buffer 1, and
13. Incubated in two changes of Tris Buffer 2 for 5 minutes each.

Detection substrate for the cells in the plates is as follows:

The alkaline phosphatase of the detection process was determined using the p-NPP substrate described above for the *in situ* hybridization detection procedure (Appendix A6.3) but also developed in the presence of 5 mM levamizole to inhibit endogenous alkaline phosphatase activity. The measurement was performed as for the *in situ* hybridization detection (Appendix A6.3).

Table A2: Primary antibodies used in immunocytochemistry

Antibody to	*Provider*	*Code*	*Dilution*	*Antigen*	*Known species cross activity*	*References*
Human osteopontin	Larry Fisher	LF-123	pAb, 1/200	Recomb. carboxyl half starts at thrombin site	Hum, Mon, Pig, Cow, She, Rat, Mouse	based on OP-10 plasmid Young et al. 1990
Human bone sialoprotein	Larry Fisher	LF-83	pAb, 1/200	YESENGEPRGDNYRA YE	Hum, Mon, Dog	Mintz et al. 1993
Human bone sialoprotein	Larry Fisher	LF-120	pAb, 1/200	recombinant Frag 1 (AA 129-281)	Hum, Mon, Pig, Dog, Rat	based on B5-5g plasmid Fisher et al. 1995
Bovine osteocalcin	Larry Fisher	LF-32	pAb, 1/200	bovine bone osteocalcin	Cow, Hum, Mon	Ingram et al. 1993
Bovine osteocalcin	Larry Fisher	BON-1	pAb, 1/200	bovine bone osteonectin	All species	Ingram et al. 1993
Type I collagen	Amrad	Col I # 90001665	mAb 1/100	Murine	Hum, Bov, Ovine	Werkmeister et al. 1989
Type III collagen	Amrad	Col III # 90001755	mAb, 1/200	Murine	Hum, Dog, Rat, Kangaroo, Porcine	Werkmeister et al. 1989
Alkaline phosphatase	Sigma Immuno Chemicals	ALP # A2951	mAb, 1/400	Mouse	Hum	
β-actin	Sigma Immuno Chemicals	β-actin # A5441	mAb, 1/200	Mouse	Hum, Bov, She, Pig, Rabbit, Cat, Dog, Guineapig, chicken Leech, Fruit fly tissue	
CD68 Clone KP1	Dako	# M0814	mAb, 1/100	Mouse		
CD68 Clone PG-M1	Dako	# M0876	mAb, 1/100	Mouse		
Human histocompatibility (HLA) complex Class II	Dako	HLA-DR # M775	mAb, 1/100	Mouse		
T-cell	Dako	CD 45RO # M0742	mAb, 1/100	Mouse		

Code: mAb = monoclonal antibody; pAb = polyclonal antibody; Hum = human; Bov = bovine; She =sheep; Mon = monkey

pAbs used in studies performed in this thesis were generously donated by Dr. Larry Fisher (Bone Research Branch, National Institute of Dental Research, National Institute of Health; Bethesda, Maryland, USA). mAbs were purchased from different sources as indicated in Table A2.

Table A3: Secondary antibodies used in immunocytochemistry

Secondary antibody used	Source and code	Dilution
Biotinylated rabbit anti-mouse IgG	Dako E0413	1/200
Biotinylated swine anti rabbit IgG	Dako E0453	1/200

A8: SCANNING ELECTRON MICROSCOPY OF CELLS

The cell cultures were prepared for scanning electron microscopy (SEM) analysis by rinsing the cells grown on the different substrata three times in 0.1 M cacodylate-buffered solution, pH 7.2 (Appendix B8) and fixed in 4% glutaraldehyde (Sigma, USA) in 0.1 M sodium cacodylate-buffered solution (Appendix B8) at 4°C for 15 minutes. Subsequently the specimens were washed with cacodylate buffer 0.1 M, pH 7.2 three times and dehydrated in ascending concentrations of ethanol, viz. 30%, 50%, 70%, 80%, 90% and 96%, finally immersed in absolute ethanol for ten minutes each, after which the specimens were immersed for 10 min each in three baths of hexamethyldisilazane (HMDS, Sigma, # H 4875). Each specimen was then air-dried for 24 hours. The dried specimens were glued onto aluminium stubs, sputter-coated with gold and examined in a CamScan MaXim at an accelerating voltage of up to 20 kV.