Interplay of the osmotic environment and a fibronectin fragment in intervertebral disc cell metabolism



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Interplay of the osmotic environment and a fibronectin fragment in intervertebral disc cell metabolism

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Abstract

Breakdown of the disc extracellular matrix is thought to arise from increased activity of matrix metalloproteinases (MMPs). Aggrecan, one of the major disc matrix macromolecules, is degraded through action of MMPs and aggrecanases and its concentration falls early in the degeneration process. Loss of the constituent glycosaminoglycans (GAGs), which are osmotically active, leads to a decrease in osmotic pressure and subsequently to a fall in tissue hydration. Apart from the major biomechanical consequences, fall in extracellular osmolarity is known to influence cell function. Apart from aggrecan, other macromolecules such as fibronectin are also broken down by MMPs. Fibronectin fragments (Fnfs) have been identified in degenerate discs. Such Fnfs have been found to stimulate production and activity of MMPs in articular cartilage but little is known about the effect of these fragments on disc cells.

The aim of the work was thus to determine whether Fnf stimulated expression of MMPs and hence induced aggrecan breakdown and loss in the disc and whether extracellular osmolarity influenced this potential response. NP cells or explants were harvested from adult bovine caudal discs. They were cultured in DMEM culture medium over a range of osmolarities with or without Fnf treatment. Profiles of gene expression of MMPs and their inhibitors and effect of changes in osmolarity on expression of selected MMPs were determined. The effect of Fnf on responses of cells and tissue explants from the central region of the disc, the nucleus pulposus (NP) and the role of changes in extracellular osmolarity in relation to GAG loss and expression of selected MMPs was then examined both at the protein level and by gene profiling using a microarray.

My results showed that expression of MMPs by disc cells is regulated by extracellular osmolarity rather than the 30 kD Fnf, with the level of some MMPs secreted by disc cells and involved in degradation of disc matrix rising as osmolarity falls. These results could explain in part the finding that MMP expression increases with degree of disc degeneration i.e. with loss of aggrecan and fall in extracellular osmolarity. These also suggested that a fall in osmolarity could induce a degenerative cascade with proteolytic digestion of aggrecan leading to a fall in osmolarity and hence a further increase in proteinase expression and matrix degradation.

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Alphabetical list of abbreviations

ADAMTS	a disintegrin and metalloproteinase domain with thrombospondin motifs
AF	annulus fibrosus
APS	ammonium persulfate
DMEM	Dulbecco's Modified Eagle's Medium
ECM	extracellular matrix
ERKs	extracellular signal-regulated kinases
FBS	foetal bovine serum
Fn	fibronectin
Fnf (FNF)	fibronectin fragment
GAG	glycosaminoglycan
IL	interleukin
IVDs	intervertebral discs
JNKs	c-Jun NH2-terminal Kinases
MAPK	mitogen-activated protein kinase
mM, µM, nM	millimole per litre, micro mole per litre, nano mole per litre
MMPs	matrix metalloproteinases
Osm	the symbol of osmole; a unit of osmotic pressure equivalent to the amount of solute that dissociates in solution to form one mole of particles
mOsm	milli-osmole
Hyper-osmolarity	≈460mOsm in extracellular matrix
Hypo-osmolarity	≈260mOsm in extracellular matrix
Iso-osmolarity	≈360mOsm in extracellular matrix
nmol	nano mole
NP	nucleus pulposus
PGs	proteoglycans
ppm	parts-per-million
RT-PCR	reverse transcription plymerase chain reaction

TEMED	N, N, N', N'-tetramethylethylenediamine
TIMPs	tissue inhibitors of metalloproteinases
TNF-alpha	tumor necrosis factor-alpha
UniGene	An experimental system for automatically partitioning GenBank sequences into a non-redundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene, as well as related information such as the tissue types in which the gene has been expressed and map location.

Chapter 1 Introduction

Intervertebral discs (IVDs) are load-bearing fibrocartilaginous tissues, situated in between the vertebral bodies of the spine (Figure 1.1). IVDs provide the source of flexibility in the vertebral column allowing extension, flexion, and torsion. The mechanical properties of the discs arise from the organization and composition of the macromolecules which make up their extracellular matrix. These molecules are constantly synthesised and turned over by a small number of resident cells.

1.1 Disc biology, organization and function

The spinal column is formed in the embryo from the central notochord and surrounding mesenchyme, which give rise to the gelatinous nucleus pulposus (NP), and the surrounding lamellae of the annulus fibrosus (AF), respectively (Figure 1.1). The edge between these two regions is very clear in the young individual (<10 years) [1]. The NP and AF are sandwiched by cartilage end-plates (Figure 1.1), a thin horizontal layer, usually less than 1mm thick, of hyaline cartilage.

The description of the intervertebral disc as a NP encircled by an AF, interposed between a superior and an inferior end-plate (Figure 1.1) is based on the anatomy of a lumbar disc. Since intervertebral discs serve both tensile and elastic functions, great variability in disc composition has been found, as the stresses and functions change, within the spine [2]. Cervical and lumber discs tended to have an elliptical cross-sectional shape, while thoracic

discs were more circular. The fibre tilt parallels that of increasing compressive stress (i.e. cervical<thoracic<lumbar NP). Cervical discs are distinctly different in morphology to thoracic and lumbar discs and these differences are evident from birth [2-6].

The intervertebral disc, like other load-bearing articular cartilages, is avascular [7]. Nutrients are supplied to the disc cells by capillaries present in the longitudinal ligaments and other soft tissues adjacent to the disc and by the capillaries arising in the vertebral bodies and terminating in loops at the boundary between the vertebral body and the cartilage end-plates [8].

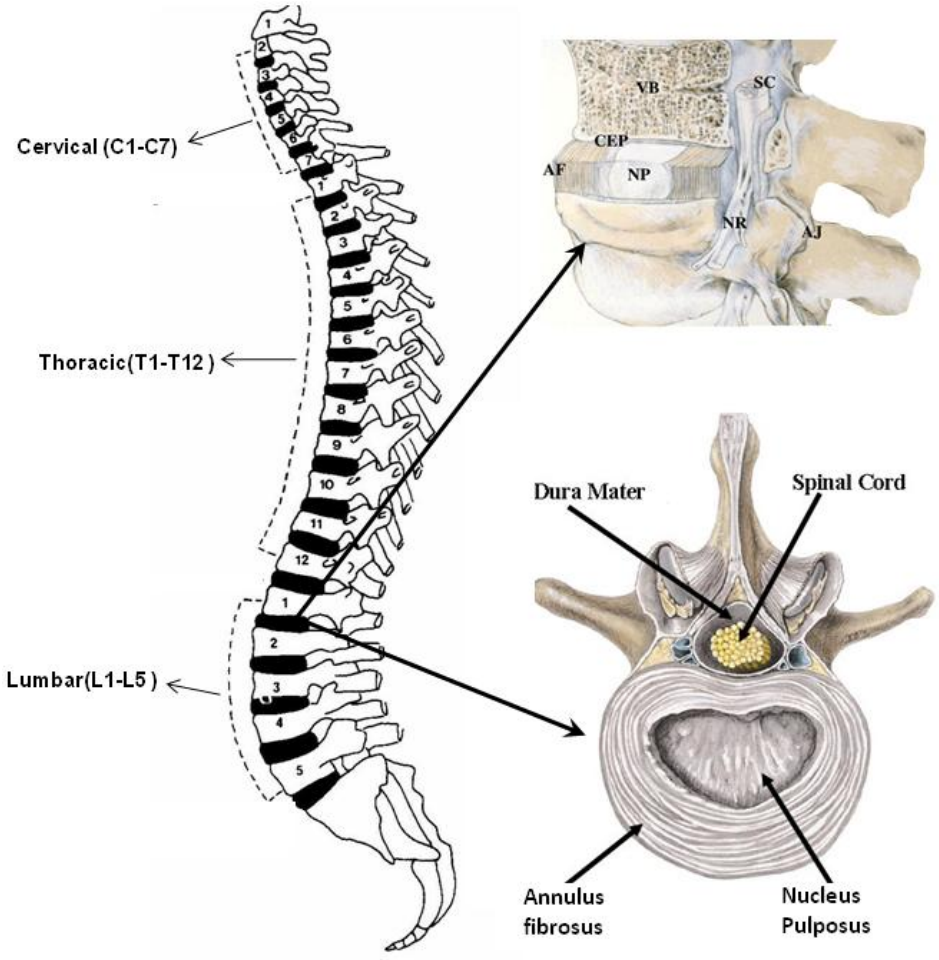


Figure 1.1 A general view of spinal columns and discs.

This figure was adapted from [1, 9] and www.ithaca.edu. Left: human spine columns and discs; Right top: the sagittal section of one of the lumbar segment; Right bottom: the transverse section of one of the lumbar segment. As shown in the figure, the human spine contains 23 discs: six in the cervical (C1-C7), twelve in the thoracic region(T1-T12), and five in the lumbar region(L1-L5).There are no discs between the Atlas(C1), Axis(C2). The two figures at the right-hand side show the organization of the disc with the nucleus pulposus (NP) surrounded by the lamellae of the annulus fibrosus (AF) and separated from the vertebral bodies (VB) by the cartilaginous end-plate (CEP). The figures also show the relationship between the intervertebral disc and the dura mater, the spinal cord (SC), the nerve root (NR), and the apophyseal joints (AJ).

1.2 Extracellular matrix

The intervertebral disc represents a particular type of cartilage tissue, which contains fibrocartilage in the annulus fibrosus and hyaline cartilage in the end plate. The nucleus pulposus seems to be unique, since this type of cartilage combines hyaline and fibrocartilage properties; the origin is also unique as it derives from the notochord [10]. Although the major components of all regions are collagen and proteoglycans [11], the tissue composition of these three anatomical zones is significantly different as discussed below (section 1.2.1; 1.2.2). Differences in organisation can be detected histologically; distinct differences in distribution of collagen types have been described [12] with collagen VI for instance concentrated in the capsule around cells of the NP. As well as collagen and proteoglycans, the disc contains many minor components including fibronectin all of which may play a significant role in maintaining disc matrix homeostasis. Figure 1.2 is a schematic showing some of the macromolecules found in the disc matrix. The elaborate ECM is made and maintained by the disc cells throughout life [13].

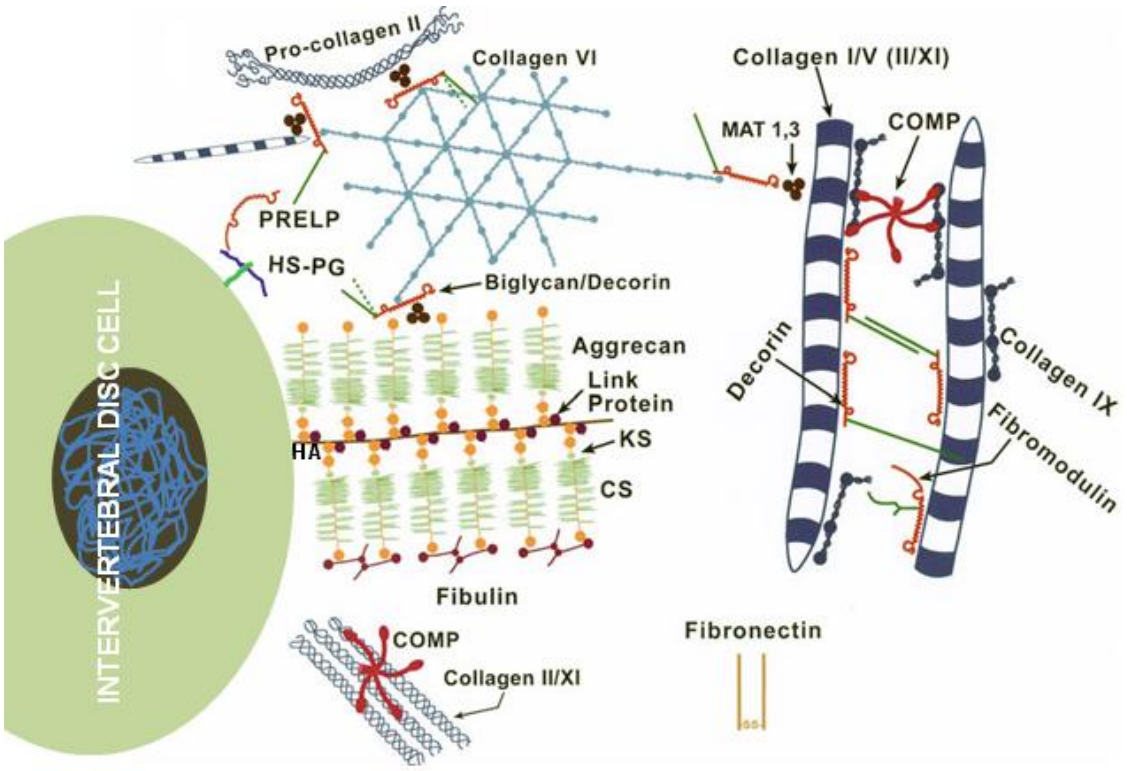


Figure 1.2 A general view of ECM organization in the intervertebral disc.

Adapted from http://www.cmb.lu.se/ctb/ and [14].

COMP-cartilage oligomeric matrix protein; CS-chondroitin sulphate; HA-hyaluronic acid; HS-PG-Heparan sulphate proteoglycan; KS-keratan sulphate; MAT-matrilin; PRELP-proline arginine-rich and leucine-rich repeat protein.

1.2.1 Water and proteoglycans

In the lumbar region the nucleus pulposus consists of a central core of matrix rich in proteoglycans and poor in collagen surrounded by the collagen-rich annulus fibrosus (Figure 1.1). In infancy the nucleus pulposus is a soft gel and occupies three-quarters of the anterior-posterior dimension of the disc [15]. The water content of the nucleus pulposus decreases from around 90% at birth to about 80% at 20 years and 70% above 60 years. The AF contains less water (60-70%), and this does not change significantly with age. The cartilage end-plate contains approximately 72% water [16]. Although dehydrating with age,

the healthy adult nucleus pulposus is still a semi-fluid material. Even in cadaveric material of older adults the nucleus still demonstrates the ability to imbibe water [6, 15]. The water content of the intervertebral discs varies with external load: when a load is applied to a disc, resulting in a stress that exceeds the osmotic pressure of the tissue, fluid is expelled, and the proteoglycan concentration and hence osmotic pressure are thus increased as fluid is lost, until an equilibrium is reached and the osmotic pressure once again balances the applied stress; when the disc is unloaded, it imbibes fluid to achieve osmotic equilibrium [17]. During daily activities, because load on the disc increases, there is a net flow of fluid out of the disc, which is reversed at night when the disc is unloaded [17]. In vivo magnetic resonance imaging (MRI) measurements indicate that the water content of lumbar discs falls by about 20-25% over the course of a day's activity [18].

Proteoglycans form 5-15% of the wet weight of the disc [19]. The proteoglycans include large, aggregating molecules (aggrecan and versican) and small, leucine-rich molecules (including decorin, biglycan, lumican, and fibromodulin). The major proteoglycan present in the disc is aggrecan. Aggrecan (the large aggregating chondroitin sulphate proteoglycan [20]) forms aggregates comprising of up to 100 monomers attached to a single chain of hyaluronic acid (HA)(Figure 1.3) [20], and additionally binds to fibulins (Figure 1.2) [21]. It is the main proteoglycan synthesized by the disc cells, and consists of a protein core (c.200kDa) where glycosaminoglycan (GAG) side-chains are covalently attached [16, 22, 23]. The GAGs, mainly chondroitin and keratan sulphate, contribute a high fixed negative charge to the disc matrix [24]. Ions distribute themselves between plasma membrane and the aggrecan-rich disc

matrix according to the Gibbs-Donnan equilibrium [25], which draws in cations, primarily sodium, and with them water; aggrecan is thus the source of the disc's osmotic properties. Because the proteoglycan content of the nucleus (especially in early life) is greater than that of the annulus [26, 27], the nucleus is more hydrated in healthy discs. Proteoglycan content decreases with age and degeneration, particularly in the nucleus, which would account for the observed decrease in water content [16].

Small, leucine-rich proteoglycans (biglycan, decorin, fibromodulin, lumican) [28] are also present in the disc [29, 30] as is the large proteoglycan versican [31]. It has been found that fibromodulin, decorin, and lumican tightly interact at the collagen surface, and especially decorin is able to cross-bridge and cross-link collagen fibres [21]. The biglycan/decorin molecules were found at the N-terminal domains of the collagen type-VI molecule [21] and they also bind to a molecule of matrilin(MAT)-1,2, or 3 [32]. Fibromodulin and lumican were indicated to play roles at different stages of collagen fibrillogenesis [21].

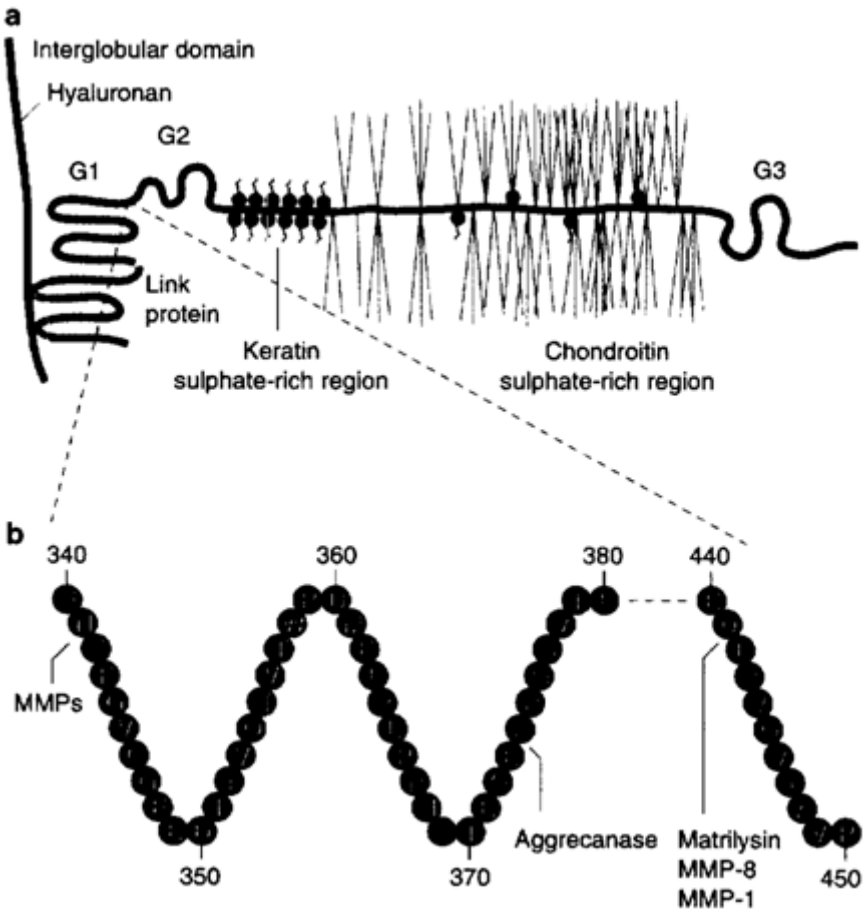


Figure 1.3 Stucture of aggrecan.

(a) Aggrecan consists of three globular regions, G1-G3, separated by linear portions of polypeptide. The interglobular domain between G1 and G2 contains sequences that can be cleaved by a variety of proteinases and so release proteoglycan fragments from the tissues. The region between G2 and G3 binds highly sulphated chondroitin and keratan sulphate residues, which provide the disc with its osmotic properties on account of their high concentration of negative fixed charges. Individual aggrecan molecules bind to hyaluronic acid via the G1 domain; this binding is enhanced if link protein is also present. (b) The interglobular domain is cleaved by different proteinases, including MMPs and aggrecanases and the cleavage points of each are shown. Adapted from [20].

1.2.2 Collagens

The collagen phenotype of the intervertebral disc is complex; at least ten collagen genes are

expressed in the intervertebral disc, including type I, II, III, V, VI, IX, X, XI, XII, and XIV [33]. Collagen types I and II comprises mostly the fibrous framework of the disc [34-36]. Both collagens are cross-linked as mature fibrils by 3-hydroxypyridinium (pyridinoline) residues, which are collagen specific products of lysyl oxidase [37, 38]. Briefly, collagen forms a strong, fibrous framework for the disc, anchors it to the vertebral bodies, and holds the cells and proteoglycans in the matrix [39]. Collagen fibres are organized randomly in the nucleus pulposus, but lie parallel within annulus lamellae, orientated at approximately 60 degrees to the vertical axis, alternating to the left and right of it in adjacent lamella (Figure 1.4) Collagen concentration is highest in the outer annulus and the lowest in the NP [40].

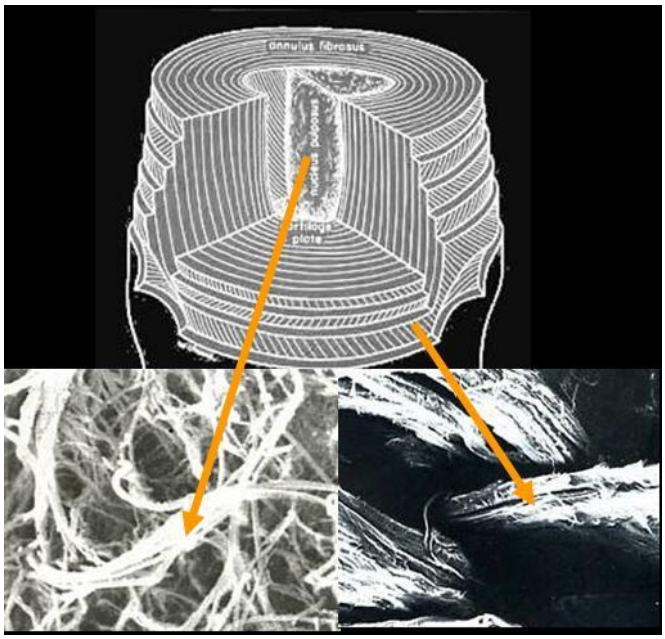


Figure 1.4 Collagen network organization of the intervertebral disc.

Above: A schematic showing the three-dimensional collagen framework of lumbar intervertebral discs; Below: scanning electron micrographs of collagen fibres in nucleus pulposus (left) and annulus fibrosus (right). Adapted from [41].

More collagen types appear to be distributed in the extracellular matrix of the disc than in any other connective tissue, and collagen types also vary across the disc: Type II collagen fibrils are found mainly in the NP, and Type I collagen fibrils are found mainly in the outer annulus [42]. Collagen IX, a minor collagen, occupying less than 1% of the disc collagen [33, 43, 44], is thought to regulate the diameter of the collagen II fibrils and also, as it forms covalent cross-links with the surface of collagen II fibrils, is thought to provide a means of stabilizing collagen networks [43]. In combination with type XI collagen, type IX is a key player in the type II/IX/XI heterofibril that is an important stabilizing element in cartilaginous tissue composed of type II collagen [45].

Other collagen types found in the disc are minor but essential. Collagen type III exists in normal disc with evidence of higher concentrations in degenerate discs [46, 47]. It can also interact with the surface of both type I and type II collagen fibrils [48]. Collagen V is presumably co-polymerized with type I collagen as in other collagen I-based tissues [33]. Type VI collagen is concentrated around cells of the nucleus pulposus and end-plate and has integrin binding sequences which suggest it forms a link between matrix and cells [12, 49]. Type X collagen is also found associated with the cells of scoliotic nucleus pulposus and end-plate [50, 51]. Apart from the function of bounding to fibril surfaces (non-covalent) [33], no more detailed information about type XII and XIV has been reported.

The importance of different collagen types to disc integrity has been demonstrated by transgenic mice. Mice with genetic mutations in selected extracellular matrix proteins, including type II collagen and type I collagen, have been shown to acquire structural and

functional matrix alterations in the intervertebral disc [52, 53]. Although a minor collagen, Type IX played an essential role in disc metabolism as collagen IX knock-out mice show early signs of disc degeneration and spinal disorders [54].

1.2.3 Fibronectin

Fibronectin (Fn) (Figure 1.5) is composed of two forms of disulphide-linked glycoprotein dimer: a soluble form found in the plasma, named plasma-Fn; and an insoluble form acting as a linker in the extracellular matrix, named cellular-Fn. The cellular form is made by fibroblasts, chondrocytes, endothelial cells, macrophages, epithelial cells as well as intervetebral disc cells [55-57].

Fn is a minor component of healthy articular cartilage and intervertebral disc. However, elevated levels are present in human osteoarthritic cartilage (OA) [58], and in canine and rabbit *in vivo* models of OA damage [59, 60]. In disc, Fn increases significantly in both nucleus and annulus tissues in areas with histological signs of mild-to-severe tissue degeneration (e.g., cleft formation and cell clustering), which indicates that a rearrangement of the extracellular matrix during disc degeneration is ongoing [61]. The increase in Fn may be due to both enhanced synthesis and enhanced retention of Fn [62].

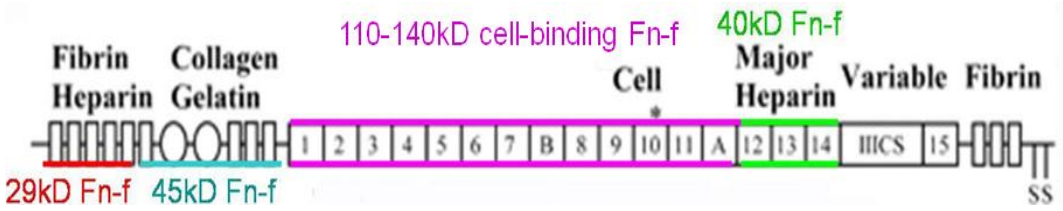


Figure 1.5 Molecular structure of Fn.

Fn is composed of type I (*rectangle*), type II (*oval*), and type III (*square*) repeats. Two extra type III repeats, EDB (*B*) and EDA (*A*), are present in cellular Fn but not plasma Fn. The IIICS region, also named the variable region, can be missing entirely or have up to 120 amino acids due to alternative splicing. Fn contains 29kD N-terminal heparin, 45kD gelatin, 110-140kD cell binding domains, and 40kD C-terminal heparin-binding domain. Fn fragments (Fnf) formed from different domains have been studied for various functions. Adapted from [63, 64].

1.2.4 Other matrix proteins

Elastin, a cross-linked polymer of tropoelastin, is an extracellular matrix protein found in several vertebrate tissues such as lungs, skin, large arterioles as well as in intervertebral discs [65]**.** The resilience of these tissues is due to the presence of elastic fibres. These are formed from an elastin core surrounded by the microfibrillar component, which mainly consists of fibrillin-1 and fibrillin-2 [66, 67]. Elastin is abundant and well organized in young and healthy discs [65] (Figure 1.6). However, with increasing age and degeneration the organization of elastin fibres changes in morphology. They appear to become more fragmented and sparse in degenerate discs (Figure 1.6) (J Yu, personal communication).

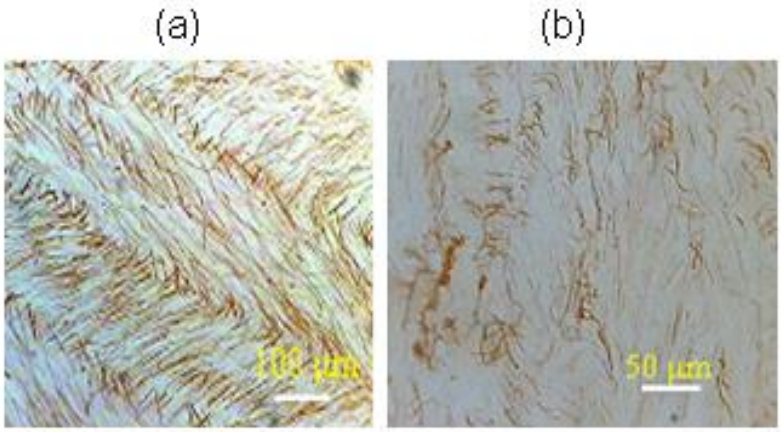


Figure 1.6 Light micrographs of elastin fibres in human discs (sagittal section in annulus).

(a): Immunostained elastin is abundant and well organized in young and healthy disc. (b) Immunostained elastin is fragmented and sparse in degenerate discs. (from J Yu, personal communication)

Also located in the disc, cartilage oligomeric matrix protein (COMP) regulates collagen fibril assembly and maintains collagen network stability by binding to collagen type IX [21]. Cartilage intermediate layer protein (CILP) is also present in the disc, and has been linked to an increased incidence of lumbar disc disease [68].

1.3 Disc degeneration

Discs degenerate much earlier than do other musculoskeletal tissues, but the causes are unclear, and may be due to a combination of factors such as accelerated aging of cells, or decreased nutritional supply; genetics is strongly involved. With degeneration and also with ageing, the normal equilibrium between synthesis and degradation is disrupted. There is a shift toward decreased matrix production and increased matrix degradation, ultimately producing a disc with diminished biomechanical properties [69].

Disc degeneration (Figure 1.7) is clinically very important as it is strongly associated with the major clinical and economic problems of low back pain [70], which lead to loss of mobility, stiffness and deformity. Clinical therapies nowadays are either conservative or surgical; there is no clear diagnosis and treatment is arbitrary for most disc problems; clinical trials have found only minor benefits of surgical procedures such as spinal funsion [71]. Therefore current biological studies mainly focus on stopping, delaying, or reversing the effects of disc degeneration.

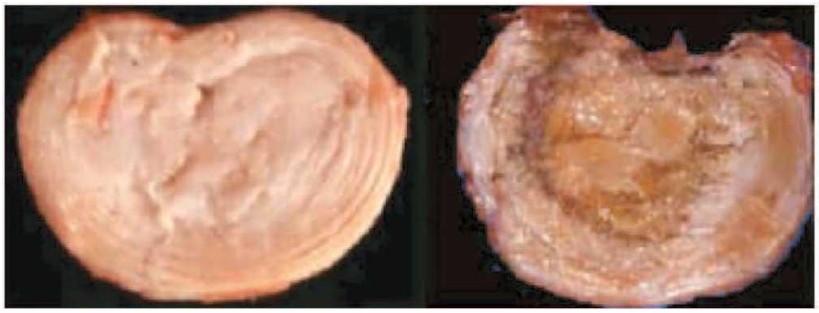


Figure 1.7 The normal and degenerate human lumbar intervertebral disc.
The figure shows a normal lumbar intervertebral disc on the left. The annulus lamellae surrounding the softer nucleus pulposus are clearly visible. In the highly degenerate lumbar disc on the right, the nucleus is dessicated and the annulus is disorganized. Adapted from [1].

1.3.1 Metalloproteinases

Normally rates of synthesis and breakdown are in balance, but in disc degeneration the balance is shifted towards degradation [72, 73] with consequent loss of matrix components, disturbances of tissue architecture and of biomechanical function leading possibly to clinical consequences [74-76]. According to the molecular structure and homology, chemical mechanism of catalytic activity, and specific inhibition, there are four classes of proteinases

(serine-, cysteine-, aspartic-, and metallo-) identified in mammalian cells [77]. However, the major enzymes responsible for this destruction are metalloproteinases, including the family of matrix metalloproteinases (MMPs), and the family of proteases with a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS). Tissue inhibitors of metalloproteinases (TIMPs) are also made by disc cells; these are endogenous inhibitors of metalloproteinase activities and thus also play an important role in regulating tissue turnover.

Both MMP and ADAMTS families have a number of members which share a common catalytic core with a zinc molecule in the active site. The MMP family comprises 23 members [78] . The MMPs can degrade all the components of the ECM which exist within the IVD. They can be divided into four main subfamilies: collagenases, stromelysins, gelatinases, and membrane MMPs. Regulation of MMP activity in tissues is complex. MMPs are secreted as pro-forms (inactive precursors), cleaved to become active, and then can be inhibited by being bound to their inhibitors, the TIMPs, in a 1:1 molar ratio [79, 80]. The ADAMTS family contains 19 members which have highly selective proteolytic activitie [81]. Six members (ADAMTS-1,-4,-5,-8,-9,-15) of this family show a particular avidity for aggrecan and thus have been termed aggrecanases [81, 82]. In vivo, aggrecanolysis probably involves both MMPs and aggrecanase activities [83]. The TIMPs (TIMP-1,-2,-3,-4) show little inhibitory specificity although TIMP-3 reportedly selectively inhibits members of the ADAMTS family, for instance, TIMP-3 is a potent inhibitor of ADAMTS-4 and ADAMTS-5 [14, 84, 85].

A number of studies have examined expression and activity of different members of the MMP

and ADAMTS families of metalloproteinases in both normal and degenerate discs [72, 73, 84-94]. MMPs and ADAMTS-4 and -5 are possibly involved in intervertebral disc development through regulating normal ECM turnover [92]. Upregulation of the MMPs and ADAMTS is well recognized in degenerating discs with significantly increased matrix degradation [82, 94]. A change in the balance between metalloproteianses and their natural inhibitors has been found and suggests a deregulation of the normal homeostatic mechanism during disc degeneration [73, 85]. However metalloproteinase expression and activity has been far more extensively studied in articular cartilage [95], in many respects a similar tissue. However, it is not clear to what extent information relating to cartilage and articular chondrocytes is applicable to disc and disc cells even though mature nucleus pulposus (NP) cells are commonly characterised as being 'chondrocyte-like'. The matrix synthesized by disc NP cells, though similar in composition, is distinctly different in organisation and properties from that of articular cartilage; differences in cell phenotype have been reported [96-98]. Although it is thus apparent that this chondrocyte-like characterisation of NP cells is an over-simplification, surprisingly little is known about what distinguishes NP cells from articular chondrocytes and how this influences the production of metalloproteinases involved in normal tissue homeostasis as well as tissue degradation.

MMP-No	**Name**	**M_r latent/active (×1000)**	**Main Substrates**
MMP-1	Collagenase 1, Interstitial collagenase	51/43	Collagen-III, I, and II
MMP-2	Gelatinase A	71/62	Gelatin, collagen-IV, V, VII, X, XI, fibronectin, elastin, PGs
MMP-3	Stromelysin-1	52/43	PGs, laminin, fibronectin, gelatine, collagen-III, IV, V, IX
MMP-4	Procollagen Peptidase	-	-
MMP-5	¾-Collagenase	-	-
MMP-6	Acid Metalloproteinase	-	-
MMP-7	Matrilysin, PUMP-1	28/19	Gelatin, PGs, Fibronectin, Elastin
MMP-8	Collagenase 2, Neutrophil collagenase	51/42	Collagen-I, II, III, Aggrecan
MMP-9	Gelatinase B	76/67	Gelatin, collagen-IV, V, X, XI
MMP-10	Stromelysin-2	52/44	Gelatin, fibronectin, collagen-III, IV, V
MMP-11	Stromelysin-3	51/46	α_1-proteinase inhibitor
MMP-12	Macrophage metalloelastase	52/20	Collagen-IV, gelatin, elastin, α_1-proteinase inhibitor
MMP-13	Collagenase-3	52/42	Collagen-II, I, II,IV, gelatin
MMP-14	Membrane-type MMP-1	64/54	Collagen-I,II,III, gelatin
MMP-15	Membrane-type MMP-2	71/61	proMMP-2,gelatin,fibronectin
MMP-16	Membrane-type MMP-3	66/56	proMMP-2
MMP-17	Membrane-type MMP-4	62/51	-
MMP-18	Collagenase 4, Xenopus collagenase	53/42	-
MMP-19	No trivial name	54/45	Collagen IV, gelatin, laminin
MMP-20	Enamelysin	54/22	Amelogenin
MMP-21	XMMP (xenopus)	70/53	-
MMP-22	CMMP (chicken)	51/42	Gelatin, casein
MMP-23	CA-MMP	-/-	-
MMP-24	Membrane-type MMP-5	63/45	proMMP-2, proMMP-9, gelatin
MMP-25	Membrane-type MMP-6, leukolysin	-/56	Collagen IV, gelatin, fibronectin, fibrin
MMP-26	Matrilysin-2, endometase	28/-	Collagen IV, fibronectin, fibrinogen, gelatin, α_1-proteinase inhibitor
MMP-28	Epilysin	59(55)/-	Casein

Table 1.1 Some of the MMPs, their names, latent and active molecular weights and substrates of the best characterized MMPs.

Adapted from [99, 100].

Protein name	Alternative names	Known substrates
ADAMTS-1	METH-1; aggrecanase-3	Aggrecan, versican and brevican
ADAMTS-2	PCINP	Procollagen I, II and III N-propeptides
ADAMTS-3	KIAA0366[*]	Procollagen II N-propeptide
ADAMTS-4	aggrecanase-1; KIAA0688[*]	Aggrecan; brevican; versican V1; fibromodulin; decorin; carboxymethylated transferrin
ADAMTS-5	aggrecanase-2; ADAMTS11	Aggrecan, versican and brevican
ADAMTS-6		-
ADAMTS-7		Cartilage oligomeric matrix protein (COMP)
ADAMTS-8	METH-2	Aggrecan, versican and brevican
ADAMTS-9	KIAA1312	Aggrecan; versican
ADAMTS-10		-
ADAMTS-12		cartilage oligomeric matrix protein (COMP)
ADAMTS-13	vWFCP	von Willebrand factor
ADAMTS-14		Procollagen I N-propeptide
ADAMTS-15		Aggrecan, versican and brevican
ADAMTS-16		-
ADAMTS-17		-
ADAMTS-18		-
ADAMTS-19		-
ADAMTS-20		-

Table 1.2 The human ADAMTS names and known substrates.
Adapted from [81, 101-103].

1.4 Disc cells

There are thought to be at least three populations of cells in the IVD each occupying a different region of the matrix [104, 105]. In bovine caudal disc, NP cells (Figure 1.8) have a chondrocyte-like appearance, being rounded and enclosed within a lacuna. The cells of the AF (Figure 1.8), on the other hand, have an elongated fibroblastic appearance, especially in the outer AF, and are orientated in the same direction as the collagen fibrils [12]. The cells of the cartilaginous endplate (Figure 1.1) produce and maintain ECM which has similar composition to that of other hyaline cartilage [7]; however, in addition to the role of

penetration of nutrients from capillary, the cartilage endplate in humans also act as the growth region for the vertebral body, whereas in most other species (including sheep and cows) there is a separate growth plate similar to that seen in longbones [106].

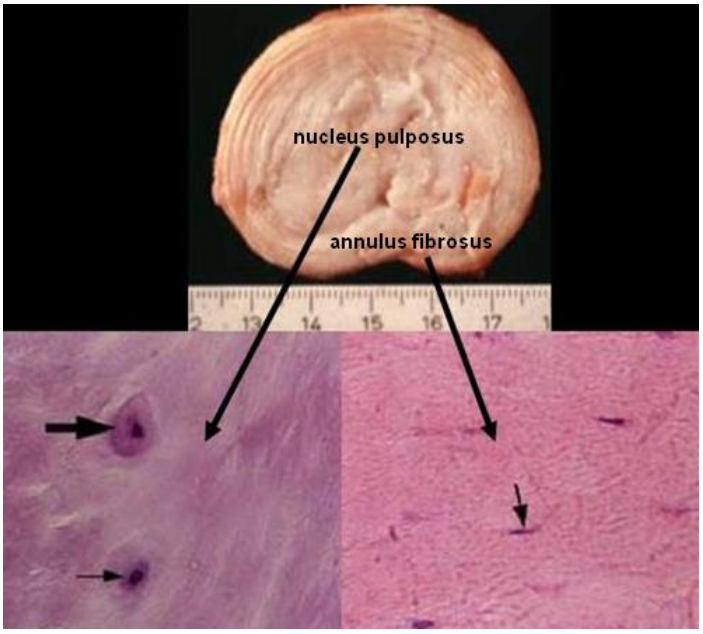


Figure 1.8 Bovine caudal disc (cross section) and light micrographs of annulus and nucleus showing differences in cell morphology.
(Above) A bovine caudal disc, in axial cross-section; (Below) Left: Nucleus cells, often in a capsule. Right: Thin elongateded annulus cells adapted from [97].

In human disc, the cell density of the NP is about $4x10^6$cells/cm^3 and of the AF is about $9x10^6$cells/cm^3 [107], which is much less than that of typical hyaline cartilage (about $1.4x10^7$cells/cm^3). Thus the mature disc is one of the most sparsely-populated cellular tissues in the body. Yet the role of disc cells is vital: throughout life they synthesize not only the extracellular matrix, but also the agents responsible for matrix breakdown, and thus they control disc tissue composition and turnover.

The mature NP cells are commonly characterised as being 'chondrocyte-like'. Like articular chondrocytes NP cells are spherical and in situ exist in isolation, embedded in a dense avascular matrix surrounded by a pericellular capsule which differs in composition from that of the inter-territorial matrix [108]. Like chondrocytes NP cells synthesize primarily type II collagen and aggrecan but also a complex pattern of other matrix macromolecules, while Type I collagen is found mainly in AF region [42]. However the matrix synthesized by disc NP cells is distinctly different in organisation and properties from that of articular cartilage. Metalloproteinase and inhibitor expression between NP cells and chondrocytes were also shown to be intrinsic to cell phenotype [109]. Other differences in cell phenotype have been reported [96, 97, 110, 111]. Although it is thus apparent that this chondrocyte-like characterisation of NP cells is an over-simplification, surprisingly little is known about what distinguishes NP cells from articular chondrocytes.

Unlike AF cells, which develop embryologically from the mesenchyme, the NP cells originally derive from notochord [112, 113], and most animals retain notochordal cells well into adulthood if not throughout life [114] but in man these notochordal cells, which produce a semi-fluid matrix with low collagen content, appear to be replaced by chondrocyte-like cells which produce the mature adult matrix. It is not known if the original population dies and is replaced by cells of mesenchymal origin from endplate and annulus, or if notochordal cells differentiate. Recently, notochordal-like markers in cells extracted form the nucleus have been reported [111, 115] and cells expressing notochordal proteins have been found in clusters in adult human and bovine discs [116, 117], further leading to speculation that the

adult NP cells are derived from the notochord. Chondrocytes, by contrast, are only of mesenchymal origin [96, 106, 118-120].

1.5 Regulation of matrix turnover

1.5.1 Physicochemical environment of disc cells

Since the disc is avascular, gradients of nutrients develop across the disc; concentrations of nutrients such as oxygen and glucose are highest near the blood supply at the disc endplates and lowest in the disc centre [121, 122]. As the disc cells respire mainly by glycolysis even in the presence of oxygen [123, 124], lactic acid is produced as the main metabolite and pH is also lowest in the disc centre because of the high lactic acid concentration in this region [122]. Disc cells are thought to be adapted to low oxygen tension [125, 126], low glucose concentration and low pH [127]. Because of the fixed negative charges on the GAGs, the cation and particularly Na^+ concentration of disc matrix is considerably higher than that of the surrounding medium or serum generating a high extracellular osmotic pressure. However the hydration of the discs is not constant: it varies with applied load [128] and, goes through a diurnal cycle with 20-25% of the disc's water content expressed during the high loads experienced during the day's activities [39]. As a result of such changes in fluid content, the extracellular osmolarity of a normal nucleus cycles between approx. 450 and 550mOsm [129]. Thus IVD cells are subjected to a complex physical environment; however, how they integrate these physical signals *in vivo* to produce matrix appropriate for their customary load is at present unknown.

1.5.1.1 Osmotic effects on disc cells

As for other cell types, isolated cells of the IVD are known to exhibit both passive and active cell volume changes following exposure to hyper- or hypo-osmotic media *in vitro* [130-132]. Osmotic stimuli have also been shown to elicit calcium transients in IVD cells that are modulated, in part, by stability of the actin cytoskeleton [131, 132]. Studies have shown that osmotic pressure altered biosynthesis of explants and isolated cells in culture [130, 133-135]. The osmotic environment of IVD cells has been shown to have a powerful effect on disc cells, as it modifies gene expression for proteins involved in cell cycle regulation, cell-cell interactions and adhesion, signal transduction mechanisms, growth factors and cytokines, and proteases [136]. Additionally, the osmotic environment does not only have an appreciable effect of gene expression but also affects responses to mechanical stimuli [137]. A recent study has shown the expression of aquaporins 1 and 3 within the normal adult human IVD types [138]. All these studies show that, as in other cell types, cell volume in the disc is regulated by transmembrane water and solute transporters over the diurnal osmotic environment of the IVD.

The MAPK (Mitogen-Activated Protein Kinase) signaling cascades (Figure 1.9) are involved in the adaptation of cells to osmotic changes in the extracellular microenvironment [139]. MAP kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis [139]. Three major groups of MAPKs have been reported in

mammalian cells—the ERKs (Extracellular signal-Regulated Kinases), the p38 MAPKs, and the JNKs (c-Jun NH_2-terminal Kinases) [140]. These MAPKs are activated by dual phosphorylation at the tripeptide motif Thr-Xaa-Tyr phosphorylates the MAPK [141].

Changes in osmolarity results in marked phosphorylation of signaling molecules (activation) in several cell types. Hypo-, or hyper- osmotic shock stimulates p38 phosphorylation in 3T3-L1 adipocytes, and human intestine 407 cells [142, 143]. Hypo-osmotic treatment led to and increased activity of Erk-1,-2 in rat astrocytes and human intestine 407 cells [144, 145]; Hypotonic conditions led to a fast activation of SAPK/JNK in human epithelial cells [146]; Hyperosmolarity also increased ERK5 activity, which is one of the least studied MAPK subfamilies [147-149]. Adaptation to prolonged changes has been studied in renal cells where activation of MAPK appears responsible for adaptive changes to osmotic stress [150, 151]. A study showed that rat nucleus pulposus cells of intervertebral discs adapt to hyperosmotic conditions by activating the ERK and p38-MAPK pathways [152].

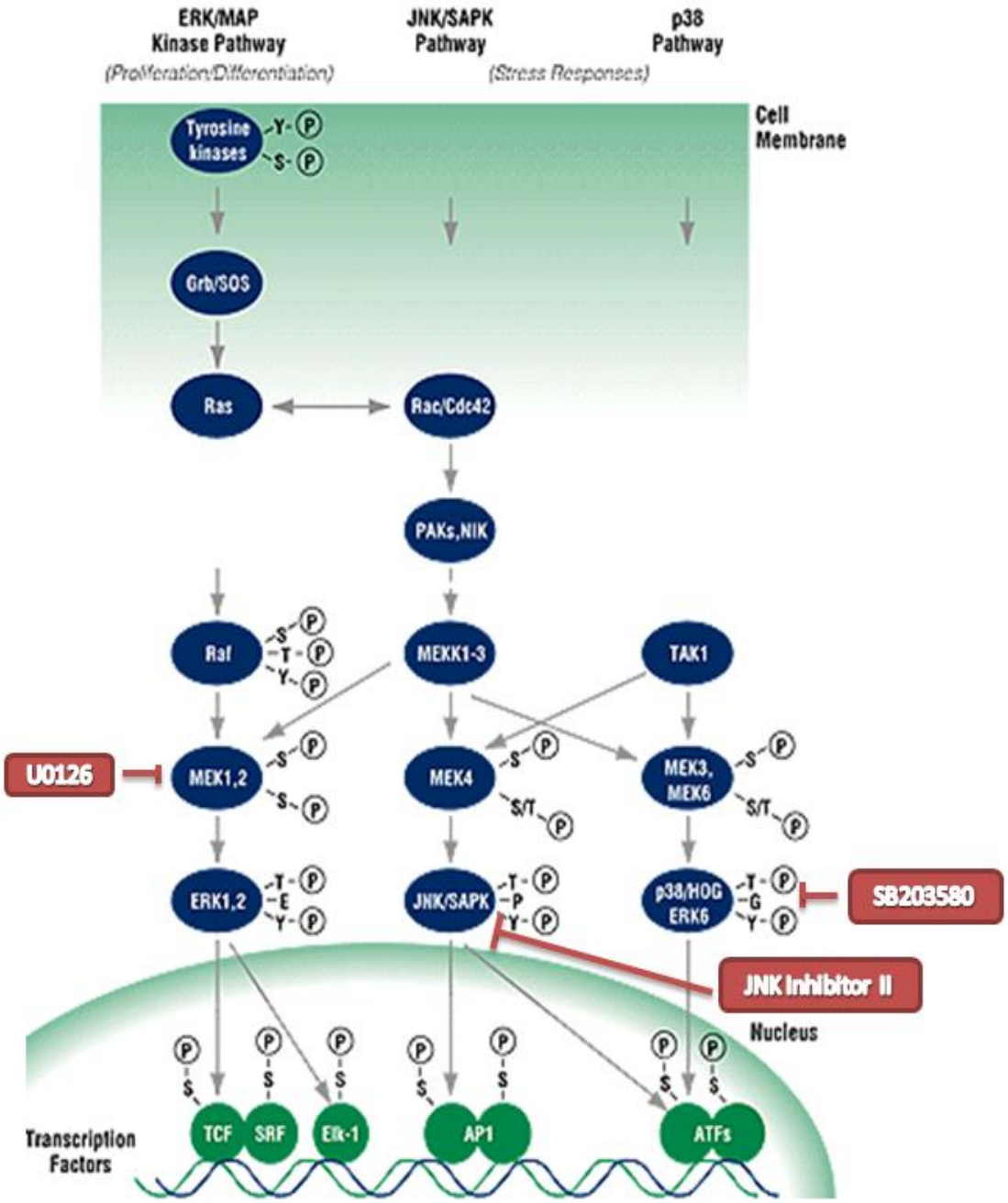


Figure 1.9 Major MAPK cascades involve specific inhibitors.

Adapted from [141]. There are three conventional cascades: MAPK/ERK, JNK and p38. Altering extracellular signals initiate initial events upon association with their respective cell surface receptors and this signal is then transmitted to the interior of the cell where it activates the appropriate cascades. Molecules associated with the signaling cascades are in blue ovals. The red rectangle indicates inhibitors used in my study (Chapter 4).

1.5.2 Matrix fragments

1.5.2.1 Fibronectin fragments

Proteolytic cleavage of fibronectin (Fn) during the process of disc and cartilage degeneration, creates fibronectin fragments (Fnfs) (Figure 1.5). Most work on Fnfs has been carried out on articular cartilage. Studies have shown that Fnfs have cartilage chondrolytic activities through their abilities to elevate MMPs expression, suppress proteoglycan (PG) synthesis, enhance rates of PG loss and upregulate catabolic cytokines. Fnf has been shown to induce a catabolic response in cartilage *in vitro* and accelerate proteoglycan degradation in bovine articular chondrocyte cultures [153, 154]. Treatment of articular chondrocytes with Fnf greatly increased release of catabolic cytokines and MMPs [154-156]. In addition Fnf significantly inhibited proteoglycan synthesis by articular chondrocytes. Fnfs thus resulted in a rapid decrease in cartilage proteoglycan content [155, 156].

Fnfs formed from different domains (29kD N-terminal heparin, 45kD gelatin, 110-140kD cell binding domains, and 40kD C-terminal heparin-binding domain) (Figure 1.5) have been studied for various functions, especially the stimulation of proteolytic activity. Most of the work has focused on a 29-kDa amino-terminal Fnf, which is the most biologically potent of the Fnfs [157]. Other Fnfs have similar effects but are slightly less active. 120kDa Fnf has been shown to enhance pro- and active-MMP-13 activity in human articular chondrocytes [158, 159]. The 110kDa Fnf also increased MMP13 production by human articular chondrocytes. 45-kDa Fnf induced MMP-13 synthesis by chondrocytes and aggrecan degradation by aggrecanases [160]. 40kDa Fnf containing the COOH-terminal heparin

binding domain induced early degradation of proteoglycans and type II collagen by MMP-3 and MMP-13 through an interleukin-1 mediated pathway [58].

There are only a few studies on Fnf signal transduction pathways that relate to catabolic pathways in chondrocytes. Fnfs are likely to interfere with native Fn signaling through the classical $\alpha_5\beta_1$ intergrin receptor [161-163]. Fnfs also upregulate inducible nitric oxide synthase (iNOS) and nitric oxide (NO) levels as well as the toll-like receptor 2 [164-166]. Recent studies have shown that increased production of reactive oxygen species (ROS) as obligatory secondary messengers in the chondrocyte Fnf signaling pathway leads to the increased production of MMPs [167]. The upregulation of MMPs was through MAP kinases [168]. Also FAK, c-src, Pyk2, NF-κ B and PKCδ were involved in a bovine cartilage Fnf mediated cartilage damage model [169]. Interestingly, a recent study demonstrated a downregulation of the Fnf-induced iNOS and COX-2 expression by dynamic compression, which showed interactions of biomechanics and cell signalling in osteoarthritis [170].

Increased Fnf levels have also been seen in human discs; their concentration increases with degree of- disc degeneration [57, 171]. Injection of the 30kDa N-terminal Fnf into rabbit discs led to a progressive degenerative process [172]. Treatment of NP cells of rabbits *in vitro* with 30kDa N-terminal Fnf led to changes in matrix proteins and gene expression similar to those seen during disc degeneration *in vivo* [173]. In NP cells the response to Fnf was less pronounced [153]. However since the role of Fnfs in disc degeneration has been far less studied, it is not clear to what extent information relating to cartilage and articular

chondrocytes is applicable to disc and disc cells.

1.5.2.2 Other matrix macromolecule degradation products

It has been increasingly recognized that breakdown of ECM molecules or cell surface molecules alters cell–matrix and cell–cell interactions and releases growth factors that were bound to the ECM, making them available for cell receptors. These degraded fragments however can feed into the degenerative cascade and play an important role in matrix turnover [174-176].

Elastin peptides produced in pathological conditions have been shown to have a wide range of biological properties. Elastin derived peptides (EDPs) formed from degradation of elastin have biological signaling properties that may contribute to a further matrix break down [177]. EDPs can upregulate MMP expression [177-180], stimulate cell proliferation [181, 182], alter Ca^{2+} fluxes and promote vasodilatation [183, 184], and induce apotosis at high concentrations [185]. In skin fibroblasts, elastin binding protein (EBP) regulated signaling pathways involved in MMP synthesis by elastin peptides activating the Serine and Threonine kinases binding to activated Ras GTPase(Raf) /MAP extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade by protein kinase A and phosphatidylinositol 3-kinase-dependent signaling. The effect of elastin peptides on MMPs production in HT-1080 tumour cells may also involve a post-transcriptional mechanism in which phospholipase C, phospholipase D and protein kinase C (PKC) could be important [174]. Degradation of elastin generates fragments that also stimulate malignant glioma cells

to synthesize tropoelastin, MMP-12, and secrete MMP-2 followed by further degradation [186].

Other ECM fragments have also been shown to play a role in matrix turnover. Chondroadherin(CHAD) fragmentation could be associated with the pathologic changes occurring in the scoliotic IVD [187]. Collagen peptides containing N-telopeptide and C-telopeptides were found in OA cartilage; although they were less active on a molar basis than Fnfs, they still enhanced proteinase release and cartilage PG depletion [188]. Type II collagen fragments differentially alter the expression of genes in bovine AF and NP cells and may be involved in the regulation of IVD homeostasis [189]. Hyaluronan fragments, in lieu of endogenous hyaluronan-chondrocyte interactions, result in the activation of inducible nitric oxide synthase [190], and induced cytokines [176]. Laminin-derived peptides promote inflammatory cell protease production and migration [176].

Other factors including biochemical events and mechanical loading effects can also switch disc cells to catabolism. Studies have implicated the failure of the nutrient supply to the disc cells with disc degeneration [191]. A decreased nutrient supply which results in a lowering of oxygen tension or of pH could impair the ability of disc cells to maintain the homeostasis of the disc's ECM and could ultimately lead to disc degeneration [126, 127, 192].

Proteinases (see section 1.3.1), cytokines, nitric oxide, and prostaglandins [193, 194] all play a role in the matrix breakdown of articular cartiage and intervertebral discs. Degenerated

herniated discs from both the cervical and lumbar spine spontaneously made increased amounts of all of these mediators [193, 194], and cells of the intervertebral discs increased the production of matrix metalloproteinases, nitric oxide, interleukin-6 (IL-6), and prostaglandin E2 when stimulated by interleukin-1 beta (IL-1β) [195]. Recent studies showed that pathologic IVD tissue expressed IL-4, IL-6, IL-12, IL-17, interferon-gamma [196, 197]; IL-1beta is reported to play a greater role than that of tumor necrosis factor-alpha (TNF-alpha) in established pathological degradation [198].

Mechanical overloading on the intervertebral disc matrix may also cause disc catabolism. It was believed that heavy load to the extent that causes structural damage, is the major cause of back diseases. Such abnormal loading producing an injury in the back triggers a signaling pathway that leads to disc degeneration [199]. However, although compression applied to the lumbar intervertebral discs of dogs for up to a year does produce microscopic changes, it does not produce degeneration in any visible form [200]. Nevertheless, many other studies have provided evidence to support the theory that loading leads to back problems. Experimental overloading [69], or injury to the disc [201, 202] can induce degenerative changes. Sustained mechanical loading impairs diffusion of nutrients entering the disc and quite possibly accelerates disc degeneration [203]. Generally speaking, the sensitivity to compression magnitude, frequency and duration should be considered when studying the effect of loading on disc catabolism [204].

1.6 Aims and objectives

The main aim of this work is to determine whether osmotic environment and a fibronectin fragment interplay in intervertebral disc cell metabolism, partly by using conventional methods to identify potential molecules, and partly by employing new techniques to gain a more unified insight to the cell behavior.

It is important to understand the regulation of matrix turnover and degradation in the IVD, and to provide useful comparative information between the cellular biology of the cartilage and the IVD in regard to expression of agents involved in matrix degradation. Therefore the aim of chapter 3 is to determine the pattern of metalloproteinase expression in disc cells and to compare it to that seen in chondrocytes as a baseline for further studies.

To identify the potential role of the physicochemical environment to IVD, it is essential to get a better insight into how IVD explants and cells behave in hypo(≈260mOsm), hyper(≈460mOsm), or iso(≈360mOsm) osmotic conditions. Therefore, the aim of chapter 4 is to investigate the role of osmotic environment in disc cell metabolism, and the signaling pathways involved.

To understand the role of the matrix macromolecule degraded product, it is important to examine the metabolism of IVD explants and cells exposed to the degraded product. Apart from this, it is also interesting to analyse the interaction of the degraded product and altered osmolarity on IVD metabolism. For this, chapter 5 aims to examine the effect of a fibronectin

fragment and the interaction of the fibronectin fragment and osmolarity on disc metabolism.

The aim of the chapter 6 is to determine the whole gene expression and the pathways involved in NP cells stimulated by a fibronectin fragment (Fnf) in differing osmotic environments. In order to test this, the affymetrix GeneChip Bovine Genome Array was used to compare changes in gene expression of NP cells to altering osmotic conditions, and the effects which may interact with Fnf treatment.

The results of this work should increase information on the role of osmotic environment interacted with the degraded product of fibronectin in disc metabolism. The study should also increase knowledge on the similarities and differences between NP cells and articular chondrocytes.

Chapter 2 Materials and Methods

2.1 General methods: cell and explants culture, and assays

2.1.1 Materials

2.1.1.1 Chemicals

Chemicals used are listed in Appendix 1 together with their suppliers.

2.1.1.2 Tissue

Bovine caudal discs and metacarpal-phalangeal joints from 18-24 month old steers were obtained within 4 hours of slaughter from the local abattoir (Mutchmeats, Witney, Oxon, UK).

2.1.2 Methods

2.1.2.1 Cell and tissue isolation

2.1.2.1.1 Dissection of disc and cartilage explants

Bovine tails and feet were cleaned thoroughly (hooves were removed) submerged in sodium hypochlorite solution (10,000 ppm chlorine) for 1 hour, then washed in water. Tissues were dissected aseptically in a class I laminar flow hood using a No. 22 surgical scalpel. Disc nucleus pulposus (NP) explants, about 120mg quantities, from the first 6 caudal discs were carefully dissected free from anulus fibrosus (AF) from bovine tails. Cartilage explants, about 100mg quantities, were thinly sliced from the metacarpal-phalangeal joint.

2.1.2.1.2 Extraction of disc cells and chondrocytes

NP and cartilage explants were enzymatically digested as previously described [130]. Briefly tissues were put into 50 ml in DMEM medium (Dulbecco's Modified Eagle's Medium) containing 20mg collagenase for 18 hrs at 37 ^{o}C. The digested tissue suspension was then filtered (50µm mesh). The cells were washed three times by repeated centrifugation (1000g for 10 mins) and resuspension in DMEM (for formula see Appendix 1).

2.1.2.1.3 Cell counting and determination of cell viability

A 50µl of the final cell suspension was mixed 1:1 with trypan blue dye (0.4% w/v in PBS), and cell numbers counted on a haemocytometer slide (BDH Laboratory supplies, Poole, Dorset, UK). According to the instruction from the company cells were counted manually for living (bright) and dead (blue) cells. All results were normalized to cell number. The cell suspensions were immediately used for cell culture unless less than 95% of cells were alive; the isolate was then discarded.

2.1.2.1.4 Encapsulation of disc cells and chondrocytes in alginate beads

The method of encapsulation of disc cells in alginate beads was originally developed by Guo [205]. Alginate maintains the phenotype of NP and chondrocytes cells [206]. NP cells and chondrocytes for culture were resuspended uniformly in 1.2% alginate solution (for formula see Appendix 2) at a density of 4 million cells/ml. Alginate beads of approximate 15µl each were formed by expressing the cell suspension through a 21G needle attached to a 5ml syringe into a beaker containing 25 ml of 102mM $CaCl_2$ solution [207]. Excess $CaCl_2$ was removed by washing the resulting alginate beads 3 times with 0.9% NaCl and twice with DMEM.

2.1.2.1.5 Dissolution of alginate beads

Alginate beads were placed in a sterile tube and the medium was aspirated. Beads were dissolved by weighing the beads, adding 3 volumes of citrate buffer (55 mM sodium citrate, 50 mM EDTA, 0.15 mM sodium chloride, pH 7.4) [205, 208] and shaking gently. The cells were thus freed and were pelleted by centrifugation for 5 min at 600g. The cells were washed with phosphate-buffered saline and pelleted by centrifugation for 5 min at 600g; supernatant was carefully removed.

2.1.2.1.6 Culture conditions

2.1.2.1.6.1 Osmolarity of culture media

The osmolarity of DMEM (Invitrogen 22320-022) was approximately 280 mOsm [209]. Osmolarity was increased to the physiological levels up to 489mOsm seen in disc NP and cartilage [129, 209]. Here we defined approximate 260mOsm, 360mOsm and 460mOsm as hypo-, iso- and hyper-osmolarity. NaCl was used to adjust the osmolarity by a rate of 2 mOsm/mM and the final osmolarity was monitored using an Automatic Micro Osmometer (Hermann Roebling MESSTECHNIK Germany). To sterilize the culture media with altered osmolarities, culture media were filtered through MILLEX-GP Filter Unit 0.22μm (MILLIPORE, Carrigtwohill, Co. Cork, Ireland).

2.1.2.1.6.2 Culture medium

Unless otherwise stated NP explants and NP cells in alginate beads were incubated using DMEM (Invitrogen, Paisley, UK – Cat. No. 22320-022) altered to the required osmolarity as described above (Section 2.1.2.1.6.1), and supplemented with 1% penicillin-streptomycin (Invitrogen, Paisley, UK-Cat No. 15140-122) and 10% foetal bovine serum (FBS) (Invitrogen, Paisley, UK-Cat. No. 10106-169). Cultures were placed in 24-well plates and maintained in

an incubator at 37^{o}C, 100% humidity, 5% CO2, 21% O2, balance N2.

2.1.2.2 Assays and outcome measurements

2.1.2.2.1 Lactic acid assay

Lactic acid production in samples was measured using a lactic acid assay kit (Appendix 1). This method is based upon the principle that lactate oxidase converts lactic acid to pyruvate and hydrogen peroxide (H_2O_2). In the presence of H_2O_2, peroxidase (type II, from horseradish) catalyzes the oxidative condensation of chromogen precursors (aminoantipyrine, N,N-dimethylaniline) to produce a colored dye with absorption maximum at 540 nm, the absorbance of which is proportional to the original concentration of lactate in the sample [210]. For quantitation of lactate in the samples, 10µl aliquots of sample medium or lactic acid standard (0-4mM) and 100µl lactate reagent were added to wells of a 96 multi-well plates. Plates were agitated gently, left at room temperature for 10-15 minutes and absorbance recorded at 540 nm on a micro-plate spectrophotometer and the concentration of the unknowns calculated from those of a standard curve.

2.1.2.2.2 Glycosaminoglycan (GAG) assay

Quantification of total sulphated GAGs in NP explant cultures, in NP cells in alginate beads and GAG release into the sample medium were assessed by using the 1, 9-dimethylmethylene blue (DMB) dye assay(Appendix 1) [211], which relies on spectrophotometric detection of metachoromatic changes in DMB when it binds to sulfate and carboxyl groups of GAG chains. However, for NP cells in alginate beads, the situation is complicated by alginate, which is an anionic polysaccharide consisting of carboxylated acid

units. The detection of GAG can be strongly interfered with by alginate especially when the GAG concentration is low. But this problem can be overcome by adjusting the pH of the dye [212]. Under low pH, carboxyl and sulphate groups will be protonated and their binding with DMB will be blocked. However, a sulphate group has a lower pKa than a carboxyl group, which means the latter is blocked by H^+ more readily than the former. So, there should be a critical pH value under which only sulphated GAG will bind with DMB while carboxylated alginate is blocked by H^+, and in this study the pH was found to be 2.3 (under pH 1.5 both GAG and alginate were completely blocked)(see Thesis of Shengda Zhou, 2005). The GAG assay of NP explant cultures, sample medium and NP cells in alginate beads were thus carried out as follows.

To assay for GAG content in NP explants, tissues were dried and digested in 100μl of sterile papain solution (250μg/ml in sodium free papain buffer, pH 6.8) at 60 ^oC for 24 hours. NP is rich in hyaluronic acid. GAGs in NP were therefore assayed using the DMB dye at pH 3.0 to eliminate the interference by other polyanions, including hyaluronic acid, DNA and RNA[213]. To assay for GAG release in the sample medium, aliquots (50μl) of the media were removed and assayed using the DMB dye at pH 6.8. To assay for GAG content of NP cells in alginate beads, 5 beads were dissolved and papain-digested (2ul papain was added to each sample) at 60 ^oC for 24 hours; GAGs were then assayed using DMB dye of pH 2.3. Briefly, 10μl chondroitin sulphate standards (2.2-10μg/ml) or digested samples, diluted to lie within the linear range of the standard curve, and 90μl DMB dye at the relevant pH were added to wells of a 96 multi-well plates. The absorbance was immediately measured at 595

nm on a micro-plate spectrophotometer and the concentration of samples calculated from the standard curve from 2.2-10 µg/ml.

2.1.2.2.3 Protein assay

Sample medium was assayed for soluble protein using the BioRad *DC* protein assay (Appendix 1), which is a colorimetric assay for protein concentration. The reaction is a modification of the well-documented Lowry assay [214].

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to color development viz, the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein [214]. Color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine [214, 215]. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

To measure the protein concentration in the sample medium, 5 µl sample or protein standard (0.2-1.5mg/ml), 25 µl of reagent A (an alkaline copper tartrate solution), and 200 µl of reagent B (a dilute Folin Reagent) were added in-order to a 96 multi-well plate. Plates were agitated gently for 5 seconds, left at RT for 15 minutes and absorbance at 750 nm on a micro-plate spectrophotometer was measured. The concentration of the unknowns was calculated from

those of the standard curve from 0-3mg/ml.

2.1.2.2.4 Zymography and reverse zymography

Zymography and reverse zymography are techniques used to analyze the activities of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in complex biological samples. The two methods are technically similar. Zymography involves the electrophoretic separation of proteins under denaturing by SDS-PAGE. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight), and SDS gel electrophoresis of samples thus has identical charge per unit mass due to binding of SDS results in fractionation by size [216-218]. Zymography is under nonreducing conditions through a polyacrylamide gel containing gelatin. The resolved proteins are renatured by exchange of the SDS with a nonionic detergent, such as Triton X-100 and the gel is incubated in an appropriate buffer for the particular proteinases under study. The gel is stained with Coomassie Blue and proteolytic activities are detected as clear bands against a blue background of undegraded gelatin [219].

Reverse zymograph was developed as a modification of zymography to detect TIMPs rather than MMPs. Initially, this was accomplished by incubating the "zymogram" with conditioned media (CM) containing MMP activity for a few hours following the Triton X-100 step. This resulted in partial degradation of the gelatin except in regions protected by TIMP activity [219, 220].

Gels were formulated as detailed in Table 2.1, modified from the table given in Susan [219]. Reagents and buffers are listed in Appendix 2. Gels were cast and set up between the glass plates of a Bio Rad Mini Protein II electrophoresis apparatus. 10µl of 10µg sample mediums were mixed with an equal volume of 2×sample buffer and incubated in a warm water bath at 55 ^{o}C for 5 min. 20µl each sample was loaded per well and 5ng MMP/TIMP (Merck Biosciences Ltd, Nottingham, UK- Cat. No.PF069) was used as positive control.

Gels were electrophoresed under non-reducing conditions at 120 V in 1×Tris Glycine SDS running buffer, until the bromophenol blue marker dye reached the bottom of the gel. After electrophoresis, SDS was removed from the gel by washing 2×10 min in 2.5% Triton X-100. Gels were then incubated in incubation buffer overnight at 37 ^{o}C. After incubation, the gels were stained with a solution of 0.25% Coomassie Blue R250 in 40% methanol and 10% acetic acid for 2 h at room temperature, and destained with 40% methanol, 10% acetic acid until the bands of lysis became clear.

Reagents[a]	Type of gel		
	10% Zymogram	15% Reverse Zymogram	5% Stacking
Acrylamide/bis (30%)	3.33ml	5.0ml	1.67ml
0.5*M* Tris-HCl, pH 6.8	-	-	2.5ml
1.5*M* Tris-HCl, pH8.8	2.5ml	2.5ml	-
Gelatin(100×)	100µl	100µl	-
Conditioned media[b], 1%SDS	-	2.35ml	-
SDS (10%)	100µl	-	100µl
ddH_2O	3.92ml	-	5.68ml
APS (10%)	50µl	50µl	50µl
TEMED	5µl	5µl	5µl

Table 2.1 Components used to produce SDS-PAGE gels for gelatin zymography and reverse zymography

These recipes are for a 10ml volume. Two minigels can be prepared from 10ml of separating gel solution and 5ml of stacking gel solution.

[a] All ingredients apart from APS and TEMED were combined initially. Polymerization was initiated by adding APS and TEMED.

[b] The conditioned media came from the culture media of a bovine skin fibroblast, which was cultured as monolayer in DMEM without FBS for 5 days.

2.1.2.2.5 Western blotting

The western blot (alternatively, protein immunoblot) is the method originated from the laboratory of George Stark at Stanford. The name western blot was given to the technique by W. Neal Burnette [221] and is a play on the name Southern blot, a technique for DNA detection developed earlier by Edwin Southern.

Figure 2.1 shows the outline protocol of the western blot technique. It uses gel electrophoresis to separate native or denatured proteins from sample medium by the length of the polypeptide (denaturing conditions). The proteins are then transferred to a membrane (nitrocellulose), where they are detected using antibodies specific to the target protein [222, 223].

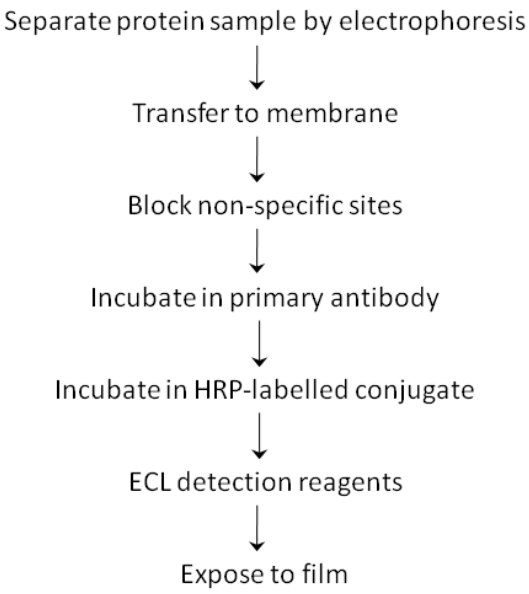


Figure 2.1 Flow diagram of western blot.

The procedure of the western blotting of my study is based on the manufacturer's instructions (Amersham ECL Western blotting detection reagents and analysis system Product Booklet). After separating protein samples by electrophoresis (see 2.1.2.2.4) at 120V for 1hr, gels were removed into transfer buffer (Appendix 2), and were washed for 15min on an orbital shaker. At the same time, membrane (GE Healthcare, UK-RPN3032D) and two thick blotting papers (GE Healthcare, UK-RPN6101M) were equilibrated in transfer buffer for at least 10min before blotting. Trans-Blot SD, Semi-Dry Electrophoretic Transfer cell (Bio-Rad, address) was used to transfer proteins. Transfer was at 15V for 30min. Non-specific binding sites were blocked by immersing the membrane in 5% non-fat dried milk and 0.1% (v/v) Tween 20 in TBS (TBS-T) (Appendix 2) for one hour at room temperature on an orbital shaker. Then the membrane was incubated in diluted primary anti-MMP3 or anti-MMP13 (1:1000) (Appendix 1) in TBS-T for 1 hour at room temperature on an orbital shaker. The membrane was rinsed 3 times for 10mins for each rinse with TBS-T. The membrane was then incubated in diluted HRP-labeled secondary antibody (1:5000) (Appendix 1) in TBS-T for 1 hour at RT on an

orbital shaker. The membrane was than rinsed 3 times 10mins with TBS-T. An equal volume of detection solution one was mixed with detection solution two to a final volume 0.125ml/cm^2/membrane. The mixed detection reagent was pipetted onto the membrane, and incubated for 1 min at room temperature. The membrane was then wrapped by cling film and placed it in an X-ray film cassette, protein side up. A sheet of autoradiography film was placed on top of the membrane. The cassette was closed and exposed for relevant time (from 1 min to 1 hour), and the film developed immediately.

2.1.2.3 Statistical analysis

Unless otherwise stated, data are presented as the mean ± the standard error of the mean (SEM) of at least three individual determinations ($n \geq 3$). Differences compared to control were examined by Student's paired *t* test. For lactate production and GAG release, the two-way ANOVA was used to assess the effects of treatment and cultured period, using the Bonferroni test as a *post hoc test.* P values of 0.05 or less were considered to be statistically significant. Significant differences for GAG remaining and MMPs production under differing osmolarity were determined using one-way ANOVA and Dunnett test with a P value of 0.05 or less considered significant.

2.2 Real-time RT-PCR methods

2.2.1 RNA extraction and quantification

For extraction of RNA, 25 beads (approximately 1 million cells) from each sample were placed in a sterile tube and the remaining medium was aspirated. Beads were dissolved by

adding 3 volumes of citrate buffer [208] and shaking gently. The cells were thus freed and were pelleted by centrifugation for 5 min at 600g. The cells were washed with phosphate-buffered saline and pelleted by centrifugation for 5 min at 600g; supernatant was carefully removed. Total RNA was isolated from these cells using a Qiagen RNeasy Mini Kit (QIAGEN, Crawley, UK) as per the manufacturer's instructions. Purified RNA was quantified by NanoDrop [224].

2.2.2 Quantitative evaluation of mRNA levels

2.2.2.1 Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesized from 0.75μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Paisley, UK) and random hexamers in a total volume of 20μl, according to the manufacturer's instructions and essentially as previously described [225]. cDNA was stored at -20 ℃ until used in downstream real-time RT-PCR.

2.2.2.2 Real-time RT-PCR

Oligonucleotide primers were designed using DNAstar (DNAstar Inc.) The primer sequences used to amplify a number of MMP, ADAMTS and TIMP bovine genes have been previously described [225] with primer sequences to other remaining family members listed in Chapter 3: Table 3.1. Relative quantitation of genes was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Metalloproteinase and inhibitor expression were determined using SYBR Green (Takara Bio Inc., Cambrex, Wokingham, UK) using the manufacturer's suggested protocol. PCR reaction mixtures

contained 2ng cDNA, 50% SYBR-Green PCR mix (Takara Bio Inc., Cambrex, Wokingham, UK) and 100 nM of each primer in a total volume of 25µl.

The 18S ribosomal RNA gene was used as an endogenous control to normalize for differences in the amount of total RNA present in each sample; 18S rRNA TaqMan primer and probe were purchased from Applied Biosystems. TaqMan mastermix reagents (Sigma-Aldrich Co., Poole, UK) were used according to the manufacturer's protocol. The calculation $2^{-(C_T \text{ gene} - C_T \text{ 18S})}$ ($2^{-\Delta C_T}$) was used as an approximate measure of gene expression to allow comparison of levels between genes. C_T is the polymerase chain reaction cycle at which a positive signal is first measurable above background.

2.2.3 Statistical analysis

Levels of mRNA for each gene were corrected using the 18S rRNA levels as described above, and all statistical tests were performed using these log ratios on the samples from a quintuplicate experiment. The significance of differences between the disc NP cells and articular chondrocytes was determined using Student's *t* test. Data presented are from a single experiment, but the pattern shown is representative of that from three individual experiments.

2.3 Microarray methods

2.3.1 RNA extraction, quality control and two-cycle amplification

RNA extraction from each sample of alginate beads is the same as that of Real-time RT-PCR (section 2.2.2.2). For each sample the quantity of total RNA was measured using NanoDrop

and the quality and RNA integrity was assessed on a BioAnalyzer (Agilent Laboratories, US). An RNA integrity number (RIN) is assigned to each sample, and only those samples passing a quality control threshold (RIN 8) are used. Using the "GeneChip 3' IVT Express Kit" (Affymetrix, UK), the labeled RNA was produced. The GeneChip 3' IVT Express Kit aRNA amplification procedure is depicted in Figure 2.2. Briefly, mRNA in the initial total RNA undergoes reverse transcription to synthesize first-strand cDNA and a T7 promoter is added to the 3' end. The cDNA is then converted into a double-stranded DNA template for transcription. *In vitro* transcription, using a T7 polymerase, synthesizes aRNA and incorporates a biotin-conguated nucleotide (cRNA is also known as amplified RNA or aRNA). The aRNA is then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate. Fragmentation of the biotin-labeled aRNA prepares the sample for hybridization onto GeneChip 3' expression arrays.

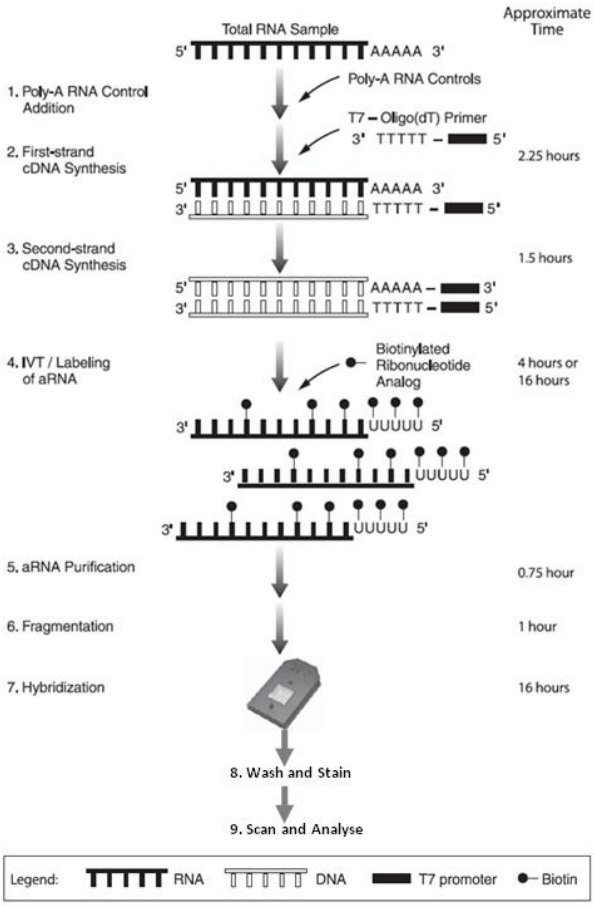


Figure 2.2 The flow diagram of GeneChip 3' IVT Express Kit aRNA amplification.

2.3.2 Affymetrix microarrays

Affymetrix arrays were chosen as they offer the broadest coverage of the transcriptome for the widest number of organisms. They are widely used by the academic community, enabling direct comparison of data with that in the literature and public databases. In my study, labeled and fragmented RNA that passed the final quality control test (24 samples, including four replicates for each condition) was then hybridized to Affymetrix GeneChip Bovine Genome Arrays Chips were washed, stained and scanned on a GeneChip Fluidics Station and Scannner. Data were extracted using the Affymetrix Command Console. Quality control was tested using Affymetrix Expression Console.

2.3.3 Data normalization and analysis

GeneSpring GX was used to normalize the data and to identify differentially expressed genes. Data were normalised using the RMA (Robust Multi-chip Average) algorithm of Irizarry. Normalisation is necessary so that multiple chips can be compared to each other, and is based on the assumption that all chips in an experiment should have approximately the same distribution of Perfect Match (PM) values. The normalisation used in RMA is quantile normalisation. The quantiles for each PM value are plotted in n dimensions, and projected onto the diagonal, resulting in the same distribution for all PM values. Once the probe-level PM values have been background-corrected and normalised, they are summarised into expression measures so that the result is a single expression measure per probe set per chip. A probe set is a collection of two or more probes that are designed to measure a single molecular species. For example, several oligonucleotides designed to hybrize to various parts of the mRNA generated from a single gene. The summarisation used relies on the assumption that observed log transformed PM values follow a linear additive model containing a probe affinity effect, a gene specific effect (the expression level) and an error term. For RMA, the probe affinity effects are assumed to sum to zero, and the gene effect (expression level) is estimated using median polishing, thereby protecting against outlier probes (SiliconGenetics, 2006).

RMA normalised data were used to identify genes that were differentially expressed (>1.5 fold difference, paired t-test $p \leq 0.05$, no multiple testing correction) in the following comparisons (Figure 2.3; Chapter 6: 6.2 Experimental plan):

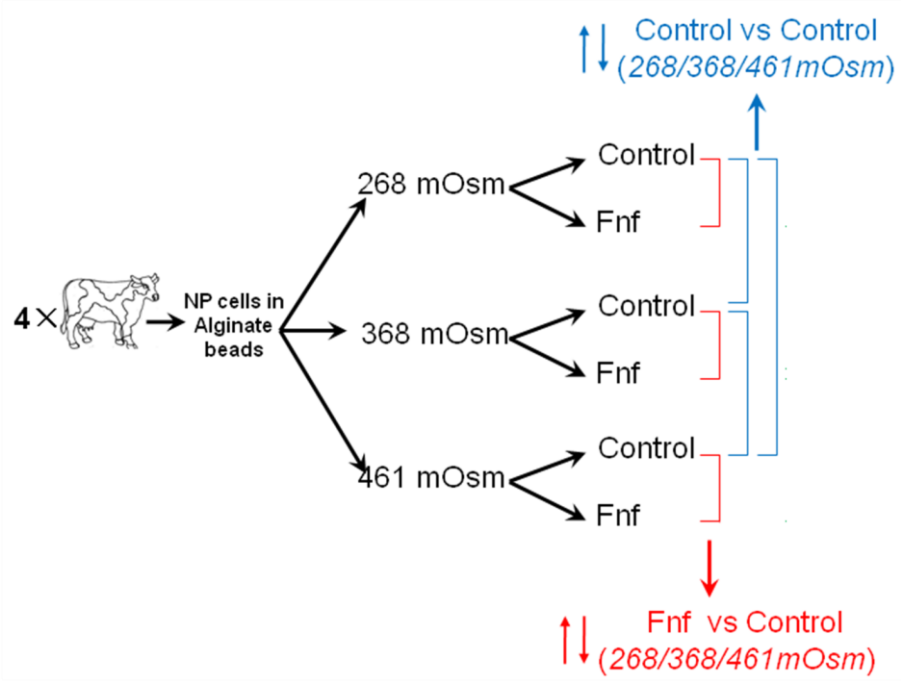


Figure 2.3 Comparisons of gene expression levels

This figure shows a diagram of the comparisons performed on the output of the gene expression chips. The output of the comparison was then statistically tested for significance (>1.5 fold difference, paired t-test $p \leq 0.05$). Each of the four replicates were hybridised to one Affymetrix GeneChip Bovine Genome Array. A total of 24 GeneChips was used.
Close bracket in blue: 268 mOsm control compared to 368 mOsm control; 268 mOsm control compared to 461 mOsm control; 368 mOsm control compared to 461 mOsm control.
Close bracket in red: 268 mOsm Fnf compared to 268 mOsm control; 368 mOsm Fnf compared to 368 mOsm control; 461 mOsm Fnf compared to 461 mOsm control.

Each sample representing one of three different osmolarities with or without fibronectin and derived from one of four different cow tails was hybridised to an Affymetrix GeneChip Bovine Genome Array. The design of the Affymetrix GeneChip Bovine Genome Array was based on content from Bovine UniGene and GenBank mRNAs. The Bovine Genome Array contains 24,027 probe sets representing over 23,000 transcripts and includes approximately 19,000 UniGene (see Alphabetical list of abbreviations) clusters. Some probe sets detect splice variants or were designed against sequences that are no longer believed to belong to different genes, therefore there are 12488 bovine probe sets on the array that have gene

ontology functions associated with them. There are also many genes that have no known function associated with them yet.

Genes were annotated with gene names, gene symbols and Gene Ontology using NetAffx, the Affymetrix annotation tool (www.netaffx.com). Some probe IDs did not have gene names. These probe IDs can be checked out by going to the Bovine genome in Ensembl (Ensembl is joint project between the EBI (European Bioinformatics Institute) and the Wellcome Trust Sanger Institute that provides a centralized resource for geneticists, molecular biologists and other researchers studying the genomes of our own species and other vertebrates and model organisms.). From Ensembl these probe IDs either hit introns or are genes that are unannotated. Here in the study, we will ignore these probe IDs, and will only focus on well-characterized genes.

A total of 24 GeneChips was used. Each comparison was performed between the chip results for each individual sample (e.g. 268 mOsm control compared to 368 mOsm control derived from the same bovine). It was therefore appropriate to use a paired t test. The results for the four bovines were statistically compared to obtain lists of genes fulfilling both cut-off criteria.

Subsequently the same comparisons were performed on RMA normalised data, and these lists were used to identify differentially expressed genes involved in the same biochemical pathways. Pathways analysis was performed using Ingenuity pathway analysis (IPA) obtained from Ingenuity Systems Company.

Chapter 3 Differential gene expression profiling of metalloproteinases and their inhibitors between bovine intervertebral disc NP cells and articular chondrocytes

3.1 Introduction

This chapter is essentially the same as that already published [109]. A number of studies have examined expression and activity of different members of the MMP and ADAMTS families of metalloproteinases in both normal and degenerate discs [72, 73, 84-91] but metalloproteinase expression and activity has been far more extensively studied in articular cartilage[95], in many respects a similar tissue. However, it is not clear to what extent information relating to cartilage and articular chondrocytes is applicable to disc and disc cells even though mature nucleus pulposus (NP) cells are commonly characterised as being 'chondrocyte-like'. Like articular chondrocytes NP cells are spherical and in situ exist in isolation, embedded in a dense avascular matrix surrounded by a pericellular capsule which differs in composition from that of the inter-territorial matrix [108]. Like chondrocytes, NP cells synthesize primarily type II collagen and aggrecan but also a complex pattern of other matrix macromolecules. However the matrix synthesized by disc NP cells is distinctly different in organisation and properties from that of articular cartilage and other differences in cell phenotype have been reported [96, 97]. Although it is thus apparent that this chondrocyte-like characterisation of NP cells is an over-simplification, surprisingly little is known about what distinguishes NP cells from articular chondrocytes and how this influences the production of metalloproteinases involved in normal tissue homeostasis as well as tissue

degradation.

In this chapter I aim to profile all of the known MMP, ADAMTS, and TIMP genes expressed by NP cells as degradation of the nucleus appears the controlling step in degradation of the whole disc. I also compare the expression profile with that of articular chondrocytes. These data will aid in the understanding the regulation of matrix turnover and degradation in the IVD. In addition, they provide useful comparative information between the cellular biology of the cartilage and the IVD in regard to expression of agents involved in matrix degradation.

3.2 Experimental plan

Experimental plan is summarized below by the schematic flow charts (Figure 3.1). The general procedures in the flow charts have been developed under Chapter 2 Materials and Methods. The primer sequences used to amplify a number of MMP, ADAMTS and TIMP bovine genes are listed in Table 3.1.

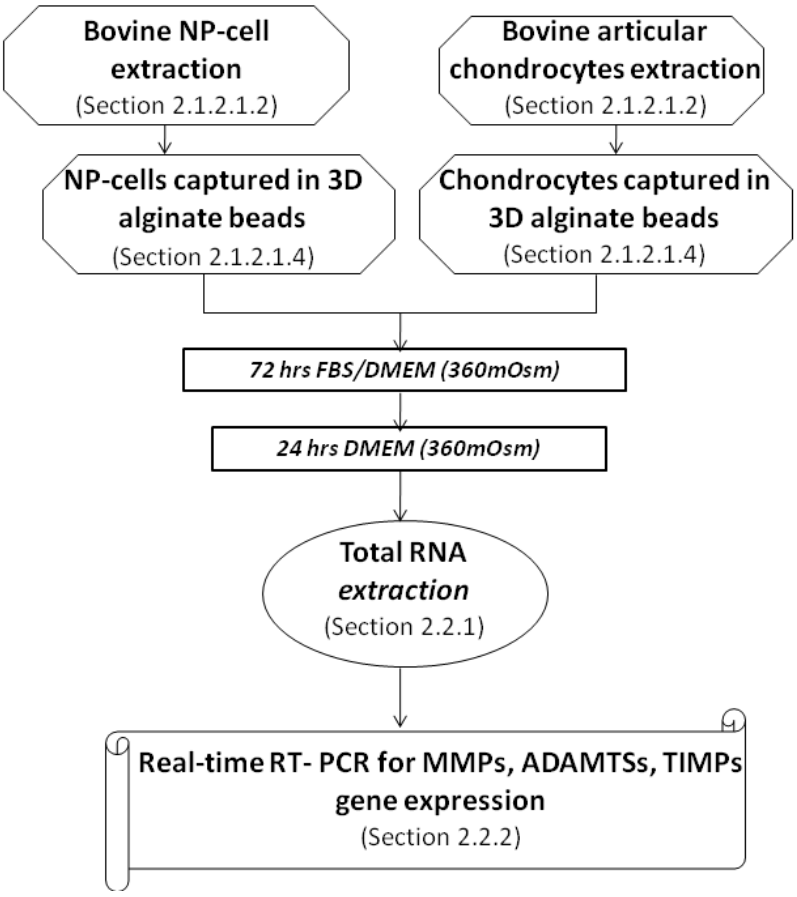


Figure 3.1 Scheme of the experimental design of differential gene expression profiling between NP cells and articular chondrocytes

Briefly bovine caudal discs and metacarpal-phalangeal cartilage were dissected from 18-24 month old (section 2.1.2.1.1). The cells of nucleus pulposus (NP) from the upper 6 coccygeal discs and of articular cartilage were extracted by enzymatic digestion (section 2.1.2.1.2). Three tails and one joint were used for each cell extraction. Three independent cell

extractions and cultures were carried out for the work described here. NP cells and chondrocytes were each cultured in alginate beads (section 2.1.2.1.4) at an osmolarity of 380mOsm by NaCl addition (section 2.1.2.1.6.1) for 72 hrs in DMEM containing 10% foetal bovine serum, followed by 24 hrs culture in serum-free DMEM. RNA was extracted from the beads and analysed for gene expression of MMPs, ADAMTSs and TIMPs by RT-PCR (section 2.2). The real time RT-PCR used here was with the help of Dr David Young in Newcastle who provided the primers, technical system and data analysis.

3.2.1 Statistical analysis

Levels of mRNA for each gene were corrected using the 18S rRNA levels as described above, and all statistical tests were performed using these log ratios on the samples from a quintuplicate experiment. The significance of differences between the disc NP cells and articular chondrocytes was determined using Student's T test. Data presented are from a single experiment, but the pattern shown is representative of that from three individual experiments.

Gene	Sequence(5'-3')	Gene	Sequence(5'-3')
MMP-7	GGAGAAGCCCAGATGTGGAG	*ADAMTS-2*	GGGCCTACCTCCAGCAGAAGC
	TGGATGATTTGGGAATAGCGA		GCCCAGCCGCACCTCGTCAC
MMP-10	TGGGCTGTCAGAAGAGTT	*ADAMTS-6*	ATCCACAACAGGCAGTATCTAA
	TTATCAGAAGTAGCTACATCAGT		CTTGCAAGTATCCTGTGTAATGA
	T		C
MMP-11	CTTTGACGCAGCCGCCACCATC	*ADAMTS-7*	CAGAGGACAGCCAGAGCCTA
	GTTCTTCTCGGAGCCCCACACCA		CAGAAACCTCATTGGTCCTCTC
	G		
MMP-15	CTGCGGCGGCGGAGGAAGC	*ADAMTS-10*	GCCAAGCCACCAGCCACCAT
	CCAAACCAGGGCCAGGGAAATA		CCCAGCCCGCGCACATCCA
	GG		
MMP-17	CTGACCAAGTGGAACAAGAGGA	*ADAMTS-12*	GGCAGGGACCATCTTTCAATACG
	AC		A
	TGTGGTGGTCGCCCGGGAAGAAG		CCCACGGCCCTTCTTCACG
	G		
MMP-19	TACCGGCCCTACTTCAAGC	*ADAMTS-13*	CTGCGGAGGCGGCGTGATTAC
	TTGCCATAGAGAGCCTGGAT		GTCTTCTCGCAGGCCTGGGTGTT
			G
MMP-20	CCTTCGATGCTGTGACGAT	*ADAMTS-14*	AGCTGATGTCCGGGAACTTGTGA
	AGAAAATCCGGTCCCTGAAG		AGAGGTCCTGGGAGCGGTGTGG
MMP-21	TCAGGCCCACTCAGAAGATG	*ADAMTS-16*	TTGCCATTGTCGCCCTGATTCTTC
	CAGGGGATTTGGGATGTGAGG		GCATATGTCCAGGCCGGTGAGTA
			A
MMP-23	CGCTTCTACCCCGTCAAC	*ADAMTS-17*	GACTACTCCGGCTGCTACGA
	GTCGAAGCAGTGGTGCAG		GCAGGTCGACGAGCACTTA
MMP-24	GGAGGGCAGGATAACCAGA	*ADAMTS-18*	CCTTGTGATAAAAACAGCTTGGA
	GTGGAAAACACGGTCTTTGACT		CGGAAAGGCTTGCTGTTATACT
MMP-25	GAGGGGACGGACCTGTTT	*ADAMTS-19*	TGGTGAATGGATTAAAGGACAA
	GGCCTCATGATGGAGTCG		CCAAATCTTCCTTGCTACATCG
MMP-27	GGTGTGGGGTGCCTGACG	*ADAMTS-20*	CCCTGCCATGGTGATTGT
	CCCTTTGGAAATCTTGGTGAATA		AAGTTCCTGGCATTCTCTGTC
MMP-28	GGCACTCGCTACTATGTGCTG		
	AGGCCTCGGGGGTAGTAG		

Table 3.1 Sequence data for bovine metalloproteinase and inhibitor primers for real-time PCR

3.3 Results

The expression of 22 known bovine MMP genes, 18 ADAMTS genes, and 4 TIMP genes at the mRNA level was determined in bovine disc NP cells and articular chondrocytes by real-time RT-PCR. The absolute levels of expression varied from experiment to experiment; results are given for only one experiment in all the figures but the pattern shown is typical for that seen in all three individual experiments. The cells were from animals of the same age and were cultured under identical conditions in alginate beads for 96 hours.

3.3.1 Summary of gene expression levels

Genes showing significant difference in expression level between NP cells and articular chondrocytes are summarised by P value and fold in Table 3.2. Expression of most MMP genes was detected in bovine NP cells and articular chondrocytes. Of all the MMP genes (Figure 3.2) quantified, MMP-3 was the most highly expressed in both NP cells and chondrocytes. In general, patterns of expression levels were similar between the two cell types (Figure 3.2); levels of MMP-9, -15, -21, -23, -25 were similar in both NP cells and chondrocytes. Low expression levels of MMP-10,-11,-17,-20 were found in both NP cells and chondrocytes. While expression of MMP-12 and MMP-27 was low in chondrocytes, it could not be detected in NP cells.

All ADAMTS genes were expressed by NP cells and articular chondrocytes, but none was highly expressed. Medium levels of ADAMTS-1,-2,-4,-5,-6-7,-8,-9,-15,-17,-18,-19,-20 were detected in NP cells and chondrocytes (Figure 3.3). The expression levels of

ADAMTS-10,-13,-14,-16 were low in both cell types.

All TIMPs with the exception of TIMP-4, were expressed by both cell types (Figure 3.4). The expression level of TIMP-1,-2 were medium in both NP cells and chondrocytes. However TIMP-3 levels were noticeably lower in NP cells than in chondrocytes.

Gene	P	Fold
Higher expression in NP		
MMP-2	<0.05	1.76
MMP-14	<0.01	1.78
ADAMTS-1	<0.05	5.61
ADAMTS-2	<0.05	3.47
ADAMTS-17	<0.01	1.92
TIMP-1	<0.01	2.94
Lower expression in NP		
MMP-1	<0.001	55.6
MMP-3	<0.001	3.34
MMP-7	<0.01	2.46
MMP-8	<0.001	2.67
MMP-10	<0.01	2.66
MMP-11	<0.05	1.64
MMP-13	<0.05	2.79
MMP-16	<0.001	8.68
MMP-19	<0.001	2.9
MMP-20	<0.01	2.36
MMP-21	<0.01	1.95
MMP-23	<0.05	3.14
MMP-24	<0.01	2.49
MMP-28	<0.01	2.28
ADAMTS-4	<0.01	5.46
ADAMTS-5	<0.001	8.23
ADAMTS-6	<0.001	1.73
ADAMTS-14	<0.01	2.34
ADAMTS-18	<0.001	2.68
ADAMTS-19	<0.01	1.87
TIMP-3	<0.05	240.16

Table 3.2 Summary of metalloproteinases whose gene expression levels differ significantly between disc NP cells and articular chondrocytes

3.3.2 Comparison of gene expression between bovine disc NP cells and articular chondrocytes

Figure 3.2 and Table 3.2 shows details of MMP expression levels in NP cells and chondrocytes. Of the MMPs, only MMP-2 and MMP-14 gene expression was higher ($p<0.05$ and $p<0.01$ respectively) in NP cells than in chondrocytes. MMP-1,-3,-8,-16, and MMP-19 expression was much lower ($p<0.001$) in NP cells while MMP-12 and MMP-27 were not detected. Of the gelatinases, expression of MMP-2 (gelatinase-1) was 1.76 fold higher in NP cells, while MMP-9 expression did not differ significantly from that in chondrocytes. The expression of MMP-1 was considerably lower in NP cells than chondrocytes (55.6 fold). All the other classical collagenases, MMP-8,-13 were also expressed at lower levels (2.67 fold and 2.79 fold respectively) in NP cells than in chondrocytes as were the stromelysins, MMP-3,-10,-11 (3.34 fold, 2.66 fold and 1.64 fold respectively). Of the membrane-type MMPs, MMP-14 (Membrane-type 1 MMP) was 1.78 fold higher in NP cells than that in chondrocytes, whilst the opposite was true for MMP-16 and MMP-24 (8.68 and 2.49 fold respectively). The expression levels of MMP-15,-17,-25 did not differ between the two cell types. Matrilysin, MMP-7, expression was 2.46 fold lower in NP cells than that in chondrocytes.

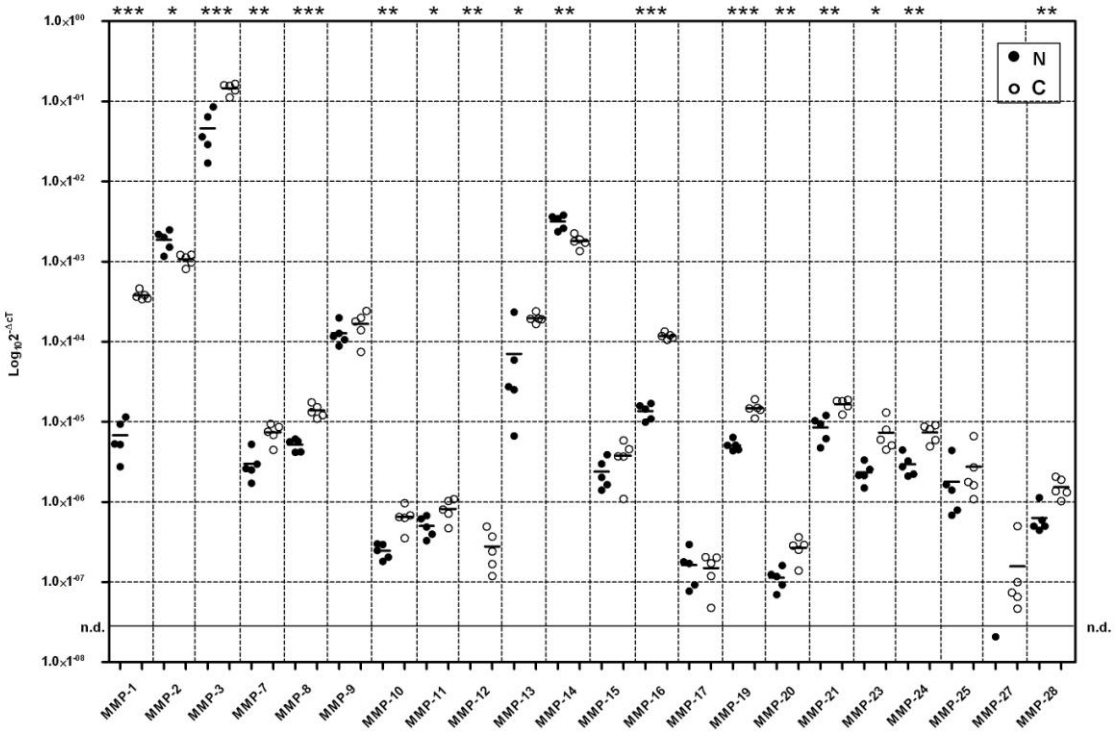


Figure 3.2 Comparative expression of mRNA, relative to 18S ribosomal RNA, for MMP genes isolated from bovine disc nucleus cells and articular cartilage.

Horizontal bars show mean results from quintuplicate of a single experiment. Samples on or below solid line were not detectable (n.d.). * represents $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for mean differences between disc nucleus cells (N, ●) and articular cartilage (C, ○).

Figure 3.3 and Table 3.2 shows details of ADAMTS gene expression levels. Of all the ADAMTS genes, only ADAMTS-1,-2 and ADAMTS-17 were more strongly expressed (5.61 fold, 3.47 fold and 1.92 fold respectively) in NP cells. ADAMTS-4,-5,-6,-14,-18, and ADAMTS-19 were all expressed at lower levels in NP cells. Of the known aggrecanases [81], expression of ADAMTS-1 was 5.61 fold higher in NP cells and ADAMTS-4 and ADAMTS-5 lower (5.46 fold and 8.23 fold respectively), whilst ADAMTS-8, ADAMTS-9 and ADAMTS-15 did not differ significantly from that in chondrocytes. Of the procollagen propeptidases ADAMTS-2 expression was 3.47 fold higher in NP cells, while ADAMTS-14

was lower. Of the remaining ADAMTS genes, expression of ADAMTS-7,-10,-12,-13,-16, and ADAMTS-20 were not significantly different between NP cells and chondrocytes.

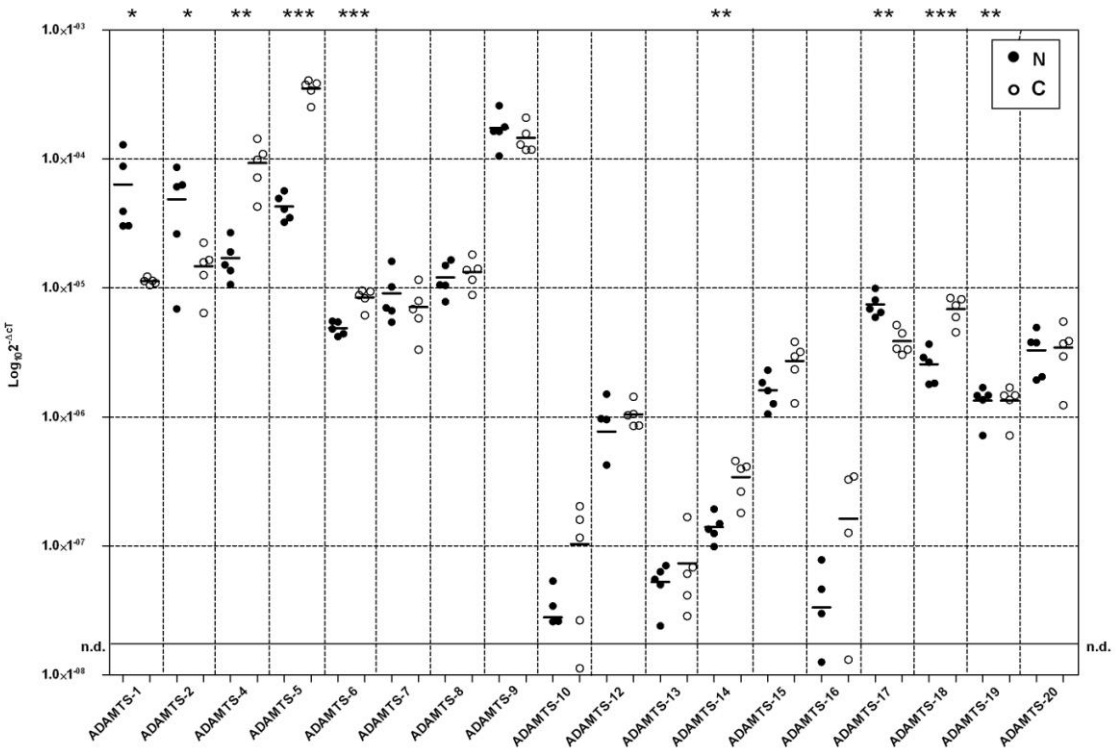


Figure 3.3 Comparative expression of mRNA, relative to 18S ribosomal RNA, for ADAMTS genes isolated from samples of bovine disc nucleus cells and articular cartilage.

Horizontal bars show mean results from quintuplicate of a single experiment. Samples on or below solid line were not detectable (n.d.). * represents $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for mean differences between disc nucleus cells (N, ●) and articular cartilage (C, ○).

Figure 3.4 and Table 3.2 gives details of TIMP expression levels. All TIMP genes expressed were seen in both NP cells and articular chondrocytes. TIMP-1 expression was 2.94 fold higher in NP cells whilst TIMP-3 expression was noticeably 240.16 fold lower. No significant difference between NP cells and chondrocytes was observed for expression of TIMP-2. TIMP-4 expression was not detected in either cell type.

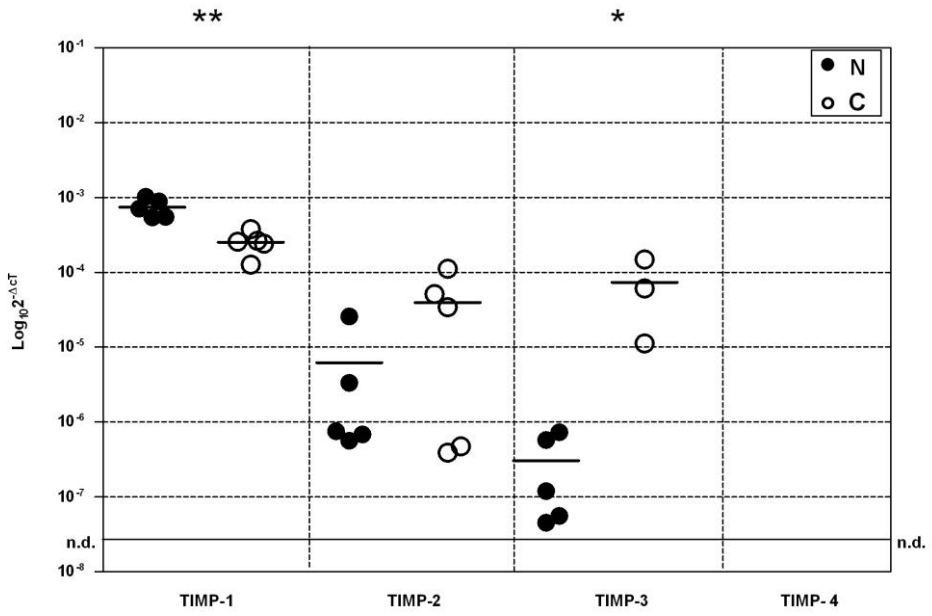


Figure 3.4 Comparative expression of mRNA, relative to 18S ribosomal RNA, for TIMP genes isolated from samples of bovine disc nucleus cells and articular cartilage.

Horizontal bars show the mean expression. Samples on or below solid line were not detectable (n.d.). * represents $p<0.05$ and ** $p<0.01$ for mean differences between disc nucleus cells (N, ●) and articular cartilage (C, ○).

3.4 Discussion

3.4.1 Differences in expression profiling of metalloproteinases and their inhibitors between disc NP cells and articular chondrocytes

This chapter reports a profile of the expression of genes from the MMP, ADAMTS, and TIMP families in cells from the nucleus pulposus of adult bovine intervertebral discs and compare the results with findings in bovine articular chondrocytes. The animals were in the identical age range and the cells were cultured under identical conditions for 96 hours. Under our culture conditions we found that there are a total of 27 genes with statistically significant differences in expression levels between nucleus cells and chondrocytes (Table 3.2). And it was noted that MMP-12 and MMP-27 genes were not detected in bovine NP cells. These differences, whether they are intrinsic or arise because of different responses to their common culture conditions, add weight to the suggestion that there are phenotypic and metabolic differences between these cell types. Furthermore the results identify proteinases that have not previously been implicated in disc metabolism, viz. MMP-11, MMP-16, MMP-19, MMP-23, MMP-28, ADAMTS-2, and ADAMTS-14. As discussed below, all these proteinases have the potential to degrade matrix components, are upregulated in degradative disorders in other tissues and thus possibly play an important role in the processes of disc degeneration.

While the roles of MMP-11, MMP-16, MMP-19, MMP-23 and MMP-28 in the disc are unexplored, there is now increasing evidence that all of these MMPs are involved in

degradation of other cartilaginous tissues and that their activity is upregulated in pathological conditions. MMP-11 (stromelysin 3) has only a weak activity toward extracellular matrix macromolecules [226] but is known to be up-regulated in osteoarthritic (OA) chondrocytes, cartilage and synovium [227, 228] and is also present a higher levels in painful and ruptured tendon [229]. MMP-16, namely MT3-MMP, is one of the 4 members of MT-MMP family, which is known to activate the zymogen of MMP-2 [230]. It plays a role in chondrogenesis and cartilage development [231, 232]. Its expression is increased in human OA cartilage [233] and synovium [228]. MMP-16 is also intensely expressed in rheumatoid (RA) synovium and synovial tissue [234, 235], and is higher in the painful compared to normal tendon [229]. MMP-19 is a novel member of the MMP family which is widely expressed in body tissues, including the synovium of normal and RA arthritic patients. Although the physiological substrates of MMP-19 are unknown, in vitro it is able to degrade both aggrecan and cartilage oligomeric matrix protein (COMP) which are found in cartilage and rat disc [96, 236]. MMP-23 expression has been shown to be up-regulated in OA cartilage when compared with macroscopically normal tissue from fracture to the neck of femur [228], however the substrates and the functions of MMP-23 in cartilage matrix turnover are as yet unknown [237]. MMP-28, a recently identified member of the MMP family is also found elevated in cartilage from both OA and RA patients, which suggests a role for the gene in cartilage degradation and/or repair [228, 233, 238]. The macrophage elastase MMP-12 is known to be expressed by chondrocytes [239] and may decrease during OA progression [228]; excessive MMP-12 expression can exacerbate articular connective tissue and cartilage degradation [240]. Virtually no studies have concentrated specifically on MMP-27, and scarce general

data are available so far. However mRNA of MMP-27 was shown to be present in bone, kidney, heart and several other tissues in the rat [241]. The high expression levels of MMP-27 in rat bone are to some degree in line with findings in human cartilage, where high expression has been reported and implicated in osteogenesis [233]. We found that both cells types expressed ADAMTS-2 and ADAMTS-14, which are procollagen N-proteinases involved in collagen biosynthesis [242], with ADAMTS-2 expression significantly higher and ADAMTS-14 expression much lower in NP than in articular chondrocytes. Both ADAMTS-2 and ADAMTS-14 expression is increased in osteoarthritic cartilage [228, 233]. Thus, even though most of these proteinases were expressed at lower levels by NP cells than chondrocytes in the cultures, the role of these MMPs in degenerate and painful discs thus warrants investigation.

TIMP-4 expression could not be detected in either bovine NP cells or articular chondrocytes. This was somewhat surprising because, although there are no reports examining the expression of TIMP-4 in IVD, expression of the gene has been detected in bovine nasal cartilage [225]. Expression of the gene has also been detected in human femoral head cartilage although reports of differential expression of the gene in OA are somewhat conflicting with one group finding an increase [243] and another a decrease [228, 233].

3.4.2 Bovine and human cartilage have similar metalloproteinase expression profiles

Obtaining healthy human NP cells for biological research is difficult. The general properties of bovine coccygeal discs, such as cell phenotype, composition, swelling pressure, and

proteoglycan synthesis are similar to those of human lumbar discs and they thus have been suggested as a suitable model for the study of the metabolic and other properties of human lumbar discs [244, 245]

Although we have no direct human disc cell data for comparison there are studies on proteinase and TIMP expression for human chondrocytes. My study on bovine articular chondrocytes found metalloproteinase expression profiles similar to those reported for human chondrocytes. When compared to the most comprehensive study of MMP, ADAMTS and TIMPS gene expression by human chondrocytes (from femoral head cartilage [228]), my data on bovine articular chondrocyte gene expression are very similar especially in regard to MMP-1, MMP-2, MMP-3, MMP-12, MMP-17, MMP-28, ADAMTS-2, ADAMTS-4, ADAMTS-5, ADAMTS-14, ADAMTS-15, ADAMTS-16, ADAMTS-17, ADAMTS-20, TIMP-2, TIMP-3 even though Davidson et al. [228] isolated RNA directly from cartilage while here chondrocytes were isolated from cartilage by sequential enzymatic digestion and cultured in alginate. This similarity between human and bovine cartilage in terms of expression patterns of metalloproteinases and inhibitors might suggest that bovine coccygeal disc cells cultured in alginate may likewise show similar expression patterns to those of human disc NP cells.

In general, the role of metalloproteinases in disc physiology and pathology is still very unclear. Further work is required to confirm that the gene expression profile reported here is reciprocated at the protein and enzymatic level and to determine changes that occur upon

initiation of disc degeneration.

Chapter 4 The role of osmolarity upon disc metabolism by NP cells encapsulated in alginate beads and NP explant

4.1 Introduction

During IVD degeneration, the loss of proteoglycan in degenerate discs [246] has a major effect on the disc's load-bearing behavior. With loss of proteoglycan, the osmotic pressure of the disc falls [19] and generates a hypo-osmotic environment. Matrix Proteinases, such as MMPs are assumed to be involved in the process of proteoglycan loss [72, 73, 84, 86, 88, 89, 91, 247, 248].

Osmolarity also affects behavior of isolated disc cells and explants as shown in a number of publications [130, 137, 249, 250]. However all these studies examined only the short-term (hours) response to osmotic shock. The effect of the osmotic environment on NP explants and NP cells encapsulated in alginate beads cultured for longer periods under different osmotic conditions has not been studied thus it is not known if these cells adapt to changes in their osmotic environment. In renal cells, activation of members of the mitogen activated protein (MAP) kinase family [141] appears responsible for adaptive changes to osmotic stress [150, 151, 251, 252] and matrix metalloproteinases were stimulated by hyperosmolarity via a JNK pathway in human corneal epithelial cells [253]. Whether these pathways are stimulated by changes in osmolarity in disc NP cells is not known.

The aim of this work is to investigate the long-term response of NP explants and NP cells to

changes in osmolarity and the involvement of MAP kinases in any short-term adaptive process.

4.2 Experimental plan

The experimental plans are summarized below in schematic flow charts (Figure 4.1). The general experimental methods used have been described in Chapter 2 Materials and Methods. The scheme for studying involvement of MAP kinases is given in section 4.2.1 and Figure 4.2).

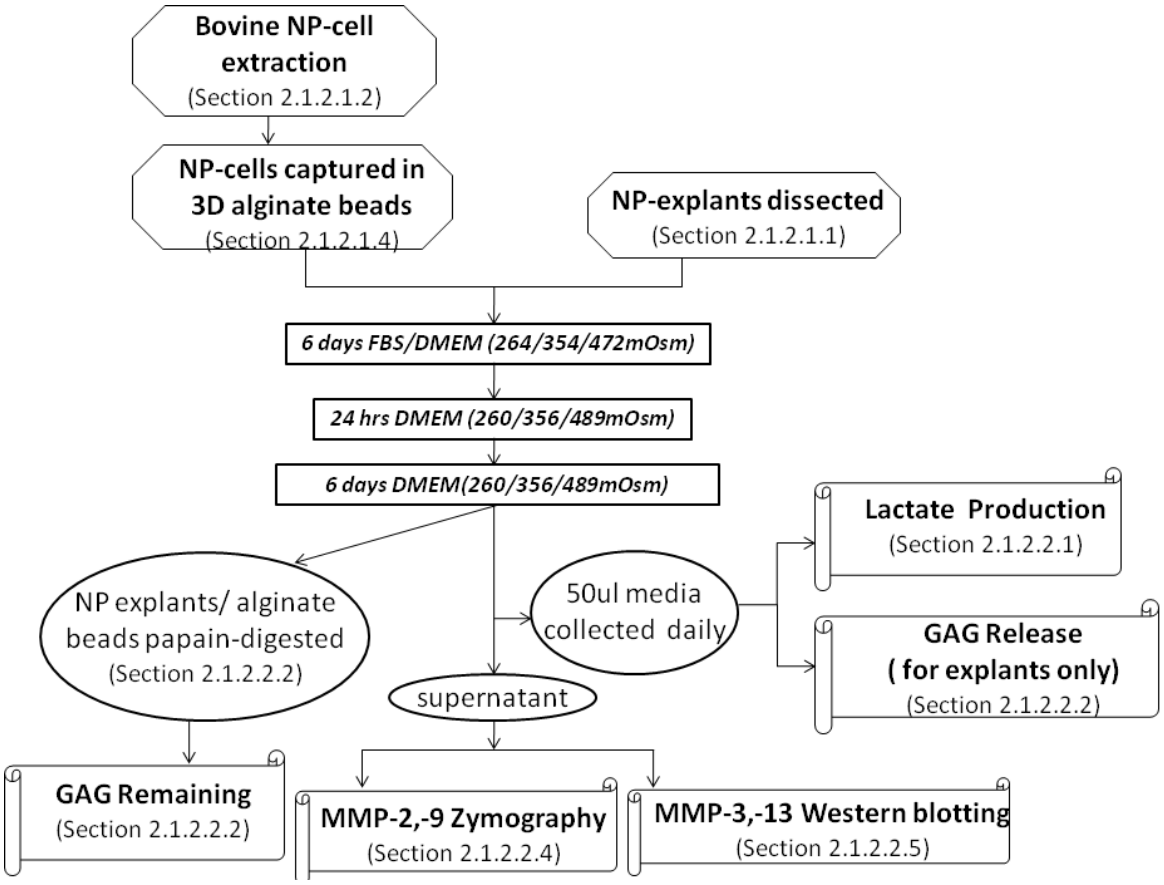


Figure 4.1 An outline of the experimental plan aimed at investigating long-term effects of changes in osmolarity on disc cells.

To examine the long-term effect of osmolarity on disc NP cells (Figure 4.1), bovine NP tissue

was dissected from bovine caudal discs from 18-24 month steers (section 2.1.2.1.1). Either cells were extracted by enzymatic digestion (section 2.1.2.1.2) or tissue was dissected into explants of about 120mg wet weight (section 2.1.2.1.1). DMEM at three different osmolarities was produced by NaCl addition (section 2.1.2.1.6.1) to cover hypo-, iso- and hyper-osmotic conditions, viz. 260, 356 and 489 mOsm. Cells were encapsulated in alginate beads (section 2.1.2.1.4) and beads and explants both cultured for six days in DMEM/FBS , and then for 24 hours in serum free DMEM at each osmolarity in order to allow samples to adapt to a serum free environment. NP slices and beads were then cultured for a further six days in DMEM. Media were collected daily for lactate (section 2.1.2.2.1) and GAG analysis (section 2.1.2.2.2) and at the end of the culture period were analysed in addition for MMP-2, -9 by zymography (section 2.1.2.2.4), and for MMP-3, -13 by western blotting (section 2.1.2.2.5). Papain digests of the remaining beads and explants were analysed for remaining GAG (section 2.1.2.2.2).

4.2.1 Effect of inhibitors to MAP kinases

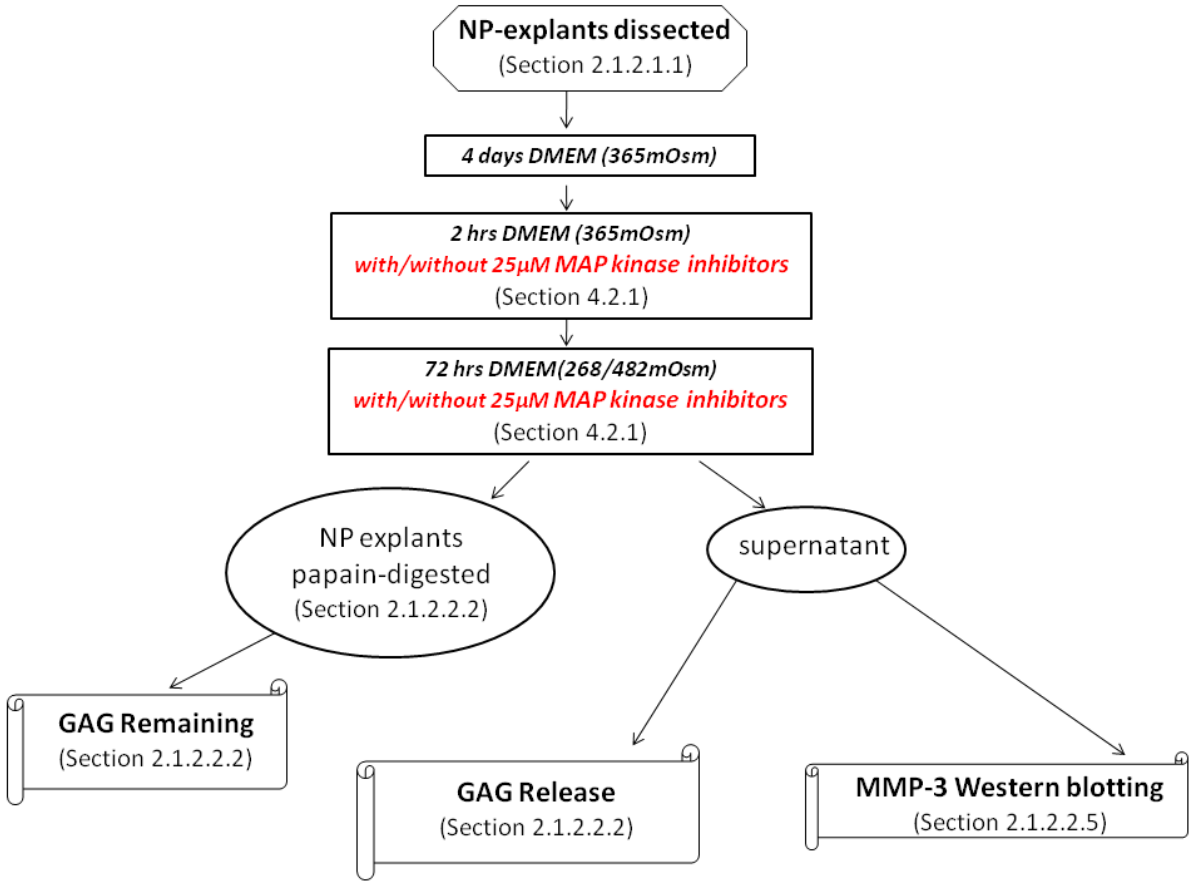


Figure 4.2 An outline of the experimental plan on the role of MAP kinases on the effect of osmolarity to disc NP cells in relation to GAG loss and MMP production.

To examine the role of MAP kinases on the effect of osmolarity to disc NP cells (Figure 4.2), bovine NP tissue was dissected from bovine caudal discs from 18-24 month steers (section 2.1.2.1.1). 120mg quantities of NP explants were pre-incubated at 365mOsmDMEM for 4 days and 2 hours prior to the end of pre-incubation, samples were treated with 25 μM of inhibitors to MAP kinases (SB203580, U0126 and JNK inhibitor II), and vehicle (DMSO). The medium was then changed to either 268 or 482mOsm DMEM with inhibitors following 72 hours exposure to altered osmotic conditions, GAG remaining from explants after papain-digestion and GAG release from sample media (section 2.1.2.2.2) and MMP-3

(section 2.1.2.2.5) from sample media were measured.

4.2.2 Statistical analysis

Data are presented as the mean ± the standard error of the mean (SEM) of at least three individual determinations ($n \geq 3$). Triplicate determinations were carried out within each individual experiment. Significant differences for lactate production and GAG release were determined using two-way ANOVA and Bonferroni post-tests; significant differences for GAG remaining and MMPs production were determined using one-way ANOVA and Dunnett test; with a P value of 0.05 or less considered significant.

4.3 Results

4.3.1 The role of osmolarity upon lactate production

Bovine NP explants and NP cells encapsulated in alginate beads were pre-incubated for 6 days in 10% FBS DMEM medium at 260, 356 or 489mOsm, before being exposed to 260, 356 or 489mOsm FBS-free DMEM for 6 more days (Figure 4.1). Culture media were changed every two days. Culture media were removed every day after the change in osmotic conditions and assayed for lactate levels.

Figure 4.3 shows rates of lactate production by NP explants (a), and NP cells in alginate beads (b), in response to incubation at 260, 356 or 489mOsm. There was no difference in the amount of lactate produced under all osmotic conditions in either culture system; lactate rate remained constant throughout the 6 day time course with the average rate being 280.7 ±111.3

nmol/million cells/hr for NP explants (based on unpublished data from Dr. Olga Boubriak of 0.0057 million cells/mg wet weight of NP), and 222.9 ± 70.3nmol/10^6 million cells/hr for NP cells. The average lactate production rates during 6 days incubation were constantly higher for cells in NP explants than that in alginate beads ($p=0.002$, 0.028 and 0.003 respectively under 260,356 and 489mOsm).

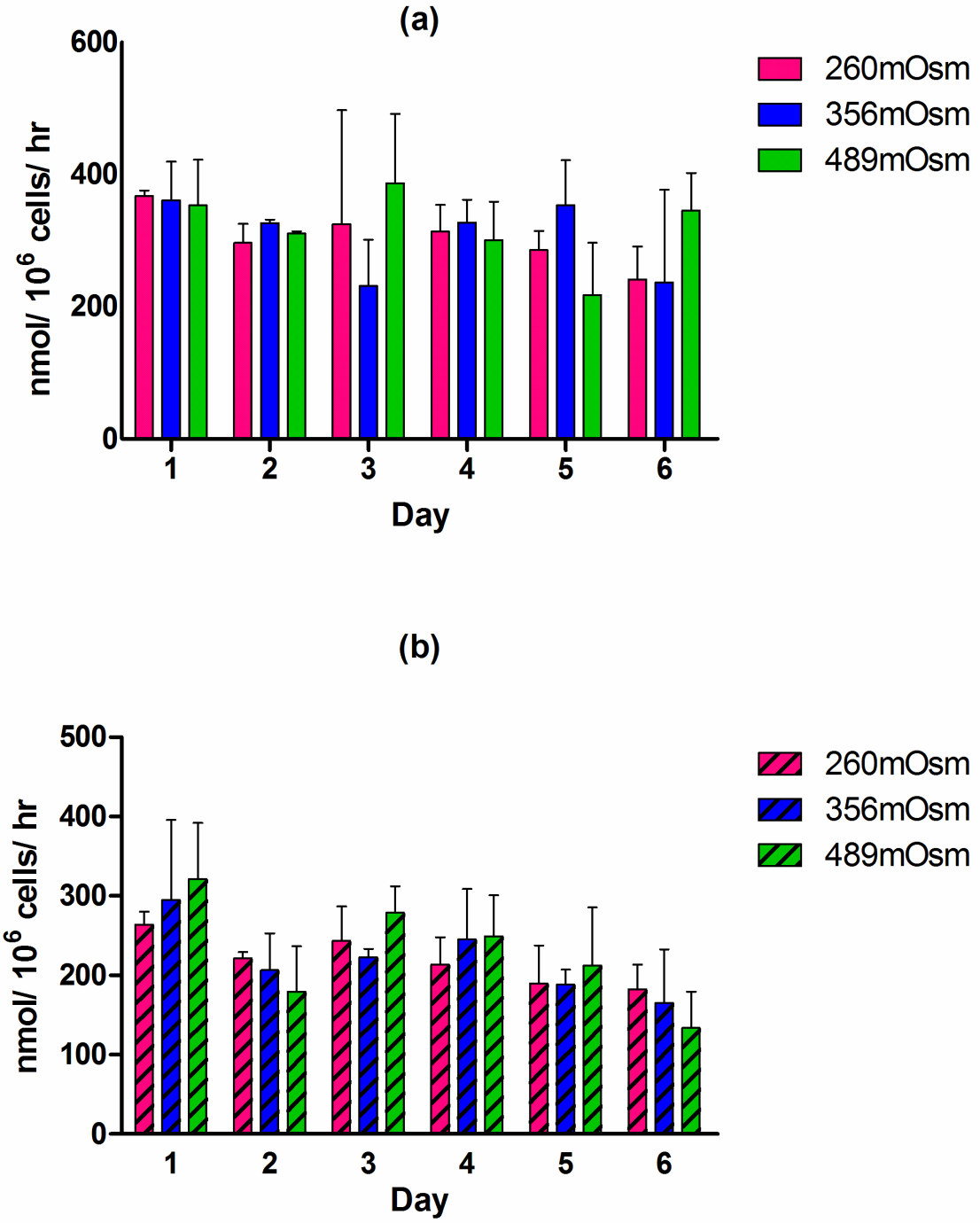


Figure 4.3 The role of osmolarity on lactate production.

Rates of lactate production over 6 days incubation for bovine NP explants (a) and NP cells encapsulated in alginate beads (b) exposed to 260, 356 or 489mOsm. Conditioned medium was removed daily and assayed for lactate. Results are given as mean ± SEM (n=3).

4.3.2 Matrix turnover

4.3.2.1 The effect of osmolarity upon total GAG release

Bovine NP explants and NP cells in alginate beads were pre-incubated for 6 days in 10% FBS DMEM medium at 260, 356 or 489mOsm, before being exposed to 260, 356 or 489mOsm FBS free DMEM for 6 more days. Culture media were changed every two days. Aliquots of 50µl media were removed daily after the onset of experimental conditions and assayed for GAG levels.

No GAG release was detected from alginate beads. Figure 4.4 shows the level of total GAG release after the initial 6 days incubation and following exposure of explants to altered osmotic conditions. Although the amount of GAG release at all differing osmotic conditions shows no differences of statistical significance, a trend of increased GAG release at 260 and 356mOsm relative to the level at 489mOsm is evident. These differences were maintained from day 2, with absolute levels of GAG increasing by approximately 2-fold relative to levels at day1, for all osmotic conditions.

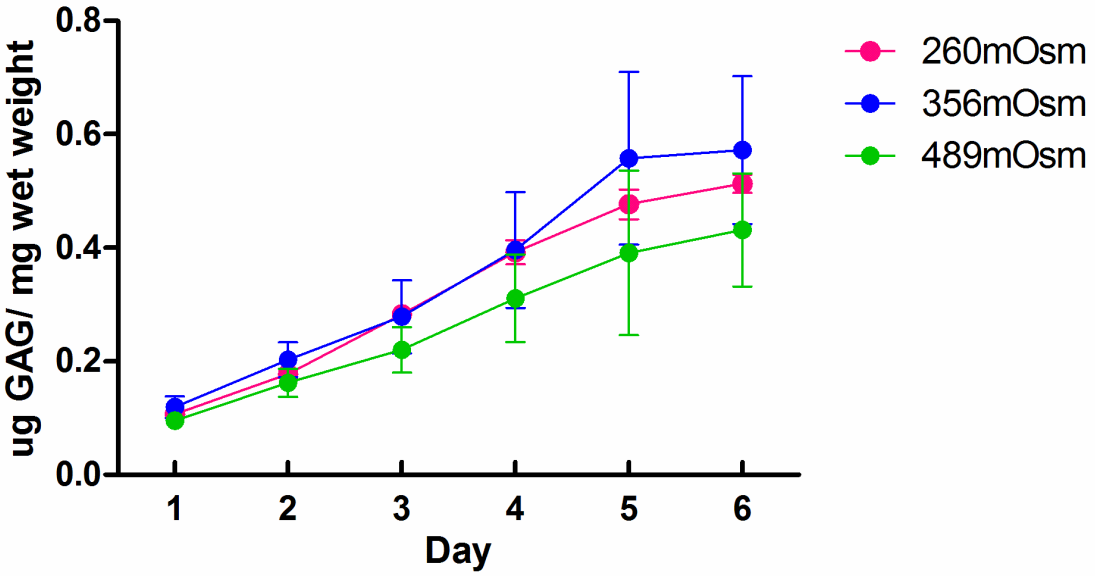


Figure 4.4 The role of osmolarity on GAG release of NP explants.

Level of accumulative GAG release from NP explants exposed to 260, 356 or 489mOsm was assayed for GAG content every day for 6 days. Results are given as mean ± SEM (n=3).

4.3.2.2 The effect of osmolarity upon GAG remaining

Bovine NP explants and NP cells in alginate beads were pre-incubated for 6 days in 10% FBS DMEM medium at 260, 356 or 489mOsm, before being exposed to 260, 356 or 489mOsm FBS free DMEM for 6 more days. Culture media was changed every two days. After six days following exposure to the altered osmotic conditions, total GAG accumulation was assayed. GAG concentration in NP explants was measured in papain digests of tissue. Alginate beads were depolymerised with citrate buffer containing papain, to release NP cells, cell attached matrix (CM) and far removed matrix (FRM) [254].

Figure 4.5 (a) shows level of total GAG remaining in NP explants following their exposure to

altered osmotic conditions. After 6 days incubation, no significant differences in the level of GAG remaining were evident although the trend seen is in agreement with results shown in Figure 4.4 that concentration of GAG remaining in the explants cultured at high osmolarity was higher than in explants cultured at lower osmolarities. (b) shows levels of total GAG remaining in NP cells in alginate beads at all differing osmotic conditions was not significantly different following 6 days culture.

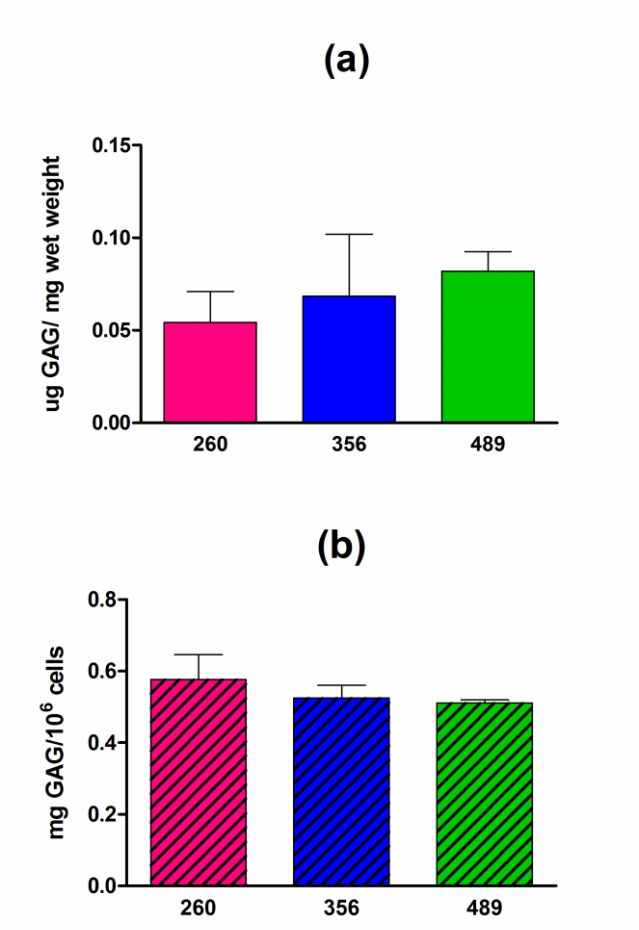


Figure 4.5 The role of osmolarity on GAG remaining.

Level of GAG remaining in NP explants (a) and NP cells encapsulated in alginate beads (b) exposed to 260, 356 or 489mOsm were assayed for GAG content after 6 days culture. Results are given as mean ± SEM (n=3).

Level of total GAG remaining in NP explants was also assayed by alcian blue staining [255].

After 6 days incubation, there was no evident difference in the level of GAG remaining in response to all differing osmotic conditions. Blinded grading of the alcian blue staining was carried out by two independent observers (Audrey Gilson and Robert Lee).

4.3.3 The effect of osmolarity upon MMP production

Accumulated expression of matrix metalloproteinases (MMPs) after culture for 3 days in DMEM FBS-free medium at 260, 356 or 489mOsm was assayed by gelatin zymography for MMP-2, -9, and by western blotting for MMP-3,-13.

Figure 4.6 shows level of MMP production by NP explants and NP cells in alginate beads following their exposure to altered osmotic conditions.

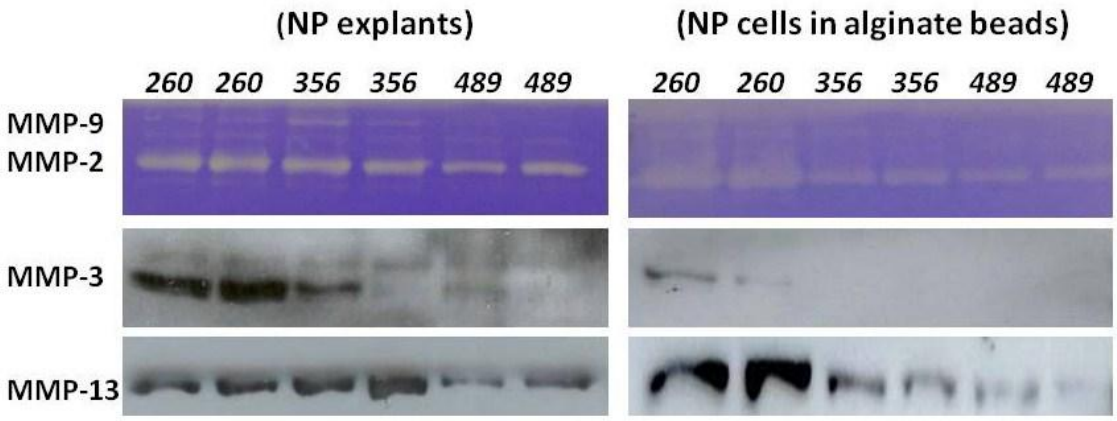


Figure 4.6 Typical zymogram and western blot showing effect of osmolarity on production of MMP2, MMP9, MMP3 and MMP13 for NP explants and NP cells cultured in alginate beads in DMEM with no FBS for 72 hours.

Figure 4.6 shows MMP expression was strongly influenced by the osmolarity of the culture medium. In general expression was greatest at 260mosm and fell with increase in osmolarity.

The effect of osmolarity was particularly evident for MMP-3 and MMP 13. MMP-9 and MMP-13 production appear to peak at 356 mOsm rather than 260mOsm for explants. MMP-3 produced by NP cells in alginate beads incubated at 356 and 489mOsm could not be detected.

The zymograms and western blots of all gels were scanned and differences in staining intensity of digitized bands were assessed using IMAGE J software; results are shown in Figure 4.7. Figure 4.7(a) shows after 3 days of explant culture, a marked decrease in the level of MMP-3 was evident in both 356 and 489mOsm medium, compared to that of 260 mOsm($p<0.01$). There was a trend toward lower production of MMP-2 at high osmolarity, while production of MMP-9 appeared maximal at 356 mOsm. While production of MMP-13 appeared independent of osmolarity, despite the large effect of osmolarity shown in the gel of Figure 4.6. Figure 4.7(b) shows level of MMPs produced by NP cells in alginate beads at the different osmotic conditions. After 3 days, a significant decrease in the level of MMP-2 was evident in response to both 356 and 489mOsm medium, compared to that of 260mOsm ($p<0.05$). There was a trend towards down-regulation of MMP-9 and MMP-13 production with increase in osmolarity but not at levels of significance. MMP-3 produced by NP cells in alginate beads incubated at 356 and 489mOsm could not be detected and hence its data are not shown in the figure.

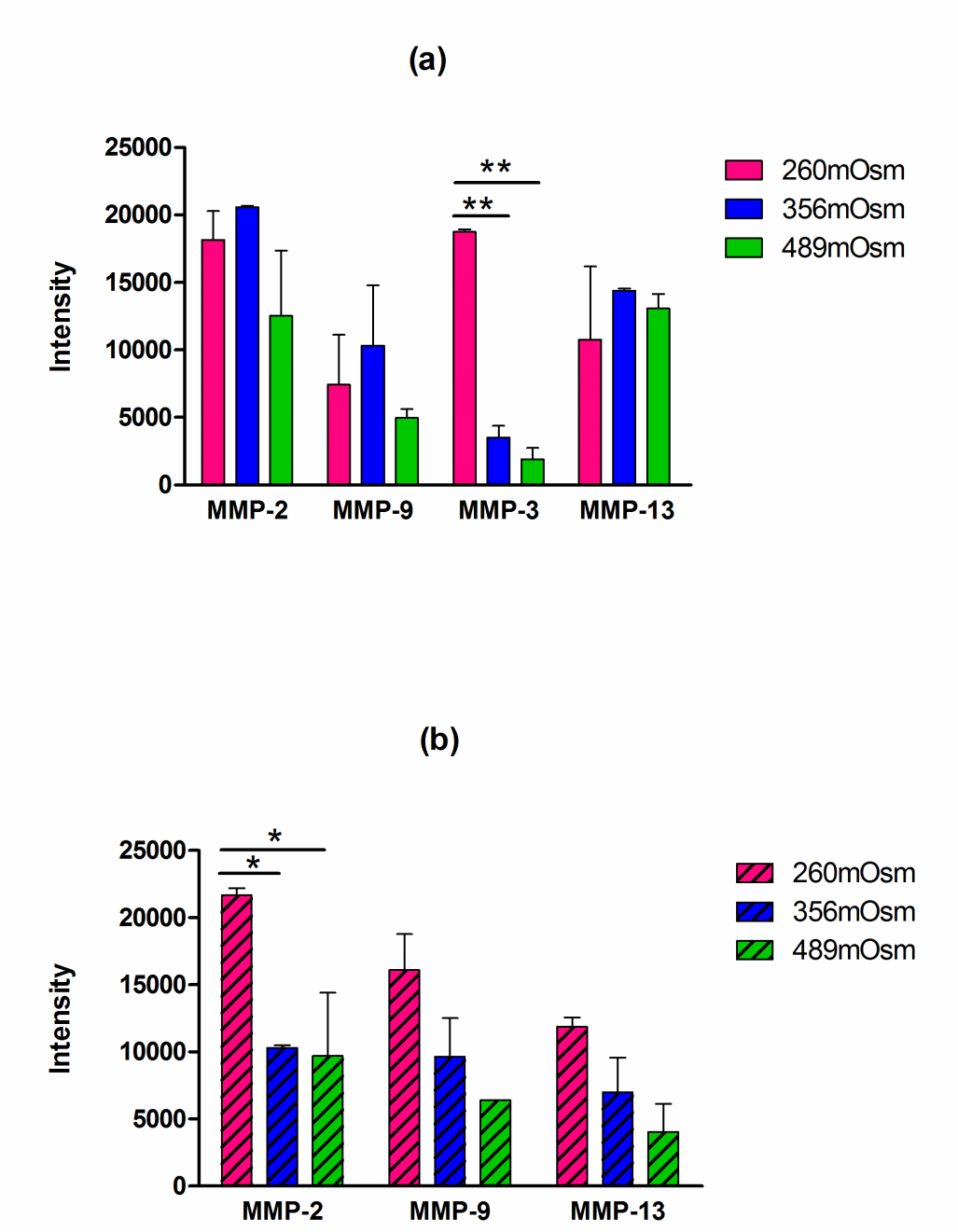


Figure 4.7 The role of osmolarity on MMPs production.

Level of MMP production from (a) NP explants and (b) NP cells encapsulated in alginate beads exposed to 260, 356 or 489mOsm for 72 hours were assayed as shown in Figure 4.6, and the zymograms and Western blots were scanned by Image J. Results are given as mean ± SEM (n=3). *p<0.05, **p<0.01.

4.3.4 The effect of MAP kinases in the adaptive response of NP explant to differing osmotic condition

4.3.4.1 The effect of MAP kinase inhibitors upon GAG release of NP explants

Figure 4.8 shows the effect of MAP kinase inhibitors, SB203580, U0126 or JNK inhibitor II, upon GAG release of NP explants in response to hyper- or hypo-osmotic conditions. After 72 hours exposure to experimental conditions, sample medium was removed and GAG concentration was measured. Under 268mOsm there was no significant effect of SB203580, U0126 and JNK inhibitor II compared to control.

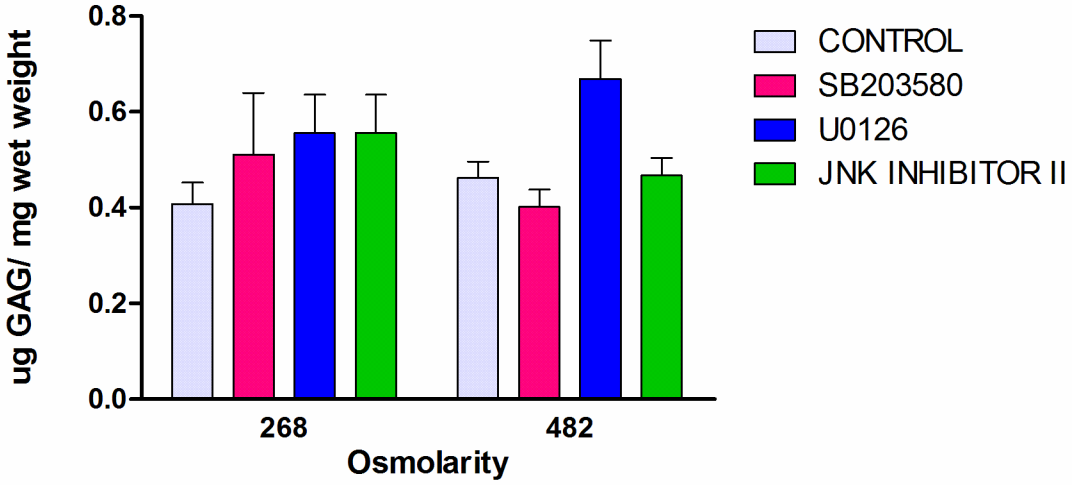


Figure 4.8 The effect of MAP kinase inhibitors upon GAG release from NP explants.
Level of GAG release by NP explants were maintained at 365mOsm for 4 days, before exposure to 268mOsm and 482mOsm, with or without the addition of MAP kinase inhibitor SB203580, U0126 or JNK inhibitor II. 72 hours after osmotic treatment GAG release was measured. Results are given as mean ± SEM (n=3).

4.3.4.2 The effect of MAP kinase inhibitors upon GAG remaining in NP explants

Figure 4.9 shows the effect of MAP kinase inhibitors, SB203580, U0126 or JNK inhibitor II,

upon GAG remaining. Following 72 hours exposure to 268mOsm and 482mOsm, GAG remaining was measured in papain digests of tissue. In response to hyper- or hypo-osmotic conditions, there was no significant difference in response to treatment by all the MAP kinases compared to control.

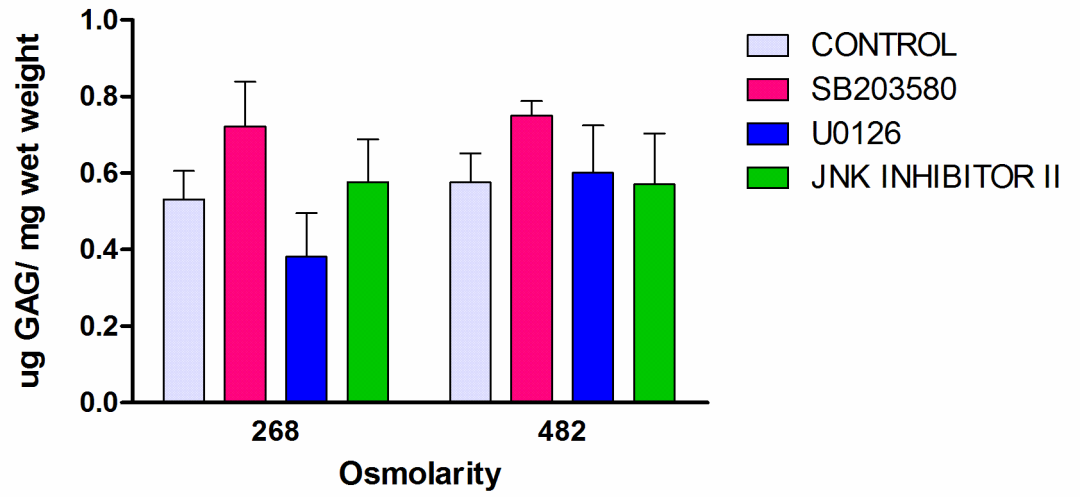


Figure 4.9 The effect of MAP kinase inhibitors upon GAG remaining in NP explants

Level of GAG remaining by NP explants were maintained at 365mOsm for 4 days, before being exposed to 268mOsm and 482mOsm, with or without the addition of MAP kinase inhibitors SB203580, U0126 or JNK inhibitor II. 72 hours after osmotic treatment GAG remaining was measured. Results are given as mean ± SEM (n=3).

4.3.4.3 The effect of MAP kinase inhibitors on MMP-3 production by NP explants

Figure 4.10 shows the results of a limited set of experiments examining the effect of MAP kinase inhibitors, SB203580, U0126 and JNK inhibitor II, upon MMP-3 production under 268mOsm or 482mOsm conditions after incubating disc explants for 72 hours in the presence of inhibitors (Figure 4.2). The control samples were incubated with DMSO only and as seen previously (Figure 4.7). MMP-3 production was greater at low than at high osmolarity. Intense stimulation, similar to that seen previously (Figure 4.7) was seen at 268 mOsm in the

presence of inhibitors of the ERK/MAP Kinase pathway and JNK pathway viz. U0126 and JNK Inhibitor (Figure 1.9) whereas no stimulation was seen using in the presence of SB203580 which inhibits the p38 MAPK pathway (Figure 1.9). MMP-3 production by contrast is repressed at high osmolarity (Figure 4.7). This repression was still evident in the presence of inhibitors of the JNK and the p38 MAPK pathway (viz. JNK-IN and SB203580 respectively), but repression of MMP-3 production was abolished in the presence of U0126, the inhibitor of the ERK/MAPK pathway. The results suggest that low osmolarity stimulates MMP-3 expression via the p38-MAPK pathway, and that high osmolarity inhibits MMP-3 expression via the ERK/MAPK pathway.

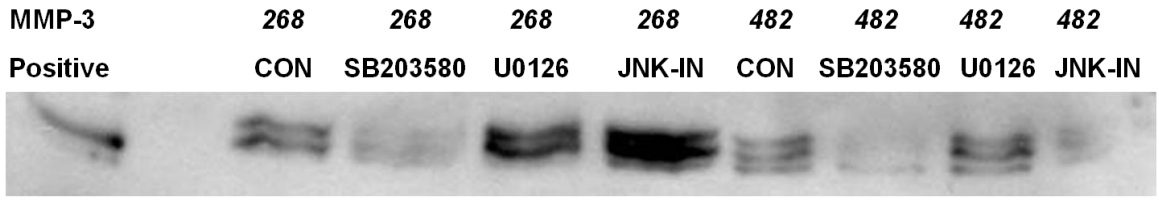


Figure 4.10 Typical western blot demonstrating the effect of MAP kinase inhibitors upon MMP-3 production by NP explants.

Level of MMP-3 productions exposed to 268mOsmand 482mOsmwere assayed by western blotting after 72 hours culture. Differences in staining intensity of digitized bands were scanned.

4.4 Discussion

The results of this chapter showed that extracellular osmolarity over the range from 260 to 489 mOsm regulates production of proteinases, particularly MMP-2 and MMP-3 by cultured NP explants and also by disc cells cultured in alginate. No effect on lactate was evident showing that osmotic change maintains cell metabolism and therefore results were not due to fall in cellular activity (see Figure 4.3). GAG loss from explants followed a trend with more GAG in medium and more loss from explants at low osmolarity (see Figure 4.4). GAG loss from beads was too low to be measured as GAGs are trapped within the alginate. In general, results showed that MMP production decreased as osmolarity increased particularly for MMP-2 and MMP-3 (see Figure 4.6 and Figure 4.7). Results of scanned gels were not significant statistically in general because of low numbers of replicates (3) and large gel-gel variations. The trend can however be seen more easily in graphs than in gels (see Figure 4.6 and Figure 4.7). Results showing more MMP expression and greater GAG loss at low osmolarity are consistent.

Studies have shown the effects of osmotic conditions on disc matrix metabolism. With changes in osmotic conditions, the genes for the small proteoglycans, biglycan, and decorin were down-regulated in notochordal pig NP cells [135]. However increasing the osmolarity resulted in an increase of the gene expression of aggrecan and collagen II in human and bovine IVD cells [137] and of a metalloproteinase(ADAMTS1) in human cells [136]. Not much is known about effect of extracellular osmolarity on MMP expression, but other studies

as well as this one suggest that the extracellular osmotic environment has differential effects on biosynthesis of proteins including MMPs.

Regulation of responses to osmolarity has not been investigated in much detail in the disc. Osmolarity affects cell volume [256]. Synthesis rates of disc matrix proteins appear to be regulated by osmolarity and correlated well with measured changes in cell volume [130]. Osmolarity also upregulates transporters and affects the intracellular ionic environment [257]; such changes in intracellular ions can trigger signaling cascades [152]. Liver cell swelling induced by hypo-osmolarity triggers the activation of MAP-kinases [258]. In other cell types, hypertonic treatment has been shown to increase activation of p38 MAP kinase, ERK1/2 and JNK [151, 259-261].

MMP expression could also be mediated by similar pathways; induction of MMP-1, MMP-2, MMP-9 and MT1-MMP in other cell types has been shown to be mediated by p38, JNK and ERK pathways [262-268]. The inhibitors SB203580, U0126 and JNK inhibitor II block the p38, MEK1/2 and JNK1/2/3 in the MAP kinase pathways respectively [269-271] (Chapter 1: Figure 1.9). Results of this study found differential effects of these inhibitors on MMP-3 production at low and high osmolarities (Figure 4.10). A limited set of results suggest that low osmolarity stimulates MMP-3 expression via the p38-MAPK pathway, and that high osmolarity inhibits MMP-3 expression via the ERK/MAPK pathway. There were no significant effects of MAP kinase inhibitors on the GAG release and GAG remaining in cultured explants (Figure 4.8 and Figure 4.9) although GAG remaining at 260 mOsm was

highest (though not at level of significance) in the presence of SB203580 which appears to block MMP-3 stimulation. GAG release will be affected by a number of different MMPs and ADAMTs (Table 1.1 and 1.2); thus, unless same pathway regulates the osmotic-dependent up-or down regulation of all these proteases in a similar manner, one would not expect any single inhibitor to have a major influence on rate of GAG release from disc explants.

This study addressed the role of osmolarity in the regulation of MMP-2 and MMP-3 NP cells. Hypo-osmolarity resulted in elevation whereas hyper-osmolarity resulted in down-regulation of MMP production; these effects were evident after 72 hours culture. The results are further evidence that hypo-osmotic environments generated during IVD degeneration appear to be involved in the progression of IVD degeneration. One of the signs of disc degeneration is loss of aggrecan [246, 272, 273]. Osmolarity therefore falls in degenerate disc. Up-regulation of MMPs seen here at low osmolarity (Figure 4.7) perhaps explains the progressive nature of degeneration, and the increase in MMP levels shown in degraded discs [248]. The complex signaling processes governing expression of MMP-3 (Figure 4.10) and other MMPs (not shown) in relation to changes in extracellular osmolarity suggests that prevention of disc degeneration through use of MMP inhibitors is unlikely to be successful.

Chapter 5 Interaction of a fibronectin fragment and osmolarity on disc metabolism by NP cells encapsulated in alginate beads and NP explants

5.1 Introduction

During intervertebral disc degeneration, accelerated breakdown of the disc extracellular matrix is thought to arise from increased activity of matrix proteinases, such as MMPs [72, 73, 84-86, 88, 89, 91, 248, 274]. Increased proteolytic activity leads to degradation of matrix macromolecules and particulary to loss of aggrecan and other proteoglycans [23, 273]; a fall in proteoglycan concentration is one of the first biochemical changes observed in degenerate discs [246]. As the extracellular osmolarity is directly related to the concentration of aggrecan (section 1.2.1 in introduction), proteolytic activity is thus also responsible for generating a hypo-osmotic extracellular environment in degenerate discs. MMPs appear to be involved in the process of altering the osmotic environment in other cell types as well [253, 275, 276].

As shown in chapater 4, a fall in osmolarity could be one factor leading to the increase in proteolytic activity seen in degenerate discs. Also, fragments of degraded extracellular matrix macromolecules have been shown to stimulate production and activity of proteolytic enzymes in articular cartilage [277]. Accumulating evidence shows that fragments of fibronectin (Fnf) exist in degraded discs [278]. Such fragments, particularly the amino-terminal-29kD Fnf, commercial form: N-terminal 30kD Fnf (section 5.2.1), found to be most proteolytically

active in cartilage [175] could possibly also stimulate production and activity of proteinases in disc [172, 173].

Here the ability of an amino-terminal-30kD Fnf to elicit catabolic responses in the disc has been investigated. I also investigate how differing osmotic conditions (pathological for the disc viz 260mOsm, physiological for the disc unloaded viz 356mOsm, and physiological for the disc under-loading viz 489mOsm), influence the responses of disc cells to Fnf both when isolated and implanted into alginate beads and also when cultured as explants.

5.2 Experimental plan

The experimental plan is summarized below in a schematic flow chart (Figure 5.1). Details of the general procedures used have been given under Chapter 2 Materials and Methods. The specific procedures using the amino-terminal-30kD fibronectin fragment treatment are given in section 5.2.1.

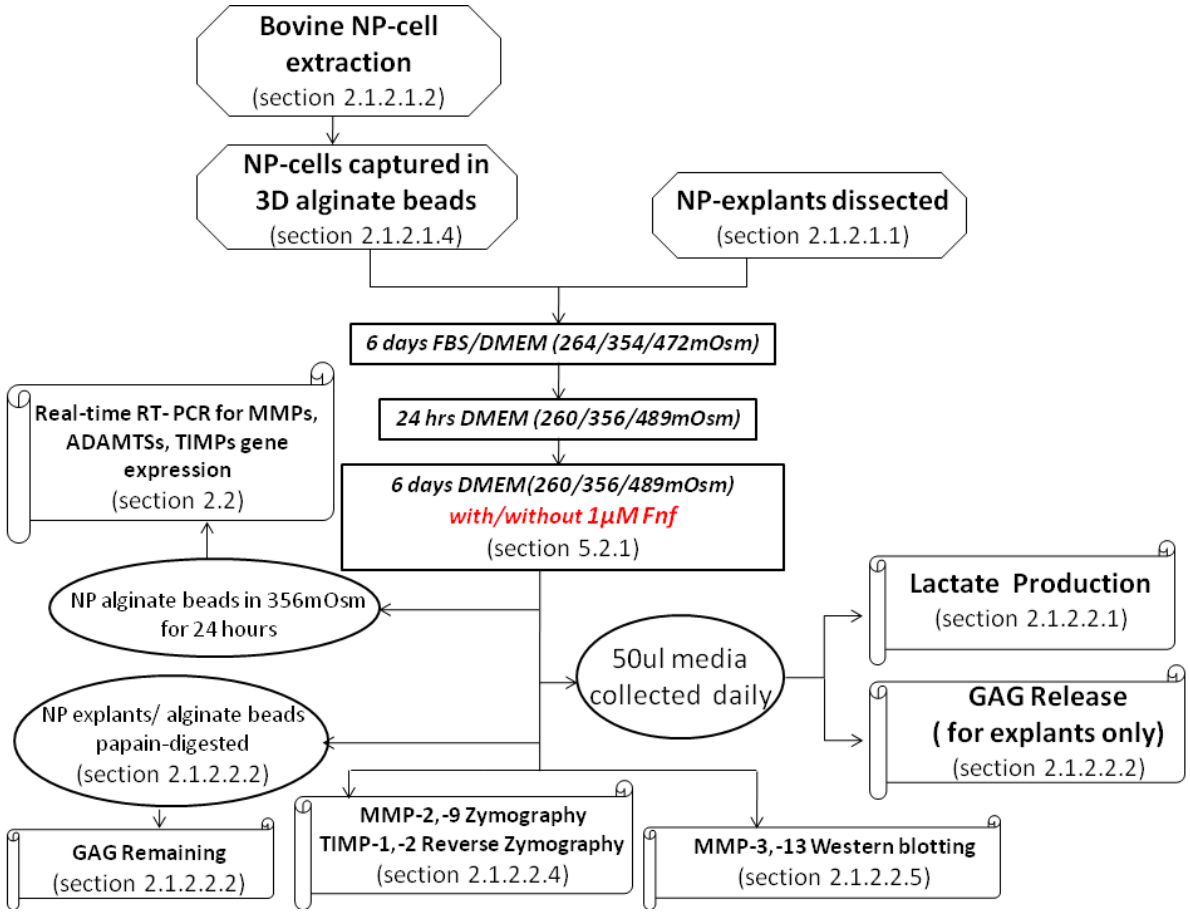


Figure 5.1 Scheme of the experimental design of the interaction of a fibronectin fragment and osmolarity on the cell metabolism by NP explants and NP cells.

Briefly, bovine NP tissue was dissected from bovine caudal discs from 18-24 month steers (section 2.1.2.1.1). Either cells were isolated by enzymatic digestion (section 2.1.2.1.2) or tissue was dissected into explants, about 120mg quantities (section 2.1.2.1.1). DMEM at three different osmolarities was produced by NaCl addition (section 2.1.2.1.6.1) to cover physiological, viz. 356 and 489 mOsm, and hypo-osmotic conditions, viz. 260 mOsm. Cells were encapsulated into alginate beads (section 2.1.2.1.4) and explants cultured for six days in DMEM containing 10% FBS at each osmolarity and then for 1 day in serum free DMEM at each osmolarity. By day six GAG loss from explants had stabilized. Serum free medium with/without 1 µM Fnf was then added (section 5.2.1) and slices and beads cultured for a

further six days. Media were collected daily from each well. The samples were stored at -20^{o}C until assayed for lactate production (section 2.1.2.2.1) and GAG release (section 2.1.2.2.2). Medium was changed every two days for up to six days. After 24 hours incubation, a representative sample of NP cells in alginate beads under 356mOsm was collected and the total RNA was extracted for determination of gene expression of MMPs, ADAMTSs and TIMPs using real-time RT PCR (section 2.2). The RT-PCR work was carried out with the help of Dr David Young, University of Newcastle. A list of primers used is given in Chapter 3 Table 3.1. After six days incubation, all the sample media were removed carefully and assayed for MMP-2,-9,-3,-13, and TIMP-1,-2 (section 2.1.2.2.4 and section 2.1.2.2.5). The remaining GAG content of each papain-digested NP explant and of the NP cells in alginate beads was also measured (section 2.1.2.2.2).

5.2.1 Fnf treatment

As shown in Figure 5.1, after six days culture in FBS/DMEM at the various osmolarities, culture medium was carefully removed from the beads and explants, and replaced with 1.5 ml of DMEM at the same osmolarity as in the initial medium but containing also an amino-terminal-30kD Fnf (Cat.no.F9911, Sigma-Aldrich, Dorset, UK) at a final concentrations of 1 μM in 1.5ml of culture medium. 1 μM Fnf was chosen because it has been extensively characterized in terms of effects on cartilage tissue [157, 168, 169, 175, 279, 280].

5.2.2 Statistical analysis

Unless otherwise stated, data are presented as the mean ± the standard error of the mean

(SEM) of at least three individual experiments (i.e. separate cell isolations and explants and cell cultures) ($n \geq 3$). Differences compared to control were examined by Student's paired *t* test. For lactate production and GAG release, the two-way ANOVA was used to assess the effects of treatment and cultured period, using the Bonferroni test as a *post hoc test.* A P value of 0.05 or less was considered to be statistically significant.

5.3 Results

5.3.1 The effect of Fnf and osmolarity upon lactate production

Media from cultured NP explants and NP cells in alginate beads were tested daily for lactate level during 6 days of culture in 260, 356 or 489 mOsm FBS free DMEM with or without Fnf treatment.

Figure 5.2 shows rates of lactate production by (a) NP explants, and (b) NP cells in alginate beads (b), in response to incubation at differing osmolarity with or without Fnf treatment. The amount of lactate produced under all osmotic conditions was similar and there was no apparent effect of Fnf treatment. Lactate production remained constant throughout the 6 day time course with the average rates being 1.93 ± 0.55 nmol/mg wet weight/hr. Similar to NP explants, NP cells in alginate beads did not significantly response to effects of either Fnf or osmolarity, even though at that both in 260 and 489 mOsm, the lactate production rate of isolated cells with Fnf or without appeared to be lower at day 6 compared to that of day1 ($p=0.13$ and $p=0.88$, respectively).

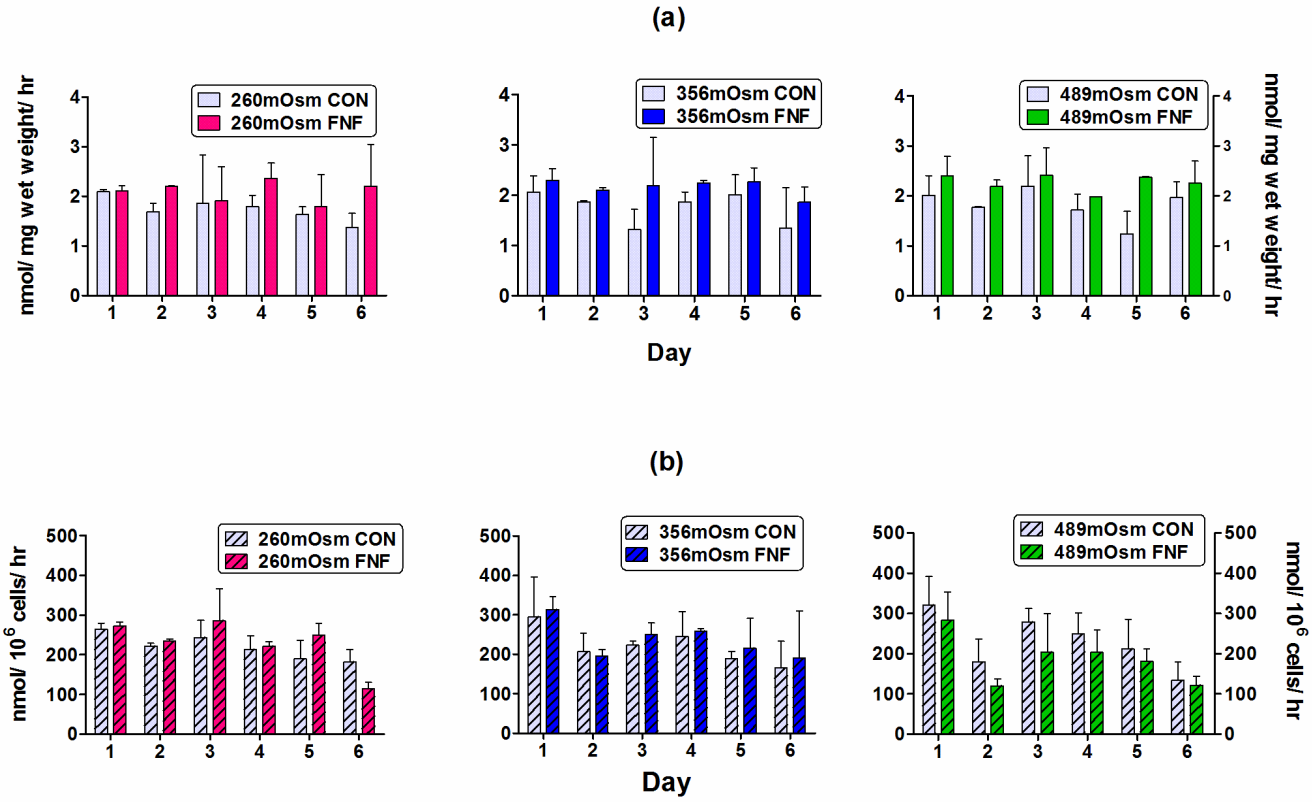


Figure 5.2 The effect of Fnf and osmolarity upon lactate production.

Rates of lactate production for 6 days in (a) bovine NP explants, and (b) in NP cells encapsulated in alginate beads exposed to 260, 356 or 489 mOsm. Medium was removed everyday and assayed for lactate levels. Results from an osmolarity time course given as means ±SEM (n=3).

5.3.2 The effect of Fnf and osmolarity upon GAG release

Media from culture of NP explants was tested daily for GAG concentration during 6 days culture in 260, 356 or 489mOsm FBS free DMEM with or without Fnf treatment.

Results are shown in Figure 5.3. The concentration of GAG in the medium in Fnf treated NP explants under 260mOsm was up to 1.3 fold greater than without Fnf present; more GAG was released in the presence of Fnf throughout the culture period, though not at the level of significance. There is no significant difference between Fnf treated and control NP explants under 356 and 489mOsm. Again there was also a trend showing increased GAG release with Fnf treatment throughout the culture period in 356 mOsm (up to 1.25 fold increase), though again not at the level of significance. At 456 mOsm, the concentration of GAG in the medium seemed very similar with or without Fnf present.

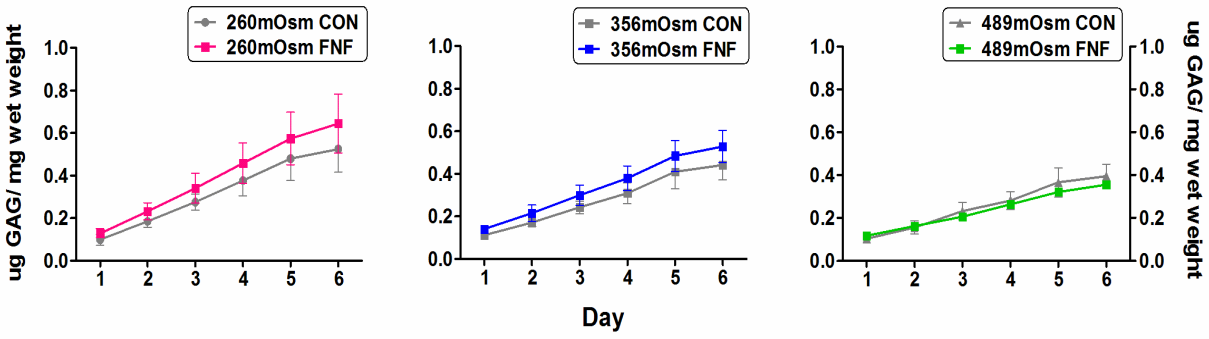


Figure 5.3 The effect of Fnf and osmolarity upon GAG release from explants.
Media from NP explants that had been cultured at 260, 356 or 489 mOsm (no FBS) with or without Fnf (1 µM) were assayed for GAG content every day for 6 days. Results are given as means ±SEM (n=5).

5.3.3 The effect of Fnf and osmolarity upon total GAG remaining

After 6 days following exposure to the altered osmotic conditions with or without Fnf treatment, total GAG remaining in NP explants or alginate beads was assayed. GAG concentration in NP explants was measured in NP explants by papain digests of tissue. Alginate beads were depolymerised with citrate buffer containing papain, to release NP cells.

Figure 5.4 shows levels of total GAG remaining in (a) NP explants and (b) NP cells in alginate beads following their exposure to altered osmotic conditions with or without Fnf treatment. After 6 days, no significant differences in the levels of GAG remaining were evident in response to Fnf treatment under any osmolarity in either culture system. However, for the explants but not beads, the amount of GAG remaining at 356 mOsm was markedly greater than that at 260 mOsm ($p=0.002$) both for Fnf treated and non-treated explants.

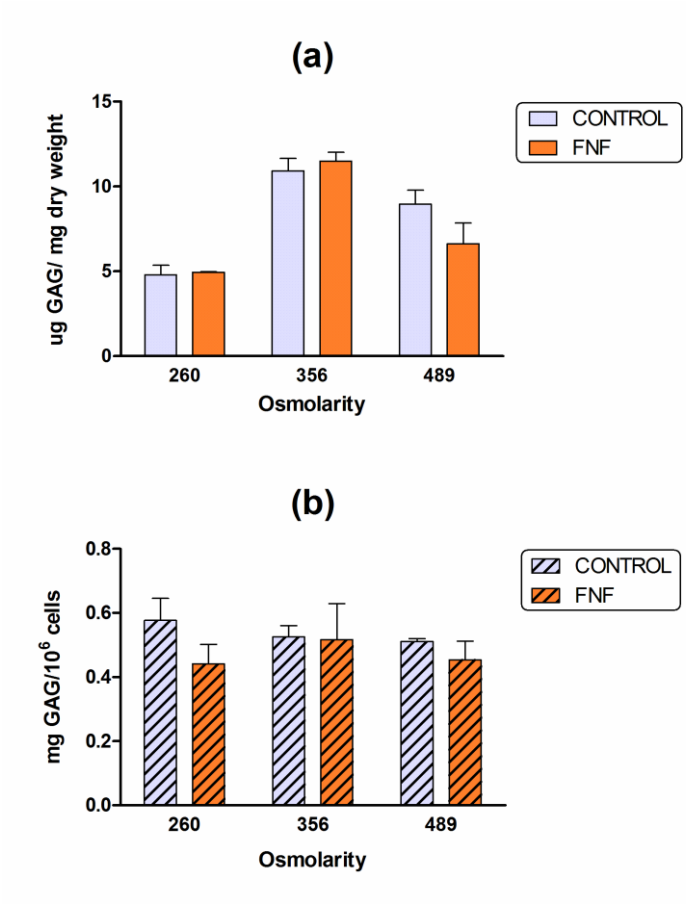


Figure 5.4 The effect of Fnf and osmolarity upon total GAG remaining.

Levels of GAG remaining in (a) NP explants and (b) NP cells encapsulated in alginate beads cultured at 260, 356 or 489 mOsm (no FBS) with or without Fnf treatment after 6 days. Results are given as means ±SEM (n=3).

5.3.4 The effect of Fnf and osmolarity on production of MMPs, ADAMTSs, and TIMPS

Gene expression of MMPs, ADAMTSs and TIMPs of NP cells in alginate beads cultured under iso-osmotic condition, viz. 360mOsm for 24 hours with or without Fnf was determined by real-time RT PCR, with the help of Dr David Young who provided the primers, technical system and data analysis; results of cultures without Fnf have been given in Chapter 3; results with Fnf addition are shown in this chapter. Proteolytic activity of MMPs and TIMPs accumulated in the medium during 3 days culture in 260, 356 or 489 mOsm DMEM was

assayed by gelatin zymography for MMP-2, -9, by reverse zymography for TIMP-1,-2, and by western blotting for MMP-3,-13. Differences in staining intensity of digitized bands were assessed using IMAGE J software.

5.3.4.1 The effect of Fnf on gene expression of MMPs, ADAMTSs, and TIMPs under iso-osmotic environment

Figure 5.5 shows MMP expression levels in NP cells with or without Fnf treatment are not significantly different. MMP-8,-10,-20,-24, -25 and MMP-27 were not detected. No detection does not mean the transcripts are not present. It could be that the PCR did not work, or that the primers do not amplify well. However, all these primers have shown positive gene expression in other systems [225, 233]. Matrilysin (MMP-7), metalloelastase (MMP-12) and MMP-28 were expressed at very low levels, near to the reliable detection limits of the PCR machine. Expression of the gelatinases (MMP-2,-9), the collagenases (MMP-1,-13), stromelysin (MMP-3), the membrane-type MMPs (MMP-14,-16), Xenopus XMMP (MMP-21), and of MMP-19 and MMP-23 did not differ with Fnf treatment.

Figure 5.6 shows that the expression level of all ADAMTSs expressed in NP cells was similar with or without Fnf treatment. ADAMTS-14-16-18-19 were not detected. ADAMTS-8 was expressed at a very low level.

Figure 5.7 gives the expression level of all four TIMPs. No significant difference was seen with Fnf treatment. TIMP-4 expression was at a very low level.

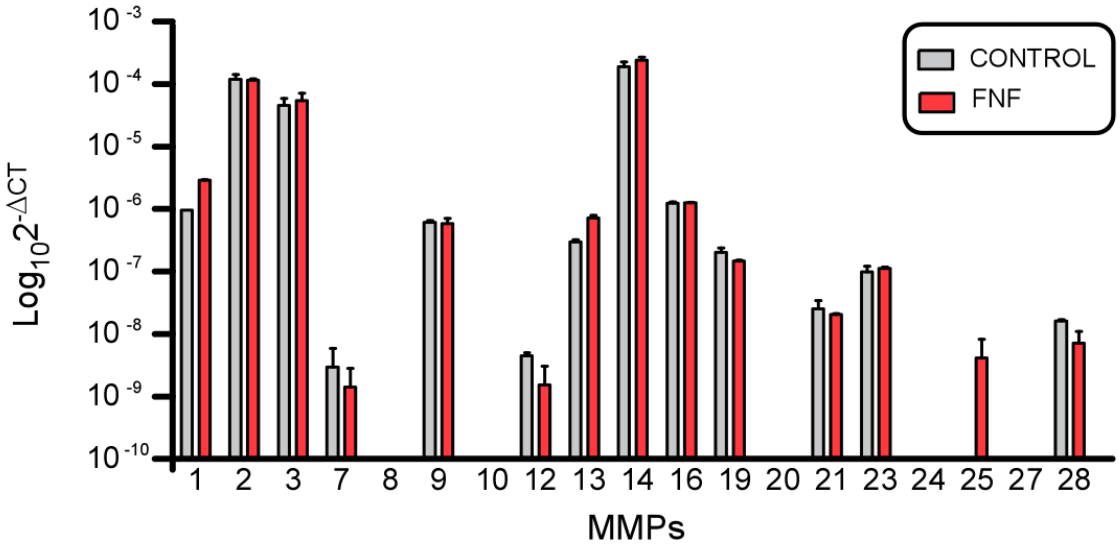


Figure 5.5 The effect of Fnf on gene expression of MMPs cultured at 356mOsm.

Comparative expression of mRNA, relative to 18S ribosomal RNA, for MMPs genes isolated from NP cells after cultured in alginate beads without FBS for 24 hours, with or without Fnf treatment. Results are given as means±SD. Samples not shown were not detected.

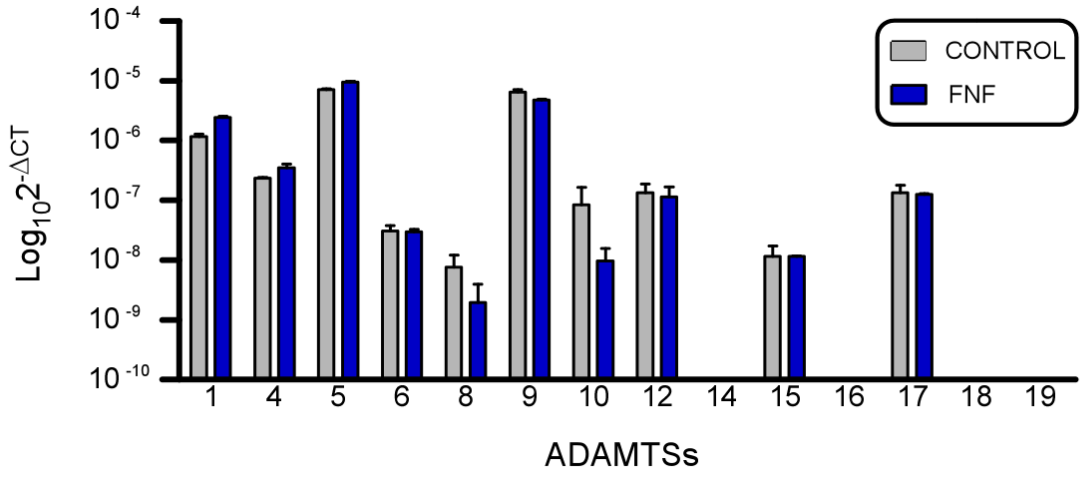


Figure 5.6 The effect of Fnf on gene expression of ADAMTSs cultured at 356mOsm.

Comparative expression of mRNA, relative to 18S ribosomal RNA, for ADAMTSs genes isolated from NP cells after cultured in alginate beads without FBS for 24 hours. Results are given as means±SD. Samples not shown were not detected.

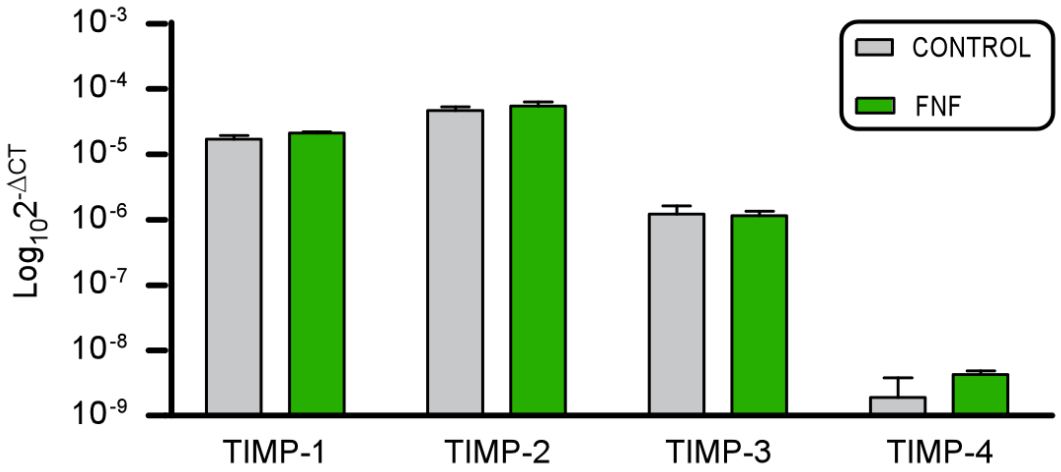


Figure 5.7 The effect of Fnf on gene expression of TIMPs cultured at 356mOsm.
Comparative expression of mRNA, relative to 18S ribosomal RNA, for TIMPs genes isolated from NP cells in after cultured in alginate beads without FBS for 24 hours. Results are given as means ±SD. Samples not shown were not detected.

5.3.4.2 The effect of Fnf and osmolarity upon MMPs and TIMPs production at the protein level

Figure 5.8 and Figure 5.9 show levels of MMPs and TIMPs producted by NP explants and NP cells cultured in alginate beads under different osmotic conditions with or without Fnf treatment.

Figure 5.8 shows typical results from zymogram, reverse-zymography and western blotting. Although at 260 and 356 mOsm, Fnf seems to upregulate MMP-3 and MMP-13, when samples were scanned and analysed using Image J, there was no significant difference in the production of MMPs between samples cultured with and without Fnf under all alter osmotic conditions. TIMP-1 and TIMP-2 were too dark to be scanned, but no osmolarity effect could be seen from the gels. There was a trend towards down-regulation of MMP production at

higher osmolarities.

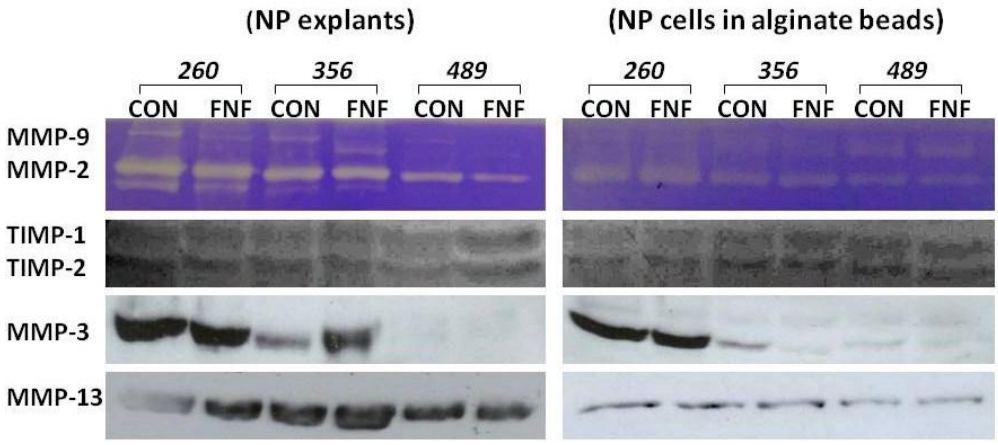


Figure 5.8 Typical zymogram, reverse zymogram, and western blotting showing the effect of Fnf and osmolarity on production of some MMPs and their inhibitors.

Levels of MMP-2,-9,-3,-13, and TIMP-1,-2 production from NP explants and NP cells encapsulated in alginate beads incubated at 260, 356 or 489mOsm were assayed by zymography after 72 hours culture.

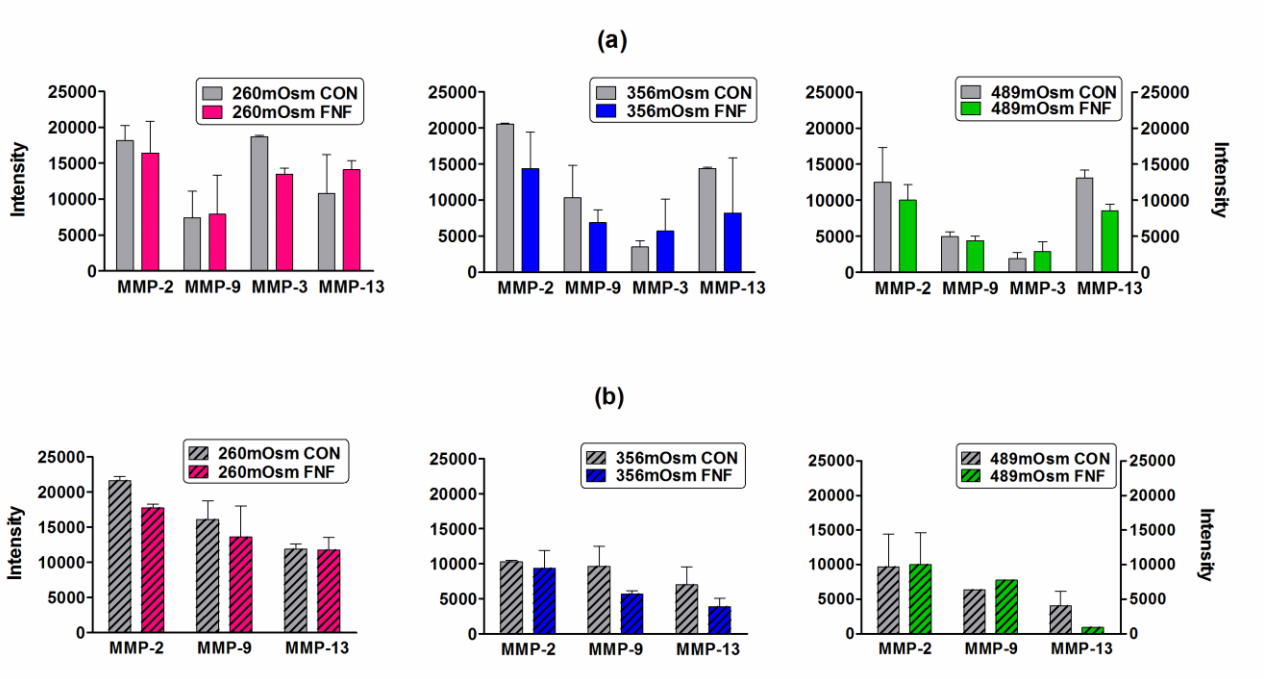


Figure 5.9 The effect of Fnf and osmolarity on MMP production.

Differences of intensity of white bands of lysis on the Coomassie blue background, and intensity of dark band of reverse zymogram and western blotting were scanned. Levels of MMP-2,-9,-3,-13 produced by NP explants (a), and MMP-2,-9,-13 by NP cells encapsulated in alginate beads (b) after culture at 260, 356 or 489mOsm were assayed after 72 hours culture with or without Fnf. Results are given as means ±SEM (n=3).

5.4 Discussion

This study has shown that Fnf exposure leads to a slight increase in GAG release from NP explants under hypo-osmotic condition, but this trend is of not at the level of significance. From the other results shown here, there is no evidence that the 30kD Fnf affects intervertebral disc energy metabolism (Figure 5.2) or metalloproteinase expression either at the gene (Figure 5.5, Figure 5.6, and Figure 5.7) or protein level (Figure 5.8 and Figure 5.9) at the level of significance although trends were seen in the protein gels shown (Figure 5.8).

This result is not in agreement with findings of others working on articular cartilage. As summarised in Table 5.1, a number of previous studies on the effect of Fnfs on articular cartilage and chondrocytes have found that Fnfs enhance gelatinolytic, collagenolytic and stromelytic proteinases and also proteoglycan release [157, 277, 281]. These degradative effects are associated with elevated release of the catabolic cytokines, TNF-alpha, IL-1beta, and IL-1 alpha. The intracellular signaling pathways have been partly delineated. Prolonged release of IL-6 and consequent chondrolysis is reported to arise through reactive oxygen species (ROS) with treatments using anti-oxidants (AOs) promoting reparative responses in Fnf and cytokine treated cartilage [155, 282]. Focal Adhesion Kinase (FAK) and MAP kinase pathways also appear to be involved in the chondrolysis process in articular cartilage [159, 164, 168]. with enhancement of FAK, proline rich tyrosine kinase 2 (Pyk2), cellular src kinase(c-src) and nuclear factor kappaB (NF-kappaB) crucial for induction of MMP upregulation and cartilage damage by Fnfs [169].

In contrast to the large number of studies on cartilage explants and chondrocytes, there are relatively few studies of the effect of Fnfs on intervertebral discs (Table 5.1) although again these all show Fnfs induce degradation. Injection of Fnfs into rabbit discs in vivo and culture of rabbit discs in the presence of Fnfs caused progressive loss of PG and down-regulation of both aggrecan and type-II collagen mRNA while MMP-9,-13 and Fas genes were up-regulated [283, 284]. Aota found that bovine NP disc cells responded to Fnf in a similar, but less pronounced manner for PG degradation [153].

However, here only minor responses of disc cells to Fnfs (Figure 5.3) were observed. Some of the possible reasons are discussed below.

(a) *Differences in the tissue sources*. Most work to date has been carried out on articular cartilage explants or chondrocytes in culture. Two of the three studies to date on discs have been on discs of the rabbit. The studies here were on cells from the bovine disc nucleus. Although these cells all produce mainly collagen and aggrecan and other molecules found in cartilaginous matrices, the cells are phenotypically distinct; a number of studies have now shown differences between these cells in expression of biomarkers at the gene and protein level [98, 115, 116, 285]. The cells of rabbits are mainly large vacuolated cells considered to be notochordal cells; these differ from the small polygonal mature bovine nucleus cells, considered to be chondrocyte-like cells [286, 287]. Also, although disc nucleus cells resemble articular chondrocytes morphologically, the properties of the tissues are very different biochemically and biomechanically. Normal human disc NP has a higher proteoglycan to

collagen ratio (27:1) (measure as GAG to hydroxyproline ratio) than hyaline cartilage and is far less stiff than articular cartilage. Moreover there are differences in matrix macromolecules produced. Disc aggrecan is more highly substituted with keratan sulphate than that of articular cartilage [288],cross-linking molecules of fibrillar collagens differ [289] as do isoforms of fibrillar collagens [290]. Moreover, while Aota et al [153] found that bovine NP cells, the same cell type tested here, did respond to Fnfs, the responses were far less marked than seen for articular chondrcytes or rabbit disc cells. Thus the extent to which cells respond to Fnfs appears to vary with cell type.

(b) *Extracellular osmolarity.* Virtually all tests carried out by others in vitro, used standard culture medium where the osmolarity is in the range 260-300 mOsm depending on medium formulation. The extracellular osmolarity of articular chondrocytes in vivo is 360-420 mOsm depending on cartilage zone [291] whereas in the disc it varies from 360-500 mOsm [129]. Here, the effects of medium osmolarity on responses to fibronectin fragment was tested with osmolarity ranging from around 260 to around 489 mOsm to simulate conditions found in the pathological to normal extracellular environments respectively. It was found that expression of some MMPs is up-regulated under hypo-osmotic conditions (Figure 4.6, Figure 4.7, Figure 5.8 and Figure 5.9). A tendency for more GAG loss from fragments incubated under hyposmotic conditions (Figure 5.3) was also observed, although not at level of significance. Thus responses to Fnf may depend on the extracellular osmotic environment and stimulation may be greater at the osmolarities used for most published studies than that seen in vivo.

(c) *The type of Fnf used*. Most previous work on the effect of fibronectin fragments (Fnfs) on

matrix degradation has been carried out by Homandberg and collaborators (Table 5.1). The Fnfs used, including the 30kD one used here, were prepared in their own laboratory. The 30kD Fnf used here is reported to be the most active Fnf in cartilage degradation [157], but was obtained commercially (see section 5.2.1). The effect of the 30kD commercial Fnf on GAG release of cartilage was thus tested. 1 μM of this fragment potently caused GAG release up to 3.5 fold, suggesting that the 30kD commercial Fnf interacted with cartilage (data not shown). This was also the case with elastin fragments (data not shown). K-elastin used by Brassart's group was prepared in his lab, but commercial k-elastin was used in the previous study, which includes effective VGVAPG sequences [177, 292]. The k-elastin experiment could not be repeated, probably due to the difference between commercial k-elastin and the in-house one. For this question, we have communicated with Debelle, a member of Brassart's group, who kindly gave us a sample of the same k-elastin as they used in their paper. This in-house k-elastin is white, rather than orange like the commercial version. No detailed information is available about the composition of these commercial fragments. Therefore there is some uncertainty about whether there are differences between the active domains in commercial and 'home-made' fragments and whether these differences play a role in regulation of MMPs and matrix turnover.

Hence cell phenotype, osmolarity of culture medium and details of fragment composition could all influence response of cells to Fnfs.

Objectives	**Papers**	**Species**	**Culture Systems**	**Fnfs**	**Results**
To observe chondrolytic effects of articlular cartilage	*[281]*	Rabbit synovial fibroblast	1^{st}-6^{th} passages in monolayer	Commercial 120kD,60kD	Enhancement of collagenase and stromelysin gene expression
	[157]	18-20 months old bovine metacarpophalangeal joints	Articular cartilage explants	In-house 29kD,50kD Fnf from human plasma Fn	Enhancement of gelatinolytic, collagenolytic proteinase and proteoglycan release
	[277]	18-20 months old bovine metacarpophalangeal joints	Articular cartilage explants	In-house 29kD Fnf from human plasma Fn	Enhancement of MMP-3 production
	[293]	18-20 months old bovine metacarpophalangeal joints	Articular cartilage explants	In-house 29kD Fnf from human plasma Fn	Higher concentration of Fnf(1uM) results in initial catabolic effects followed by the later anabolic effects; lower concentration(1nM) results in continuous anabolic effects
	[294]	18-20 months old bovine metacarpophalangeal joints	Articular cartilage explants	In-house 29kD Fnf from human plasma Fn	Cartilage damage can be caused by short exposures to Fnf and that exposure makes the cartilage resistant to further damage by amplifying anabolic processes

Table 5.1 Previous studies on Fnf effects

Objectives	Papers	Species	Culture Systems	Fnfs	Results
To analyze the mechanism of chondrolytic effects of articular cartilage	*[155]*	Normal human knee	Articular cartilage explants	In-house 29kD Fnf from human plasma Fn	Early chondrolytic events are associated with elevated release of the catabolic cytokine, TNF-alpha, IL-1beta, and IL-1alpha; later period of slowing PG loss and MMP-3 release is associated with slowed release of these cytokines, but prolonged release of IL-6
	[282]	18-20 months old bovine metacarpo phalangeal joints	Articular cartilage explants	In-house 29kD Fnf from human plasma Fn	Fnf mediates cartilage chondrolysis through reactive oxygen species(ROS), and anti-oxidants(AOs) promote reparative responses in Fnf and cytokine treated cartilage
	[159]	Normal human ankle and knee ariticular cartilage	Primary chondrocytes in monolayer	Commercial 120kD Fnf	MAP kinase signaling is involved in increased production of MMP-13
	[164]	Nomal human femoral and knee cartilage	1st passage chondrocytes in monolayer	Commercial Fnfs	Focal adhesion kinase(FAK) and MAP kinase induced activation of human articular chondrocytes.
	[168]	Bovine articular cartilage	Cartilage explants and chondrocytes in monolayer	In-house 29kD,50 kD and 140kD Fnf	Fnfs upregulated MMP-3 and MMP-13 through MAP kinases
	[169]	Bovine articular cartilage	chondrocytes in monolayer	In-house 29kD,50 kD,140k D Fnf and native Fn	Enhancement of FAK, proline rich tyrosine kinase 2(Pyk2), cellular src kinase(c-src) and nuclear factor kappaB(NF-kappaB)are crucial in Fnfs mediated signaling in MMP upregulation and cartilage damage.

(Continued) Table 5.1 Previous studies on Fnf effects

To observe chondrolytic effects of inverterbral discs	*[283]*	Rabbit intervertebral discs	Animal model in vivo	Commercial 30kD Fnf	Progressive loss of PG and down-regulation of both aggrecan and type-II collagen mRNA
	[153]	Bovine tail and articular cartilage	Disc cells and articular chondrocytes in alginate beads	In-house Fnf mixture	NP cells in disc respond to Fnf in a similar, but less pronounced manner for PG degradation
	[284]	Rabbit nucleus pulposus of intervertebral discs	1st passage in alginate beads and NP explants	Commercial 30kD Fnf	MMP-9,-13 and Fas genes were up-regulated and TypeII collagen and aggrecan genes were down-regulated.PG content was reduced.

(Continued) Table 5.1 Previous studies on Fnf effects

Chapter 6 Regulation of NP cells gene expression in response to a fibronectin fragment treatment interacted with osmotic shock

6.1 Introduction

In our studies to date, we found that addition of Fnf to disc cells or disc explants resulted in only minor increases in expression of MMPs (Figure 5.9) or GAG loss (Figure 5.3) suggesting that Fnf does not induce expression of degradative signals in disc cells. However, my results cannot explain the report of Anderson et al who found injection of Fnf into discs in vivo induced degenerative changes [172] and the findings reported by others that Fnf induced degradative responses in bovine disc cells cultured in vitro [153]. One possibility for the differences between my results and these reports, is that Fnf affects pathways other than those we have investigated here. Thus to provide a more complete overview of the effects of Fnf on disc cells, I undertook a microarray study [136] using a relatively newly developed bovine microarray platform (Chapter 2: section 2.3.2).

NP cells respond to altered osmotic pressure with changes in expression of metalloproteinases MMP-2 and MMP-3 at least (See Chapter 4: Figure 4.7). In other cell types [136, 137, 295], including notochordal disc cells, a wide range of genes are transcriptionally activated following exposure to hypo- or hyper-osmotic conditions [296]. In addition, the amino-terminal-30kD fibronectin fragment (Fnf) stimulates catabolic activity in cartilage [277]. Thus examining the interactions of these two environmental signals on activity of disc cells could provide information of interest.

Microarray analysis is a powerful technique to study the relative gene expression of thousands of transcripts in one single experiment. Recently, researchers have used this technique to increase more comprehensively knowledge about the cellular and molecular changes seen in human [136] or pig [135] intervertebral disc cells, in response to altered osmolarity.

Here I further study the effects changing of osmolarity on gene expression in NP cells and hypothesize that extracellular osmolarity may interact with Fnf treatment. I have therefore investigated how Fnf influenced gene expression by disc cells. I also investigated the effects of differing extracellular osmolarities ranging from those seen pathological discs to normal active discs (268 - 461mOsm) on the responses of disc cells to application of Fnf. I studied bovine disc cells encapsulated in alginate beads and used microarray technology (Chapter 2 section 2.3) to examine the effects of changes in the extracellular environment on gene expression. As in earlier studies [135, 136] it was found that disc cells are very responsive to alterations in extracellular osmolarity and many genes are up-or down regulated.

6.2 Experimental plan

The experimental plan is summarized below using a flow chart (Figure 6.1). The general procedures used have been described in Chapter 2: Materials and Methods. Briefly, NP cells were extracted from adult bovine caudal discs by collagenase digestion (Section 2.1.2.1.2). They were cultured in alginate beads in DMEM (Section 2.1.2.1.4) to maintain phenotype for 6 days in the presence of serum; cells were then cultured for 1 day without serum and then for

a further 2 days in serum free DMEM with/without Fnf (Section 5.2.1) over a range of osmolarities. Osmolarity was altered by NaCl addition (Section 2.1.2.1.6.1) from that seen in severely degenerate discs (standard DMEM, no NaCl addition) to that seen in normal discs (268-461 mOsm). Total RNA was extracted (Section 2.2.1) from the cells and gene expression analysed using the Affymetrix GeneChip Bovine Genome Array (described in Section 2.3). Each sample representing one of three different osmolarities with or without a fibronectin fragments and derived from one of four different cow tails was hybridised to an Affymetrix GeneChip Bovine Genome Array. A total of 24 GeneChips were used.

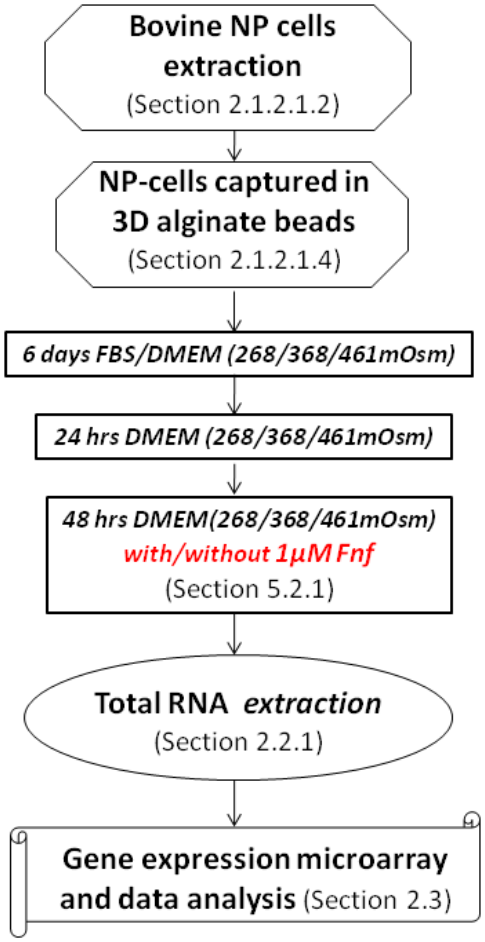


Figure 6.1 An outline of the experimental plan aimed at investigating gene expression of NP cells under differing osmotic conditions and any interaction of osmolarity with Fn fragments.

6.3 Results

The ways of investigating gene expression levels have been discussed in Chapter 2 Materials and Methods 2.3, Figure 2.2 and Figure 2.3. The work on microarrays was done in collaboration with Ms S Lee, Dept of Physiology, Anatomy and Genetics. A paired t test ($p < 0.05$) was used to identify differentially expressed genes which had a ≥ 1.5 fold change between any two conditions selected. Peart *et al.* [297] and Raouf *et al.* [298] declare genes to be differentially expressed if they show a fold-change of at least 1.5 and also satisfy $p < 0.05$ after adjustment for multiple testing. These combination criteria typically find more biologically meaningful sets of genes than *p*-values or fold-change alone. Ingenuity pathway analysis (IPA) was conducted on the comparisons of the effect of osmolarity.

6.3.1 Correlation between the effects of osmolarity conditions and Fnf treatment

6.3.1.1 Correlation Plot

As mentioned above (section 6.2), four biological replicates for each condition were analysed using GeneSpring GX (Agilent Technologies UK Limited.). A correlation plot is available which calculates the correlation coefficient for each pair of arrays and then displays these in visual form. The correlation coefficient is calculated using Pearson Correlation Coefficient [299]. Figure 6.2 shows the correlation across all the arrays. In this data set there is no sample showing a strong difference from others in the same group showing that samples have been collected and cells grown in a reproducible manner.

The Pearson correlation plot is also useful in showing an overview of the similarities between different experimental groups. Samples which are identical have an identical correlation coefficient and are shown in bright red. Samples which are very different have a very different correlation coefficient and are shown in black. For example, when the 268mOsm control (268C) samples are compared to the 268mOsm Fnf (268F) samples, the correlation coefficient is high and the interaction is shown in red indicating that the responses were very similar. When the 268mOsm control samples are compared to the 461mOsm control samples the black coloration indicates a greater dissimilarity.

The plot uses different shades of red to indicate the degree of correlation, the brighter the red, the more similar the samples are and the higher the correlation coefficient. It can be seen that there is more overall similarity between the Fnf (F) and control (C) samples than between samples at different osmolarities. It can also be seen that F1 and C1 and F2 and C2, etc are highly correlated which looks as though the Fnf had very little effect for the most part. In other words, the greatest differences are between the osmotic conditions. In particular, gene expression at 268mOsm is very different from that at 461 mOsm. It is noticeable that a bright red square is in the center which means a consistent correlation between 368 C and 368 F. While at 461 mOsm, the correlation between C and F is lower compared to those at 268 mOsm and 368 mOsm.

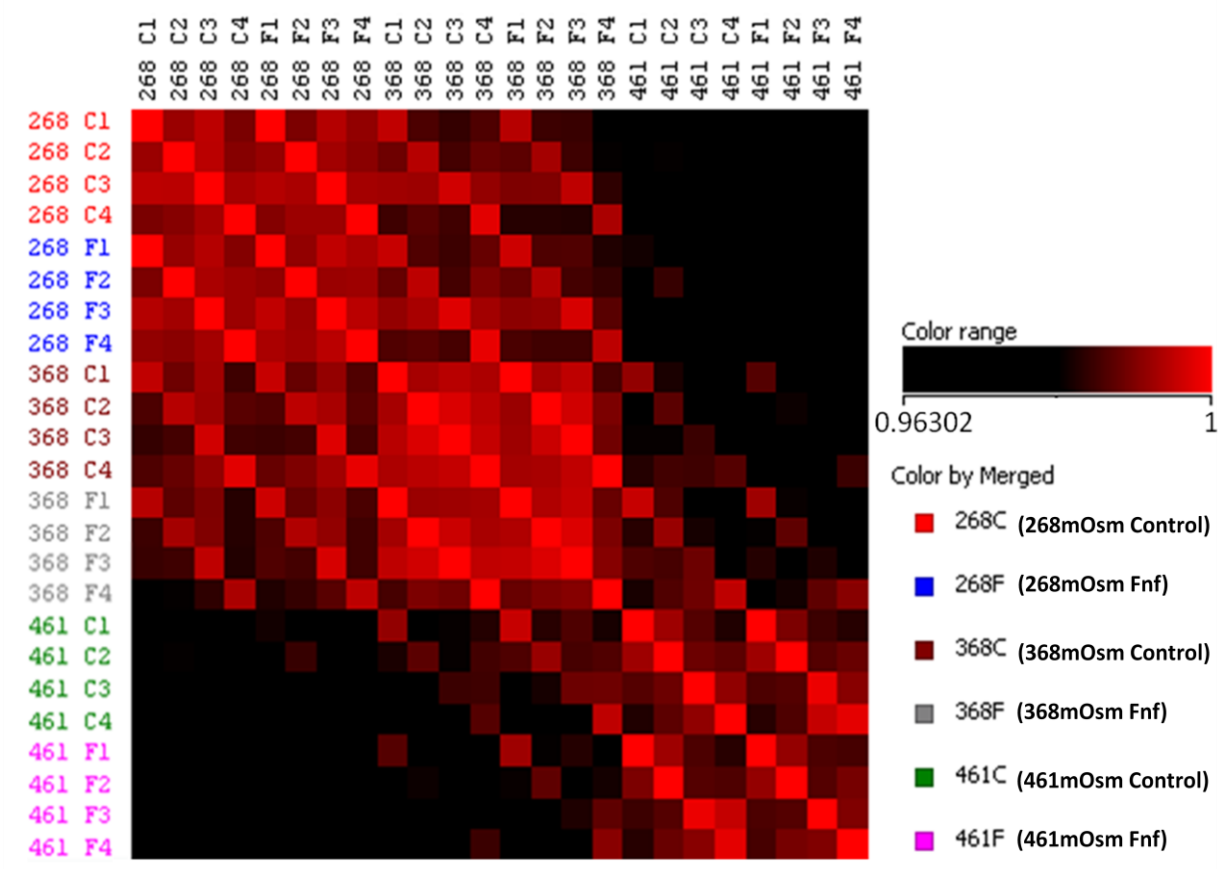


Figure 6.2 A correlation plot shows the correlation between samples across all the arrays.

The Pearson Correlation Coefficient shows the correlation across all the arrays. Values on the correlation plot varied from 0.96302 to 1; where 0.96302 is minimal correlation (represented in black) and 1 was a perfect correlation (represented in red). Four biological replicates for each condition have been used (C1, C2, C3, C4, F1, F2, F3, F4). The graphic is symmetric about the diagonal. The correlation between two samples is the same, irrespective of the order in which the two samples are viewed. The order of the color corresponds to the order of the strength of any correlations, from the least to the greatest.

6.3.2 The effect of osmolarity on gene expression by NP cells

A Venn diagram, generated by GeneSpring GX (Agilent Technologies UK Limited.), was used to show the relationships between 3 different gene lists, which contain 446, 544, and 1375 genes respectively in 268C vs 368C, 368C vs 461C and 268C vs 461C (see Appendix 4,

5, and 6 respectively). Figure 6.3 shows the Venn diagram consisting of three symmetrically placed mutually intersecting circles comprising a total of eight regions. The regions in bright red (268C vs 368C), bright blue (368C vs 461C), and bright green(268C vs 461C) consist of 74, 105, and 679 probes respectively which are up- or down- regulated in one group only and no others; the three regions in dark green, light brown, and purple consist of 17, 341, and 274 probes respectively which are in two groups but not in the third while the brown region in the centre consists of 81 probes, differentially expressed in all three comparisons.

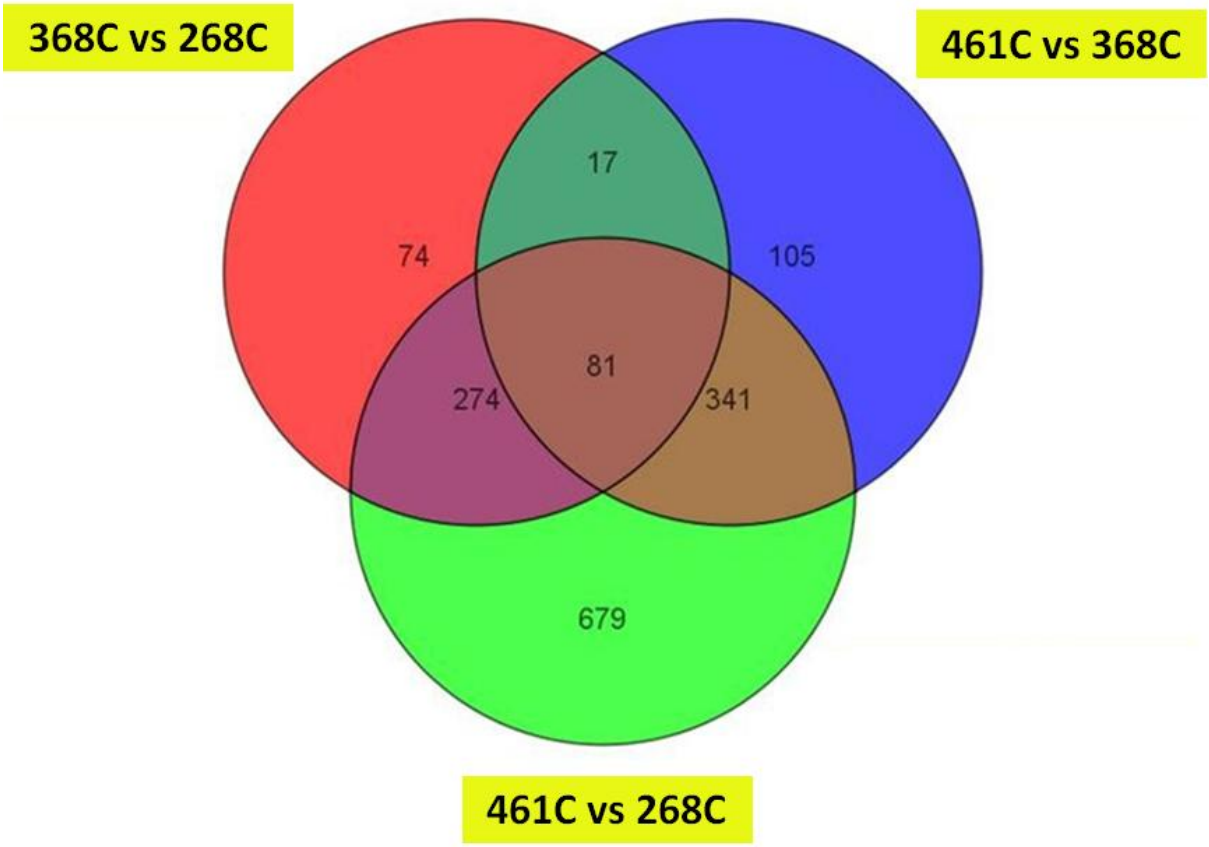


Figure 6.3 A Venn diagram comparing genes expressed at different osmolarities.

Control groups (C), no Fnf treatment. The combined area of three sets 368C vs 268C (368C compared with 268C; in bright red) and 461C vs 368C (461C compared with 368C; in bright blue) and 461C vs 268C (461C compared with 268C; in bright green) is called the union of three comparisons on differing osmolarity conditions. The union contains all probe expressions identified as being significantly up- or down-regulation that are either in 368C vs 268C, or 461C vs 368C, or 461C vs 268C, or all three. The areas in both or in all, where the two or three groups overlap, are called the intersection of three comparison groups.

368mOsm compared to 268mOsm

A total of 446 probe sets (the bright red region in Figure 6.3) were reported as expressed at a significant different level (314 probes up-regulated and 132 probes down-regulated) in 368C vs 268C mOsm (see Appendix 4).

The 20 genes with the highest fold-change were in the categories below (Table 6.1). Among those genes associated with the cartilage and discs, were COL11A2 and LECT1 which are upregulated under higher osmolarity at 368mOsm compared to 268mOsm, and MMP13, B3GALT2, GPNMB, NID1, and IGFBP4 which were downregulated under higher osmolarity. COL11A2 encodes the pro-alpha2 (XI) chain which combines with pro-alpha1 (XI) and proalpha1 (II) collagen chains to form a procollagen molecule and cross-linkage of these procollagen fibrils results in formation of type XI collagen fibers, which is a part of the normal structure of the cartilage and discs (http://ghr.nlm.nih.gov/gene/COL11A2). LECT1 encodes leukocyte cell derived chemotaxin 1, which may contribute to the rapid growth of cartilage prior to the replacement of cartilage by bone during endochondral bone development (http://www.lsbio.com/products/GeneDetail.aspx?LSID=137232). MMP13 encodes matrix metalloproteinase 13, which is collagenase 3 and plays a role in degrading collagens particularly in osteoarthritis [300] (http://www.genecards.org/cgi-bin/carddisp.pl?gene=MMP13). B3GALT2 encodes beta 1, 3-galactosyltransferase, which is involved in the biosynthesis of the carbohydrate moieties of glycoproteins, including structural molecule of collagens (http://www.genecards.org/cgi-bin/carddisp.pl?gene=B3GALT2). GPNMB encodes osteoactin, which enhances the repairing process in bone fracture, demonstrated by its high expression during chondrogenesis and osteogenesis[301]. NID1 encodes nidogen-1, which is associated with laminin and binds to collagen IV and perlecan (http://www.genecards.org/cgi-bin/carddisp.pl?gene=NID1). IGFBP4 encodes insulin-like growth factor (IGF) binding protein 4, binding of which prolongs the half-life of the IGFs and alters their interaction with cell surface receptors (http://www.ncbi.nlm.nih.gov/gene/3487). IGFs

are important in cartilage growth and maintenance [302-306]. The functions in cartilage and disc of other genes are not well understood and needed to be further explored.

368C vs 268C			
Fold Change up-regulated			
Gene	**Fold Change**	**Function of encoded protein**	**Probe Set ID[a]**
CD96	5.5	maybe involved in adhesive interactions of activated T and NK cells	Bt.20940.1.S1_at
THRSP	4.2	may play an important role in lipogenesis	Bt.15842.1.S1_at
LYZ	4.2	bacteriolytic function	Bt.209.3.S1_at
PLXNC1	3.9	transmembrane receptor for semaphorins	Bt.26968.1.S1_at
ID1	3.6	negative regulation of transcription	Bt.1730.1.A1_at
PKP2	3.5	may regulate beta-catenin signaling	Bt.21547.1.A1_at
COL11A2	3.5	fibrillar collagen	Bt.2303.1.S1_at
LOC512486	3.5	GTPase	Bt.9296.1.A1_at
LECT1	3.3	chondromodulin	Bt.1509.1.S1_at
GDPD1	3.3	glycerophosphodiester phosphodiesterase	Bt.26100.1.S1_at
Fold Change down-regulated			
Gene	**Fold Change**	**Function of encoded protein**	**Probe Set ID**
MMP13	4.5	collagenase 3	Bt.26268.1.A1_at
B3GALT2	3.3	galactosyltransferase	Bt.7447.1.A1_at
GPNMB	3.0	osteoactivin	Bt.9807.1.S1_at
PLAU	2.6	fibrinolysis	Bt.2519.1.S1_at
NID1	2.6	cell-matrix adhesion	Bt.8106.1.S1_at
GJB2	2.5	gap junction assembly	Bt.9760.1.S1_at
LRRC17	2.4	bone homeostasis	Bt.4886.1.A1_at
FABP4	2.4	lipid transport protein in adipocytes	Bt.97.1.S1_at
IGFBP4	2.2	skeletal system development	Bt.5237.1.S1_at
LGALS1	2.2	apoptosis	Bt.5472.1.S1_at

Table 6.1 Table of the 20 genes that are the most differentially expressed in the 368C mOsm compared to 268C mOsm.

[a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID.

461mOsm compared to 368mOsm

A total of 544 probe sets (the bright blue region in Figure 6.3) were reported as expressed at a significant different level (242 probes up-regulated and 302 probes down-regulated) in 461C vs 368C mOsm (see Appendix 5). The 20 genes with the highest fold-change, were in categories below (Table 6.2). Among those genes associated with the cartilage and discs, were MMP1, DKK1, ADAMTS1 and MMP9 which were upregulated under higher osmolarity at 461mOsm compared to 368mOsm, and GPNMB and IGSF10 which were downregulated under higher osmolarity. MMP1 encodes matrix metalloproteinase 1, which cleaves collagen types I, II, III, VII and X (http://www.genecards.org/cgi-bin/carddisp.pl?gene=MMP1). ADAMTS1 encodes ADAM metallopeptidase with thrombospondin type1 motif 1, which cleaves aggrecan and involves in its turnover (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ADAMTS1). MMP9 encodes matrix metalloproteinase 9, which may play an essential role in local proteolysis of the extracellular matrix (http://www.genecards.org/cgi-bin/carddisp.pl?gene=MMP9). DKK1 encodes dickkopf homolog 1, which play an important role in vertebrate development, where it can locally inhibit Wnt regulated processes such as antero-posterior axial patterning and affect matrix anabolic and catabolic metabolism in chondrocytes[307]. IGSF10 encodes immunoglobulin superfamily member 10, which may be involved in the maintenance of osteochondroprogenitor cells pool (http://www.genecards.org/cgi-bin/carddisp.pl?gene=IGSF10). GPNMB was described above. The functions in the cartilage and discs of other genes are not well understood and need to be further investigated.

461C vs 368C			
Fold Change up-regulated			
Gene	**Fold Change**	**Function of encoded protein**	**Probe Set ID[a]**
MMP1	11.6	collagenase 1	Bt.72.1.S1_at
CLCA2	5.8	chloride channel modulator	Bt.15970.1.A1_at
DKK1	5.1	negative regulation of Wnt receptor	Bt.13880.1.S1_at
C5	4.7	activation of MAPK activity	Bt.5622.1.S1_at
JAKMIP1	4.1	janus kinase and microtubule interacting protein 1	Bt.19810.1.A1_at
CA2	4.0	carbonate anhydrase	Bt.22854.1.S1_at
ADAMTS1	3.4	aggrecanase	Bt.12010.2.S1_at
LTBP1	2.9	transforming growth factor beta receptor	Bt.20820.1.A1_at
MMP9	2.6	gelatinase B	Bt.4714.1.S1_at
ANG	2.6	angiogenesis	Bt.20883.1.S1_at
Fold Change down-regulated			
Gene	**Fold Change**	**Function of encoded protein**	**Probe Set ID**
GPNMB	7.1	osteoactin	Bt.9807.1.S1_at
FGL2	3.9	fibrinogen-like 2	Bt.9202.1.S1_at
IGSF10	3.4	ossification	Bt.26774.1.S1_at
FHOD3	3.4	actin cytoskeleton organization	Bt.24819.1.S1_at
LRRC17	3.2	bone homeostasis	Bt.4886.1.A1_at
SPARCL1	3.1	unknown function	Bt.5153.1.S1_at
KCNE4	3.1	potassium voltage-gated channel	Bt.22411.1.A1_at
VNN1	3.0	inflammatory response	Bt.28243.1.S1_a_at
PTGDS	2.9	prostaglandin biosynthetic process	Bt.1645.1.S1_at
CA9	2.9	carbonate anhydrase	Bt.27842.1.S1_at

Table 6.2 Table of the 20 genes that are the most differentially expressed in the 461C mOsm compared to 368C mOsm.

[a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID.

461mOsm compared to 268mOsm

A total of 1375 probe sets (the bright green region in Figure 6.3) were reported as expressed at a significant different level (725 probes up-regulated and 650 probes down-regulated) in 461C vs 268C mOsm (see Appendix 6). There were more different probes detected in the comparison of 461C vs 268C mOsm, compared to that of 368C vs 268C and 461C vs 368C. The 20 genes with the highest fold-change, are mentioned in Table 6.3. Among those genes associated with the cartilage and discs, were MMP1 and DKK1 which were upregulated under higher osmolarity at 461mOsm compared to 268mOsm, and GPNMB, NID1 and B3GALT2 which were downregulated under higher osmolarity. These genes were mentioned as above. The functions in the cartilage and discs of other genes are not well understood and need to be further determined.

461C vs 268C			
Fold Change up-regulated			
Gene	**Fold Change**	**Function of encoded gene**	**Probe Set ID[a]**
MMP1	16.0	collagenase 1	Bt.72.1.S1_at
DKK1	12.0	negative regulation of Wnt receptor	Bt.13880.1.S1_at
CLCA2	10.6	chloride channel modulator	Bt.15970.1.A1_at
CD96	10.3	maybe involved in adhesive interactions of activated T and NK cells	Bt.20940.1.S1_at
ID1	8.2	negative regulation of transcription	Bt.1730.1.A1_at
LYZ	7.5	lysozyme	Bt.209.3.S1_at
THBD	6.9	thrombomodulin	Bt.471.1.S1_at
CA2	5.8	carbonate anhydrase	Bt.22854.1.S1_at
C5	5.8	activation of MAPK activity	Bt.5622.1.S1_at
ARG2	5.6	arginase type II	Bt.8624.1.S1_at
Fold Change down-regulated			
Gene	**Fold Change**	**Function of encoded gene**	**Probe Set ID**
GPNMB	20.0	osteoactin	Bt.9807.1.S1_at
LRRC17	7.8	bone homeostasis	Bt.4886.1.A1_at
NID1	6.0	cell-matrix adhesion	Bt.8106.1.S1_at
B3GALT2	5.5	galactosyltransferase	Bt.7447.1.A1_at
FGL2	4.8	fibrinogen-like 2	Bt.9202.1.S1_at
PTGDS	4.7	prostaglandin biosynthetic process	Bt.1645.1.S1_at
GCSH	4.6	glycine catabolic process	Bt.4195.1.S1_at
LGALS1	4.5	apoptosis	Bt.5472.1.S1_at
GCLC	3.8	cysteine metabolic process	Bt.19499.1.S1_at
CA9	3.8	carbonic andydrase	Bt.27842.1.S1_at

Table 6.3 Table of the 20 genes that are the most differentially expressed in the 461C mOsm compared to 268C mOsm.

[a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID.

Comparison of all osmolarities as a heat-map

44 genes of the gene expression data on the 81 probes (see Appendix 3) that are altered in all comparisons (see Figure 6.3, the region in the centre in brown) have been clustered according to functions. Gene functions were integrated from the Ingenuity Pathway Analysis system, Genecards (http://www.genecards.org/) and Gene (http://www.ncbi.nlm.nih.gov/gene) and shown as a heatmap (see Figure 6.4) in GeneSpring GX. These probes can be divided into 2

groups: genes that increase in signal with increasing osmolarity and genes that decrease in signal with increasing osmolarity. 9 unknown function probes (MID1, LRRN1, FRY, LRRC17, TMEM220, CFHR5, LIMA1, LOC782642 and MOXD1) and 28 unnamed probes have not been clustered in the heatmap. There are no genes that show a peak or trough of expression at the 368mOsm. Samples from the same osmolarity cluster together showing similarity between samples of the same type. The data also show that the 268mOsm and 368mOsm expression profiles, although clearly different, are more similar to each other than to 461mOsm.

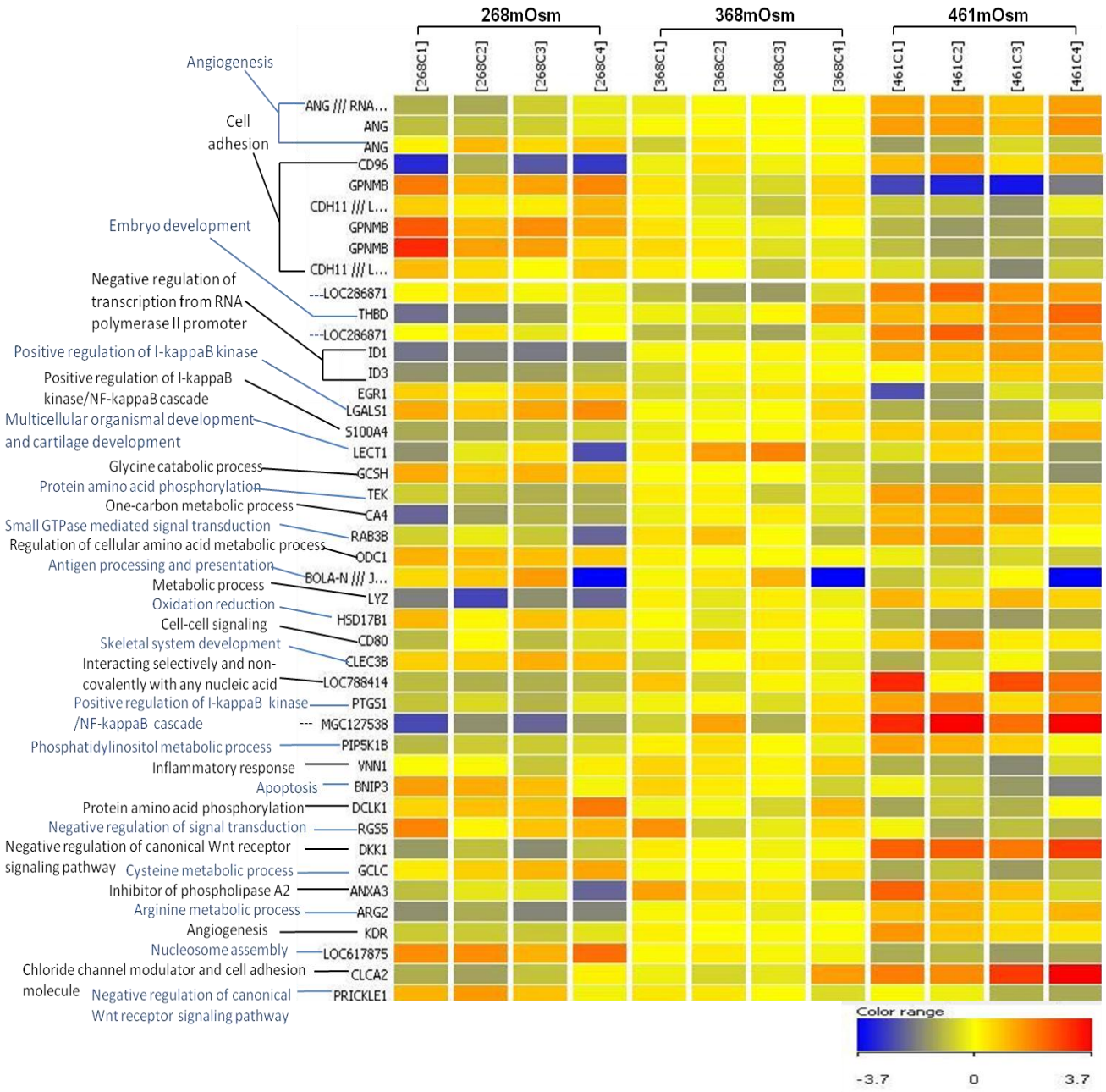


Figure 6.4 A cluster heatmap shows 44 probes simultaneously expressed in all three osmotic comparison.

The heatmap represents a two-dimensional array of data: the level of expression of 44 genes(Y axis) with their biological terms (either in black or blue for the sake improving legibility within limited line spacing) across a number of comparable samples(X axis) from all three osmolarities (268mOsm, 368mOsm and 461mOsm). The legend shows the normalised signal values ranging from -3.7 to 3.7, where red is the highest and blue is the lowest. '---': categorized literature findings not available for this identifier. This may be because this identifier refers to an uncharacterized gene (e.g. EST) or other identifier for which there is no known findings content.

6.3.3 The effect of Fnf on NP cells gene expression at differing osmolarities

The order-three Venn diagram (Figure 6.5) consists of three symmetrically placed mutually intersecting circles comprising a total of eight regions. Basically three gene lists: 268F vs 268C mOsm (Table 6.4), 368F vs 368C mOsm (Table 6.5) and 461F vs 461C mOsm (Table 6.6) were compared and there is not much difference between the Fnfs and controls.

A total of 10 probe sets were reported as expressed at a significant different level (2 probes up-regulated and 8 probes down-regulated) in 268F vs 268C mOsm. A total of 12 probe sets were reported as expressed at a significant different level (10 probes up-regulated and 2 probes down-regulated) in 368F vs 368C mOsm. A total of 12 probe sets were reported as expressed at a significant different level (all up-regulated) in 461F vs 461C mOsm.

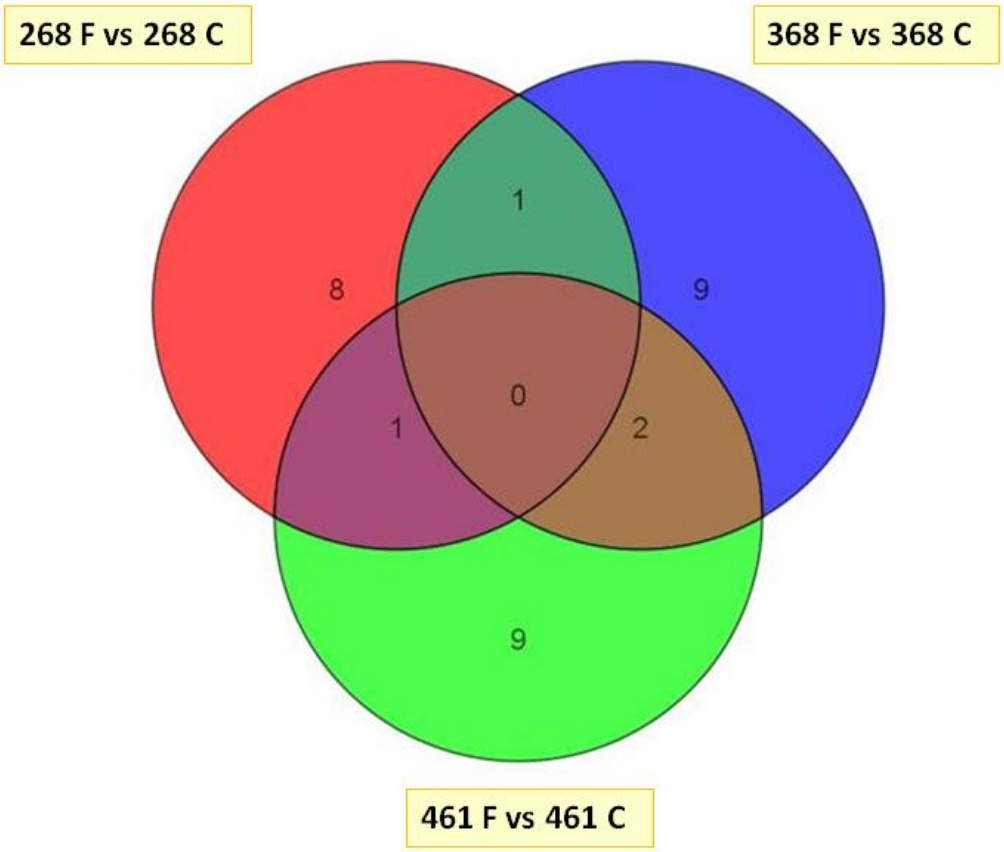


Figure 6.5 A Venn diagram comparing gene expression in samples cultured at 268, 368 and 461 mOsm with F (Fnf) treatment to their respective C (Control) samples cultured at the same osmolarities without Fnf .

The combined area of three sets 268F vs 268C (in bright red) and 368F vs 368C (in bright blue) and 461F vs 461C (in bright green), the union of the three comparisons, contains all probe expression identified as significantly up- or down-regulation in 268F vs 268C or 368F vs 368C or 461F vs 461C (or all three). The areas overlapping, the intersection of three comparison groups, do not contain any probes.

6.3.3.1 Effect of Fnf on gene expression at 268 mOsm

With Fnf treatment (F), a total of only 6 annotated probe sets were reported as expressed at a significantly different level (2 probes up-regulated and 4 probes down-regulated) in 268F compared to 268C mOsm. A list of all the genes with fold-change ≥ 1.5 fold are given in Table 6.4. The functions of these genes in the cartilage and discs are not well understood and

need to be further explored.

Gene	Fold change	Function of encoded gene	Probe Set ID[a]
GCSH	1.5	glycine catabolic process	Bt.4195.1.S1_at
C5	1.6	activation of MAPK activity	Bt.5622.1.S1_at
LOC617875	1.7	nucleosome assembly	Bt.9531.1.S1_at
HIST1H1C	1.6		Bt.2728.1.S1_at
MGC134574	1.8	---	Bt.17517.1.S1_at
TMEM220	1.5	---	Bt.28251.1.S1_at

Table 6.4 Table of the genes that are differentially expressed at 268F mOsm compared to 268C mOsm.
This table lists the genes that were statistically significantly altered (paired t test $p<0.05$ and had a ≥ 1.5 fold change) in Fnf samples compared to controls at 268 mOsm. Fold change in red: up-regulation; in black: down-regulation. [a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID. Unless stated, gene functions were integrated from Genecards (http://www.genecards.org/) and Ingenuity Pathway Analysis System.

6.3.3.2 Effect of Fnf on gene expression at 368 mOsm

With Fnf treatment (F), a total of only 6 annotated probe sets were reported as expressed at a significantly different level (all of the 6 probes up-regulated) in 368F compared to 368C mOsm. A list of all the genes which were significantly up or down regulated (fold-change $\geq$ 1.5) together with their functions are given in Table 6.5. Among those genes associated with the cartilage and discs, were ADAMTS1, MMP13, MMP1 and DKK1, and their functions were detailed in section 6.3.2. These genes related to matrix degradation and turnover were all upregulated with treatment with Fnf at 368 mOsm demonstrating that Fnf may play a role in disc matrix metabolism under resting physiological conditions (low external loads, viz.

368mOsm. The other genes were all upregulated, however their functions in the cartilage and discs are not well understood and need to be further investigated.

Gene	Fold change	Function of encoded gene	Probe Set ID[a]
LOC286871	2.0 1.9	unknown function	Bt.12010.2.S1_at Bt.12010.1.S1_at
C5	2.0	activation of MAPK activity	Bt.26268.1.A1_at
ADAMTS1	1.7 1.6	aggrecanase	Bt.19356.1.A1_at Bt.20820.1.A1_at
MMP13	1.6	collagenase 3	Bt.19204.1.S1_at
MMP1	3.7	collagenase 1	Bt.11625.1.S1_at
DKK1	2.2	negative regulation of Wnt receptor	Bt.72.1.S1_at
LTBP1	1.5	transforming growth factor beta receptor	Bt.594.1.S1_at

Table 6.5 Table of the genes that are differentially expressed at 368F mOsm compared to 368C mOsm.

This table lists the genes which were statistically significantly upregulated (paired t test $p<0.05$: ≥1.5 fold change) in Fnf samples compared to controls at 368 mOsm. Fold change in red: up-regulation; in black: down-regulation. [a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID. Unless stated, gene functions were integrated from Genecards (http://www.genecards.org/) and Ingenuity Pathway Analysis System.

6.3.3.3 Effect of Fnf on gene expression at 461 mOsm

With Fnf treatment (F), a total of only 9 annotated probe sets were reported as expressed at a significantly different level (all of the 9 probes were up-regulated) in 461F compared to 461C mOsm. A list of all the genes which were significantly up or down regulated (fold-change ≥

1.5) together with their functions are given in Table 6.6. Among those genes associated with the cartilage and disc, were MMP1, DKK1, and ASPN. ASPN encodes asporin, which is a critical regulator of TGF-beta in articular cartilage and plays an essential role in cartilage homeostasis and osteoarthritis pathogenesis[308]. Functions of MMP1 and DKK1 were mentioned in section 6.3.2. These genes were all upregulated with treatment with Fnf at 461 mOsm implying that Fnf may play a role in disc matrix metabolism in normal active physiological conditions (medium loads) viz. 461mOsm. The other genes were all upregulated, however their functions in the cartilage and discs are not well understood and need to be further determined.

Gene	Fold change	Function of encoded gene	Probe Set ID[a]
NOV	1.7	regulation of cell growth	Bt.13482.1.S1_at
MMP1	2.1	collagenase 1	Bt.72.1.S1_at
HIST1H1C	1.5	nucleosome assembly	Bt.2728.1.S1_at
DKK1	2.1	negative regulation of Wnt receptor signaling pathway	Bt.13880.1.S1_at
	2.3		Bt.13880.2.S1_at
CXCL2	2.1	chemotaxis	Bt.610.1.A1_at
	1.9		Bt.611.1.S2_at
HDC	1.6	histidine decarboxylase	Bt.18146.1.S1_at
ASPN	1.6	regulator of TGF-beta in articular cartilage	Bt.24211.1.A1_at
RARRES1	1.6	negative regulation of cell proliferation	Bt.24933.1.S1_at
SLCO4A1	1.6	ion transporter	Bt.27240.1.A1_at

Table 6.6 Table of the genes that are differentially expressed at 461F mOsm compared to 461C mOsm.

This table lists the genes that were statistically significantly altered (paired t test $p<0.05$: fold change ≥ 1.5) in Fnf samples compared to controls at 461 mOsm. Fold change in red: up-regulation; in black: down-regulation. [a]The Probe Set ID refers to the DNA microarray probe set identifier corresponding to that gene. See Chapter 2 section 2.3.3 to find out how to get information from the Probe Set ID. Unless stated, gene functions were integrated from Genecards (http://www.genecards.org/) and Ingenuity Pathway Analysis System.

6.3.4 Pathway analysis

The Ingenuity Pathways Analysis (IPA) software was used to reveal pathways on differentially expressed genes across the different osmolarities without Fnf treatment. The aim is to identify entire signaling cascades or pathways that contained a significant number of genes that were altered across two different osmolarities. The pathways identified can provide a good clue to the function of NP cells under particular osmolarity environment. The pathway analysis was not conducted on the effect of Fnf is because there were insufficient genes expressed when ±Fnf was compared at each osmolarity and thus IPA was unable to process the analysis.

There were 68 different biological processes identified as significantly different when considering those genes that were expressed at a higher osmolarity in all comparison conditions. The highlight canonical pathways include nitrogen metabolism, arginine and proline metabolism, urea cycle and metabolism of amino groups, lysine biosynthesis, and polyamine regulation in colon cancer. In nitrogen metabolism, nitrogen is a critical chemical element in both proteins and DNA, and thus every living organism must metabolize nitrogen to survive. However only 3 molecules in this study out of 133 were associated with nitrogen metabolism (see Appendix 7). Even fewer significant genes were involved in other pathways: (3/183) with arginine and proline metabolism; (2/80) with the urea cycle and metabolism of amino groups; (1/66) with lysine biosynthesis; and (1/22) with polyamine regulation in colon cancer. Since none of these pathways are of particular relevance to the disc, or can give significant results or information, their analysis was not pursued further.

It was surprising that, although many genes (see Result 6.3.2) were significantly different across vary osmolarity levels, none are meaningful to the disc. One of the reasons might be that the IPA system is based on data in the literature and there is as yet too little published information at the cell level on effects of osmolarity on chondrocytes or disc cells to relate gene expression of these cells to signaling pathways.

6.4 Discussion

A microarray based approach was used to discover changes in gene expression in NP cells incubated at different osmolarities and with Fnf treatment. The greatest changes were across the different osmolarities indicating that genes encoding a broad functional range of proteins are regulated by osmotic conditions. However relatively few genes showed altered expression as a result of adding Fnf to cell cultures.

In this study, when comparing gene expression of higher- to lower-osmotic conditions, viz. either 461C mOsm or 368C mOsm compared to 268C mOsm, we saw that osmolarity influenced expression of a number of agents involved in matrix turnover and degradation. Our results showed hyperosmolarity led to downregulation of the MMP-2 and MMP-3 genes (Table 6.7) consistent with the downregulation at the protein level described earlier in this thesis (Chapter 4 section 4.3.3). MMP-13 was also down regulated under hyper-osmolarity; the same trend in protein production was shown in Chapter 4 section 4.3.3. The downregulation of MMP2, 3, and 13 under hyper-osmotic conditions has also been seen

in a similar study carried out in human NP-cells.[249]. By contrast, expression of MMP1, MMP9, MMP11, MMP16, ADAMTS1, ADAMTS5, TIMP1 and TIMP3 (Table 6.7) were all upregulated at hyper-osmolarity. MMP-1 also known as interstitial collagenase or collagenase-1, is an enzyme that in humans is encoded by the MMP1 gene. MMP-1 has been identified in degenerated discs and its expression has been considered to be a possible initial event in disc alterations [94, 309, 310]. My study has shown that MMP-1 was upregulated more than 10 fold when the osmolarity increased from 268 mOsm or from 368 mOsm to 461 mOsm. The upregulation of ADAMTS1 and ADAMTS5 following hyper-osmotic load confirmed the data of other studies [136, 249]. The discrepancy of metalloproteinase gene expression under differing osmolarities may reflect different functions in matrix turnover of different metalloproteinases, the role of which in altered osmolarity are currently unknown.

	461C vs 268C	368C vs 268C	461C vs 368C
MMP1	16↑	-	11.6↑
MMP2	2.2↓	1.7↓	-
MMP3	1.9↓	1.7↓	-
MMP9	3.1↑	-	2.6↑
MMP11	2.3↑	1.9↑	-
MMP13	3.1↓	4.5↓	-
MMP16	1.6↑	-	-
ADAMTS1	2.8↑	-	3.4↑
ADAMTS5	-	-	2↑
TIMP1	1.9↑	-	1.7↑
TIMP3	1.9↑	1.9↑	-

Table 6.7 Table of the metalloproteinases genes that are differentially expressed (fold change) at differing comparisons.

This table lists the genes that were statistically altered (paired t test p0.05 and had a ≥1.5 fold change) in higher osmolarity compared compared to lower osmolarity; ↑: upregulation; ↓: downregulation; **-** : no difference.

Also of interest are four genes, GPNMB, DKK1, CLCA2, and CD96 in 81 probes the

expression of which in all three osmotic comparisons (see Figure 6.4, and Appendix 3) was identified as being significantly different with big fold changes (≥10), taken from 268C vs 461C. Although the functions of these genes in discs have not ever been studied, it may indicate that the protein production encoded by these genes was altered when the disc cells are under hyper-osmotic conditions, viz 461 mOsm.

GPNMB (Glycoprotein (transmembrane) nmb), also known as osteoactivin, showed a 20-fold decrease in gene expression in hyper-osmotic conditions. The GPNMB gene is known to be upregulated in osteoarthritic cartilage and associated with bone formation, which is a complex process involving the expression of specific growth factors and regulatory proteins that control cell proliferation, differentiation, and maturation [311]. GPNMB, involved in osteoblast differentiation (Entrez Gene), was found to be overexpressed 3- to 4-fold in rats with osteopetrotic bones when compared to normal rat long bones [312]. Furthermore osteoactivin mRNA was primarily localized in cuboidal osteoblasts lining bone surfaces [312]. Osteoactivin is expressed at highest levels in primary osteoblasts and thus may account for the imbalance in activity of osteoblasts and osteoclasts in osteopetrosis [312]. Overexpression of osteoactivin in an independent, weakly bone metastatic breast cancer cell model significantly enhanced the formation of osteolytic bone metastases in vivo [313]. In my study, the downregulation of GPNMB under hyper-osmotic condition, suggests that GPNMB not only exists in NP cells in discs, but may also play an important but unknown role in regulating cell function of disc cells.

DKK1 (Dickkopf homolog 1) showed up to a 12-fold increase in gene expression in hyper-osmotic condition. This gene encodes a secreted protein with two cysteine rich regions and is involved in embryonic development through its inhibition of the Wnt/β-catenin signaling pathway, a major signal transduction pathway of Wnt proteins, which can strongly affect matrix anabolic and catabolic metabolism in chondrocytes [307] (Entrez Gene). DKK1 inhibited cartilage matrix homeostasis toward catabolism [314]. Also as an extracellular inhibitor of osteoblasts, DKK1 was elevated in rheumatoid arthritis [315], while down-regulation of DKK1 promoted osteogenic differentiation [316]. Expression of DKK1 contributes to cartilage deterioration and is a potent factor in osteoarthritis pathogenesis [317]. In this study, DKK1 upregulation with increasing osmolarity and with Fnf treatment suggests that DKK1 play important roles in the regulation of discs matrix homeostasis.

CLCA2 (Chloride channel accessory 2) was also up-regulated with high fold change (10.6 fold). The protein encoded by this gene belongs to the calcium sensitive chloride conductance protein family (Entrez Gene). In addition to their function as chloride channels or channel modulators, some CLCA family members function as cell adhesion molecules and tumor suppressor proteins [318, 319]. It has also been proposed that CLCA proteins are membrane anchored or secreted metal-dependent hydrolases [320]. So far, no studies on the function of CLCA2 has been done in cartilage and disc, although in my study higher levels of expression of CLCA2 in hyper-osmotic condition suggests a specific role for CLCA2 in the development and homeostasis of disc cells.

CD96 was 10.3-fold up-regulated. CD96 (also termed Tactile, for T cell activation, increased late expression) was identified as a T cell-specific receptor that is highly upregulated upon T cell activation. CD96 is an Ig superfamily member with three Ig-like domains [321]. CD96 shares sequence similarity with CD226, Drosophila amalgam, the melanoma antigen MUC-18, members of the carcinoembryonic antigen (CEA) family, Necl-5 and the neural cell adhesion molecule (NCAM) [321, 322]. CD96 is generally absent from non-hematopoietic cell types such as carcinoma cell lines or fibroblasts with the exception of some intestinal epithelia [321, 323]. There are no reports concerning CD96 in the intervertebral disc. It may however have a novel function in disc metabolism.

A few genes showed altered expression with Fnf treatment compared to controls. These included some of the metalloproteinases, including MMP-1, MMP-13 and ADAMTS-1. However the fold changes were so low that they are not really significant. The microarray results are mostly in accord with my prior results on Fnf effects. In general, there was no evidence to show that Fnf play a role in intervertebral disc cell metabolism, viz. lactate production, metalloproteinase expression both at gene and protein level (Chapter 5:section 5.3) and gene screening for all known genes (Chapter 6: section 6.3.3). However, the Fnf played an important role in physiological or pathological environment in cartilage [175], its inconsistent action in discs may be due to cell type-related differences.

An unanticipated finding was that genes related to the extracellular matrix proteins were not found to be differently expressed at all three osmotic comparisons. The proteins encoded by

these genes including collagen, aggrecan, decorin, biglycan, fibromodulin, lumican, fibronectin and elastin are the main products of disc cell biosynthesis. However, it was found that NP cells expressed higher levels of ACAN, aggrecan gene, at 461 mOsm compared to lower osmotic environments (see Appendix 4 and 6). COL11A2 gene, which encodes collagen alpha-2(XI) chain, by comparison, was up-regulated at 368 mOsm when compared to 268mOsm. Type XI collagen is normally found in cartilage, and also in the NP of the discs. Thus the bovine NP cells appear to maintain matrix biosynthesis under altering osmotic conditions. As mentioned gene expression for metalloproteinases was changed with osmotic alterations, while the matrix genes adjusted themselves to the changing environment. These results were consistent with the data by Boyd [136]. This may suggest that hyper-osmotic environment may induce matrix protein expression.

It is desirable to validate microarray results using mRNA quantitation techniques. Studies show that correlation between qRT-PCR and RMA normalized microarray data are generally strong (r=0.89). However there were poor correlations between the two methods for 13% of 48 human genes [324]. Therefore future work should focus on the validating microarray data using qRT-PCR.

In summary, my studies analyzed gene expression in bovine NP cells grown in alginate culture under altered osmotic environment in the absence or presence of Fnf. Overall, the microarray has shown osmotic environment have major effects on NP-cell metabolism. Osmotic alterations appear to be more critical for the onset or progression of an imbalance of IVD matrix turnover than the effect of Fnf. Although the majority of the genes regulated by

osmotic stimuli have poorly understood functions in the intervertebral disc, a number of genes identified in this study were related to metalloproteinase activity, which is essential in the physiological and pathological process of intervertebral disc matrix metabolism.

Chapter 7 General discussion

The main aim of this thesis was to study the effect of fibronectin fragments (Fnf) on matrix turnover of intervertebral discs. Fnfs either initiate or amplify catabolic processes involved cartilage pathology [175]. As Fnf are seen in increasing concentrations in degenerate discs [57, 171], my hypothesis was that Fnf might also be involved in degradative processes in the disc. I first determined the pattern of metalloproteinase expression in discs cell and found differences compared to that seen in chondrocytes (Chapter 3).

However, using standard assays, it was found that the effects of Fnf on discs compared to those found in cartilages were only minor (Chapter 5). I adjusted osmotic conditions, to determine if the effects of Fnf on discs interacted with the osmotic environment (Chapter 5), but were still unable to detect the stimulation effects of Fnf on discs under differing osmolarity. Instead, osmolarity seemed to have large effects on disc matrix turnover. To further clarify whether there is really no effect of Fnf on discs, or methods employed to characterise the role of Fnf limited the responses observed, I used a gene microarray study to gain a more thorough insight into responses of disc cells when exposed both to Fnf and different levels of osmolarity (Chapter 6). Similar to the results at the protein level, gene microarray data have shown osmolarity rather than Fnf plays an important role in disc metabolism.

7.1 Osmotic variations are important for altering the matrix turnover of intervertebral discs

As noted in Chapter 1: section 1.2.1, the osmolarity of the disc is governed by GAGs, which are highly negatively charged. The negative charges attract free cations, such as Na^+ to balance the negative charges. As these cations are osmotically active, water is imbibed into the matrix to equilibrate osmotic pressures between disc and external solution. This creates a swelling pressure that enables the matrix to withstand compressive forces. Hence the hydration of the discs is not however constant as it varies with applied load [128] and goes through a diurnal cycle during the day's activities [39]. As a result of such changes in fluid content, the extracellular osmolarity of a normal nucleus cycles between approx. 450 and 550mOsm [129]. The osmotic environment of IVD cells has been shown to have a powerful effect on notochordal nucleus disc cells, as it modifies gene expression for proteins involved in cell cycle regulation, cell-cell interactions and adhesion, signal transduction mechanisms, growth factors and cytokines, and proteinases [136], but there is no detailed information on how changes in the osmotic environment affect mature disc nucleus cells such as those found in bovine discs.

By looking at both protein and gene level, we found that NP cells of disc respond to altered osmotic pressure with changes in expression of metalloproteinases: MMP-2, MMP-3 and MMP-13 at least (see Chapter 4: Figure 4.7; and Chapter 6: Table 6.7). Bovine NP cells significantly upregulated the MMP3 gene, and human NP cells also showed a tendency to upregulate MMP1, 2, 3 and 13 at the gene level under hypoosmotic conditions [249]. One of

the signs of disc degeneration is loss of aggrecan [246, 272, 273] which leads to a low osmotic environment of disc cells. The finding that MMPs responsible for matrix degradation are up-regulatd perhaps explains the progressive nature of degeneration, and the increase in MMPs levels shown in degraded discs [248]. However, I also found that gene expression of MMP1, MMP9, MMP11, MMP16, ADAMTS1 and ADAMTS5 were all upregulated at hyper-osmolarity (Chapter 6: Table 6.7) but as TIMP1 and TIMP3 were also upregulated under these conditions, it could be that activity of these MMPs was not changed. Moreover expression at the gene level does not necessarily reflect the protein level and the osmotic alterations could affect protein production differently to that seen at the gene level [325].

By using the gene microarray to further explore the whole genome picture of the effect of osmolarity on disc cells metabolism, we have identified that osmolarity influenced expression of a number of agents involved in matrix turnover and degradation. Especially, four genes (GPNMB, DKK1, CLCA2 and CD96) were identified as big fold changes in all comparison. GPNMB gene is known to play a role in a complex process involving the expression of specific growth factors and regulatory proteins that control cell proliferation, differentiation, and maturation [311]. DKK1 gene encodes a protein involved in inhibition of the Wnt/β-catenin signaling pathway, a major signal transduction pathway of Wnt proteins, which can strongly affect matrix anabolic and catabolic metabolism in chondrocytes [307]. The protein encoded by CLCA2 gene functions as a chloride channel or channel modulator, and as a cell adhesion molecule and tumor suppressor protein [318, 319]. CD96 was identified as a T cell-specific receptor and is generally absent from non-hematopoietic cell

types fibroblasts [321, 323]. In this study, CD96 was highly expressed and regulated by osmotic environment in NP cells. Although the functions of these genes in disc have not ever been studied, the big changes in expression of these genes under altering osmotic conditions may suggest roles of these encoded proteins on disc turnover.

Microarray results showed that hyper-osmotic environment results in higher levels of ACAN, the aggrecan gene (Chapter 6: 6.4, Appendix 4 and 6), which is in accord with our results on measurements of GAG (Chapter 4: Figure 4.4, 4.5) or those of another study which found that the sulphated GAG synthesis rates of disc cells, either in the tissue or isolated from it, increased about 200% when extracellular osmolarity was maintained at 420-450mOsm [130]. However, change of osmolarity caused by compression appear to affect GAG chain length rather than the core protein [325], so the GAG synthesis and GAG assay which focus on concentration of sulphated GAG would not necessarily be in agreement with results of core protein gene expression; in addition changes in gene expression may not reflect changes in protein expression.

The osmotic effects on disc turnover imply that it is important to choose an appropriate osmotic condition to optimize the activity of disc cells in any culture systems. Since the extracellular osmolarity of a normal nucleus cycles around 25% during each 24 hours [129], IVD cells are subjected to a complex osmotic environment; however, how they respond to these osmotic signals *in vivo* to produce matrix appropriate for their customary load is at present unknown and so far there is no study which has investigated how such osmotic cycles

affect matrix turnover. The osmolarity of commercial DMEM culture medium is 280 mOsm [209], which represents the low osmolarity seen during disc degeneration and should be used with care when the cell behavior is to be observed physiologically. To attain a higher osmotic media, either NaCl or sucrose or other sugar such as mannitol can be applied, because the biological response to changes in osmolarity does not appear to be mediated by ionic concentration changes, but rather depends directly on extracellular osmotic pressure [130].

7.2 Differences between disc cells and articular cartilage chondrocytes

Disc NP cells are commonly characterized as being "chondrocyte-like" and share many similarities with chondrocytes; they have a similar morphology in the tissue, existing in a capsule [12, 46, 108], and produce a matrix consisting of mainly of collagen II and aggrecan and containing the same spectrum of other macromolecular components [23, 34, 61, 65, 68]. They are also similar in physio-pathological situations. The extracellular osmolarity of normal discs and cartilage is affected by daily activities, for discs it cycles between 450 and 550mOsm over 24 hours [129]; whereas for cartilage it cycles between 350-450mOsm over each period of prolonged activity rather than diurnally [209]. The effects of osmolarity are very similar on the cell behaviour and matrix production in cartilages and discs. Osmolarity appears to affect cell volume at least in the short term (hours) with cell swelling observed at hypo-osmolarity and shrinkage under hyper-osmotic conditions, while sulphated GAG synthesis rates fall under hypo-osmotic conditions and increase under hyper-osmolarity [130, 326]. Studies have also shown similar osmotic regulation of gene expression in intervertebral disc cells and chondrocytes [135, 327]. During degeneration, the most significant biochemical change to occur in discs is loss of proteoglycan, which is responsible for a fall in

the osmotic pressure of the disc matrix and so a loss of hydration [1, 246]; later osteoarthritis is also characterised by proteoglycan loss from the cartilage, which further exacerbates osmotic perturbations [328].

Little is known about what distinguishes NP cells from articular chondrocytes and how this influences the production of metalloproteinases involved in normal tissue homeostasis as well as tissue degradation. Therefore I aimed to determine the different patterns of metalloproteinase expression in discs cells compared to that seen in chondrocytes (Chapter 3). The study revealed useful comparative information of gene expression of metalloprotainases between discs and cartilage: under identical environmental conditions, NP cells expressed considerably more MMP-2 and MMP-14 than chondrocytes, but expression of MMP-12, and MMP-27, by NP cells was not observed; this difference is a potential marker for distinguishing between NP cells and chondrocytes. Although both cell types express similar MMP patterns in general, articular cartilage remains intact well into adulthood while in humans, disc degeneration can be observed histologically in NP as early as in the second decade of life and the earliest signs of disc degeneration appear on magnetic resonance imaging in the nucleus pulposus soon after the disappearance of notochordal cells [329, 330]. These age-related changes in composition and structure of the intervertebral disc occur earlier and to a greater extent than in any other source of cartilage [331].

Metalloproteinases have an essential role during growth and in normal tissue homeostasis in the adult; murine gene deletion studies have shown that the lack of a given metalloproteinase

can exacerbate cartilage destruction and subsequent arthritis [332]. Differences in expression could lead to differences seen in aggrecan degradation between disc and cartilage; unlike the situation in articular cartilage, many of the aggrecan molecules in normal discs are proteolytically degraded soon after biosynthesis [333, 334] and yet have a long half-life (decades) and are osmotically active [335]. While important for normal turnover, genes of the metalloproteinase family and their inhibitors also play an essential role in the deregulation of the normal homoeostatic mechanism in disc [74, 85, 336] as well as in cartilage [95] and in the consequent degradative processes. To determine whether the differences in the gene expression profiles reported here are involved in the different patterns of degradation seen in these two tissues, it is first necessary to determine whether differences in MMP expression at the gene level are reciprocated at the protein and enzymatic level.

Unlike studies on cartilage explants and chondrocytes, my studies on disc have not shown the ability of 30kD N-terminal fibronectin fragment to stimulate proteoglycan degradation at either the protein or gene level (Chapter 5 and Chapter 6: 6.3.3). These results are in agreement with a study which found that the response to Fnf was less pronounced in NP cells than in articular chondrocytes, while annulus fibrosus cells were unresponsive to fibronectin fragments except at very high concentrations [153]. Work on chondrocytes indicates that the 29-kd Fnf forms a 150kD complex with a cell membrane protein on fibroblasts [175] and that this process is necessary for signaling. Whether disc cells have the necessary receptor at the cell surface is at present unknown; lack of the receptor could be one reason why disc cells respond poorly to the Fnf.

While the activities of the 29kD Fnf may still play only a minor role in disc matrix degradation, other matrix component fragments could initiate degradation. It has recently been shown that other fibronectin fragments (40kD, 45kD, 110kD, and 120kD) and/or other degraded matrix macromolecules (elastin peptides, chondroadherin fragments, collagen peptides, hyaluronan fragments and laminin-derived peptides) affect cartilage matrix turnover. [58, 158-160, 176, 177, 190]. These fragments could also affect disc matrix turnover and it would be useful to explore them in this tissue as well.

7.3 Future directions

Extracellular osmolarity is only a small part of a very complex physical-chemical environment. Understanding how disc cells integrate and respond to osmotic changes in their extracellular microenvironment in relation to other factors such as variations in extracellular pH, mechanical stresses such as pressure and stretch and oxygen tesion will be fundamental for understanding the mechanisms involved in the maintenance of healthy discs. Associated with this, and of particular interest, is the differential regulation of these parameters in degenerative diseases. My studies have characterised the transcriptional response of disc NP cells to osmotic alteration by using a gene microarray. It is important to further verify the genes which were expressed significantly differently under osmotic perturbations both by real-time PCR and at the protein level. These genes include the noted four (Chapter 6: Figure 6.4 and Appendix 3) and metalloproteinases genes (Chapter 6: Table 6.7) which appeared particular responsive to alterations in osmolarity. Verification of gene microarray results could include the use of real-time PCR for the confirmation at gene level and western blotting

or immunohistochemistry at the protein level. Once the candidate genes are confirmed, their function should be further explored in disc cells and other cartilages, both by tracking their expression in response to extracellular signals other than osmolarity, and by knockout studies or by gene silencing [337, 338].

The degraded fragment system has yet to be fully understood. These fragments have been suggested to play a role in maintaining normal tissue metabolism [188, 293], as well as in catabolic cytokine pathways [155]. The involvement of these fragments in the complex pathways of disc homeostasis could be helpful both for understanding disc degradation and for promoting repair.

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Appendix 1 Chemicals

Chemicals	Suppliers
Dulbecco's Modified Eagle's Medium (Cat. No. 22320-022, with 25mM Hepes, 1mM sodium pyruvate, 1000mg/L glucose and pyroxidine) Antibiotic/antimycotic solution (Cat No. 15240-062) Dulbecco's Phosphate Buffered Saline (D-PBS) (Cat. No. 14190-094) SeeBlue Plus2 Pre-Stained Standard(Cat.No.LC5925) Fetal bovine serum (Cat. No. 10106-169)	Invitrogen 5 Fountain Drive Paisley Scotland
Alginic acid, Sodium salt from brown algae (Cat. No. 71238)	Fluka Biochemika The Old Brickyard New Rd,Gillingham,Dorset,UK
1,9-Dimethylmethylene blue-chloride(Cat. No. 341088)	Sigma-Aldrich Company Ltd., Poole Dorset, UK
MMP/TIMP Positive Control,Human Fibroblast(Cat.No.PF069)	Merck Biosciences Ltd. Boulevard Industrial Park,Padge Road,Beeston, NOTTINGHAM,NG9 2JR
Lactate assay kit (Cat.No.735-10)	Trinity Biotech Plc. Bray. Co. Wicklow,Ireland
DC Protein Assay (Cat. No.500-0116)	BIO-RAD
Rabbit polyclonal to MMP3 (Cat. No. ab53015)	Abcam, Cambridge, UK
Rabbit polyclonal to MMP13-Hinge region(Cat.No.ab39012)	
Polyclonal Goat Anti-Rabbit immunoglobulins/HRP(Code-Nr.P0448)	DakoCytomation,Denmark
Amersham ECL Western blotting detection reagents and analysis system(RPN2209)	GE Healthcare, UK
Hyperfilm ECL(RPN2103K)	
Hybond ECL Membrane(RPN3032D)	
Hybond Blotting Paper(RPN6101M)	
SB 203580(Cat.No. 559389)	Calbiochem,UK
JNK Inhibitor II(Cat.No.420119)	
U0126(Cat.No.662005)	

All other chemicals were from the Sigma (Sigma-Aldrich Company Ltd., Poole Dorset, UK)

Appendix 2 Solutions

1.2% Alginate solution:
1.2g alginic acid
0.9g NaCl
NaCl was added to 100ml ultrapure water and heated to 60-65^{o}C on a hotplate stirrer. Alginic acid was then added slowly over 2-5 minutes. This was covered with parafilm and left for 1-2 hours. It was then filter sterilized (pore size 0.2μm) and stored at room temperature.

Citrate buffer:
1.617g sodium citrate
1.8g EDTA
0.876g NaCl
This was made up to 100ml with ultrapure water. The pH was adjusted to 7.4 using NaOH. The solution was filter sterilized (pore size 0.2μm) and stored at room temperature.

Glycosaminoglycan assay reagent:
21mg 1,9- dimethylmethylene blue
5ml absolute ethanol
2.0g sodium formate
1L ultrapure water was added, and adjusted pH to relevant requirements by formic acid. Solution was kept in brown bottle at room temperature and discarded after one month.

Zymography/ Reverse Zymography Buffers:

Solution/Buffer	Contents	Recipe	Storage
Incubation Buffer (Developing Buffer, Enzyme Buffer)	50mM TRIZMA, pH7.6; 10mM $CaCl_2.2H_2O$, 50mM NaCl, 0.05% Triton X-100)	For 1 L: 6.06g Tris base, 1.47g CaCl2, 2.92g NaCl, 0.5ml Triton X-100; Make up to 800ml with HPLC water, pH to 7.6 with HCl. add water to final volume of 1L	4^oC
5 x Running Buffer (Laemmli Buffer)	125mM TRIZMA, pH 8.3, 1.23M Glycine, 0.5% S.D.S	For 1L: 15.1g Tris base, 94g Glycine, 5g S.D.S. Make up to 800ml with HPLC water, pH to 8.3. Add water to final volume of 1L.	Room temperature
2 x Non-reducing buffer (Sample Buffer)	For 8ml: mix 2.8ml dH2O; 1ml 0.5M Tris-HCl, pH 6.8; 0.8ml glycerol; 3.2ml 10% (w/v) S.D.S; 0.2ml 0.2% (0.04g)bromophenol blue	For 16ml: 2ml 0.5M Tris-HCl, 1.6ml Glycerol, 3.2ml 20%(W/V)S.D.S, 0.4ml 0.2% bromophenol blue, (0.08g), 8.8ml dH_2O	Room temperature
Stain Buffer	0.25%Coomassie Blue R250, 40% methanol and 10% acetid acid		Room temperature
30% Acrylamide solution	29.2% Acrylamide; 0.8% N'N'-Bis-methylene acrylamide		Dark 4^oC

Western blot Solutions:

Transfer buffer:

100ml of 10×Tris/Glycine, 200ml methanol made up to 1L with distilled water

10×Tris/Glycine:

25mM TrisBase

192mM Glycine

pH8.3. No need to adjust pH

10×Tris-Buffered Saline (TBS)

20mM TrisBase

500mM Nacl

Ajusted pH with 5.0M HCl to pH7.4

Appendix 3 Genes from 53 probes that are altered in all comparisons that increase or decrease in signal with increasing osmolarity (28 unnamed probes deleted)

Cluster Name	Gene	Gene Symbol	Fold Change			Probe Set ID
			(268C vs 368C)	(368C vs 461C)	(268C vs 461C)	
acute inflammatory response and chronic inflammatory response	vanin 1	VNN1	***1.5***	3.0	2.0	Bt.28243.1.S1_a_at
angiogenesis	angiogenin, ribonuclease, RNase A family, 5	ANG	***1.6***	***2.6***	***4.1***	Bt.20883.1.S1_at
			1.7	***2.4***	***4.1***	Bt.12722.1.A1_at
			1.6	1.8	3.0	Bt.29009.1.A1_at
antigen processing and presentation	MHC class I antigen /// MHC Class I JSP.1	BOLA-N /// JSP.1	1.6	1.6	2.5	Bt.3805.1.S1_at
apoptosis	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	1.7	2.2	3.7	Bt.26692.1.S1_a_at
cell adhesion	CD96 molecule	CD96	***5.5***	***1.9***	***10.3***	Bt.20940.1.S1_at
	glycoprotein (transmembrane) nmb	GPNMB	2.8	7.1	20.0	Bt.9807.1.S1_at
			3.0	2.3	6.7	Bt.9807.2.S1_at
			3.0	2.2	6.7	Bt.9807.3.S1_at
	cadherin 11, type 2, OB-cadherin (osteoblast) /// similar to Cadherin-11 precursor (Osteoblast cadherin) (OB-cadherin) (OSF-4)	CDH11 /// LOC790083	1.6	1.8	2.8	Bt.2696.2.S1_at
			1.6	1.7	2.7	Bt.2696.1.A1_at
cysteine metabolic process	Glutamate-cysteine ligase, catalytic subunit	GCLC	1.6	2.3	3.8	Bt.19499.1.S1_at
estrogen biosynthetic process	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	1.6	2.4	3.8	Bt.22879.1.S1_at
female pregnancy	uterine milk protein	LOC286871	1.8	***6.7***	***3.7***	Bt.24199.1.A1_s_at
			2.2	***7.0***	***3.2***	Bt.594.1.S1_at
	thrombomodulin	THBD	***2.9***	***2.3***	***6.9***	Bt.471.1.S1_at

Fold change in bold: up-regulation; not in bold: down-regulation

Cluster Name	Gene	Gene Symbol	Fold Change			Probe Set ID
			(268C vs 368C)	(368C vs 461C)	(268C vs 461C)	
glycine catabolic process	glycine cleavage system protein H (aminomethyl carrier)	GCSH	2.1	2.2	4.6	Bt.4195.1.S1_at
metabolic process and cell wall macromolecule catabolic process	lysozyme (renal amyloidosis)	LYZ	***4.2***	***1.8***	***7.5***	Bt.209.3.S1_at
multicellular organismal development and cartilage development	leukocyte cell derived chemotaxin 1	LECT1	***3.3***	1.8	***1.9***	Bt.1509.1.S1_at
negative regulation of signal transduction	Regulator of G-protein signaling 5	RGS5	1.6	2.2	3.5	Bt.28182.1.A1_at
negative regulation of transcription from RNA polymerase II promoter	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	***3.6***	***2.3***	***8.2***	Bt.1730.1.A1_at
	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	***2.4***	***1.5***	***3.6***	Bt.5230.1.S1_at
	early growth response 1	EGR1	1.5	2.5	3.8	Bt.22265.1.S1_at
nucleosome assembly	histone H4-like	LOC617875	3.1	2.4	7.2	Bt.9531.1.S1_at
one-carbon metabolic process	carbonic anhydrase IV	CA4	***2.7***	***2.0***	***5.5***	Bt.555.1.S1_at
ovarian follicle development	kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	***1.6***	***1.7***	***2.8***	Bt.13860.1.S1_at
phosphatidylinositol metabolic process	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	***1.8***	***1.6***	***2.9***	Bt.20732.1.A1_at
polyamine biosynthetic process	ornithine decarboxylase 1	ODC1	1.9	1.5	2.9	Bt.7133.1.S1_at
positive regulation of I-kappaB kinase	lectin, galactoside-binding, soluble, 1	LGALS1	2.2	2.1	4.5	Bt.5472.1.S1_at
Cluster Name	**Gene**	**Gene Symbol**	**Fold Change**			**Probe Set ID**
			(268C vs 368C)	**(368C vs 461C)**	**(268C vs 461C)**	
prostaglandin biosynthetic process	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	***1.6***	***2.5***	***4.0***	Bt.2151.2.A1_at
protein amino acid phosphorylation and cell-matrix adhesion	TEK tyrosine kinase, endothelial	TEK	***1.8***	***2.3***	***4.1***	Bt.4390.1.S1_at
protein amino acid phosphorylation and	doublecortin-like kinase 1	DCLK1	2.1	1.8	3.6	Bt.14572.1.A1_at

intracellular signaling cascade						
skeletal system development	C-type lectin domain family 3, member B	CLEC3B	2.0	1.6	3.1	Bt.1366.1.S1_at
T cell costimulation	CD80 molecule	CD80	***1.6***	***1.5***	***2.5***	Bt.27986.1.S1_at
small GTPase mediated signal transduction and protein transport	RAB3B, member RAS oncogene family	RAB3B	***2.1***	***1.7***	***3.5***	Bt.388.1.S1_at
urea cycle and arginine metabolic process	arginase, type II	ARG2	***2.9***	***1.9***	***5.6***	Bt.8624.1.S1_at
T cell costimulation	hypothetical protein MGC127538	MGC127538	***3.9***	***10.2***	***40.3***	Bt.2577.1.S1_at
inhibitor of osteoblast differentiation	Dickkopf homolog 1 (Xenopus laevis)	DKK1	***2.4***	***5.1***	***12.0***	Bt.13880.1.S1_at
chloride channel modulator and cell adhesion molecule	chloride channel accessory 2 (CLCA2)	CLCA2	***1.8***	***5.8***	***10.6***	Bt.15970.1.A1_at
Interacting selectively and non-covalently with any nucleic acid	similar to RNA-binding protein 35A (RNA-binding motif protein 35A)	LOC788414	***2.1***	***3.9***	***8.2***	Bt.27030.1.S1_at
Cluster Name	**Gene**	**Gene Symbol**	**Fold Change**			**Probe Set ID**
			(268C vs 368C)	**(368C vs 461C)**	**(268C vs 461C)**	
Inhibitor of phospholipase A2, also possesses anti-coagulant properties. Also cleaves the cyclic bond of inositol 1,2-cyclic phosphate to form inositol 1-phosphate	annexin A3	ANXA3	***2.4***	***1.6***	***3.8***	Bt.6775.1.S1_at
Interacts with PPFIBP1 in a calcium-dependent mode	S100 calcium binding protein A4	S100A4	***2.0***	***1.8***	***3.6***	Bt.5493.1.S1_at
Interacting selectively and non-covalently with zinc (Zn) ions	prickle homolog 1 (Drosophila)	PRICKLE1	1.7	1.6	2.8	Bt.18126.2.S1_at
Unknown function	midline 1 (Opitz/BBB syndrome)	MID1	***1.6***	***1.8***	***2.9***	Bt.16611.1.A1_at
	leucine rich repeat neuronal 1	LRRN1	***1.7***	***1.7***	***2.9***	Bt.17579.1.A1_at
	furry homolog (Drosophila)	FRY	***1.8***	***1.5***	***2.7***	Bt.8327.1.S1_at
	leucine rich repeat containing 17	LRRC17	2.4	3.2	7.8	Bt.4886.1.A1_at
	transmembrane protein 220	TMEM220	2.0	2.0	4.0	Bt.28251.1.S1_at
	Complement factor H-related 5	CFHR5	1.8	1.5	2.8	Bt.17612.2.S1_at

	LIM domain and actin binding 1	LIMA1	1.5	1.6	2.4	Bt.10730.1.S1_at
	similar to Mal, T-cell differentiation protein /// mal, T-cell differentiation protein	LOC782642 /// MAL	***2.5***	***2.2***	***5.3***	Bt.4060.1.S1_at
	monooxygenase, DBH-like 1	MOXD1	***2.9***	***1.7***	***5.0***	Bt.20519.1.A1_at

Fold change in bold: up-regulation; not in bold: down-regulation

Appendix 4 Genes from 315 probes that are altered in 368mOsm compared to 268mOsm that change in signal with increasing osmolarity (131 unnamed probes deleted)

Cluster Name	Gene	Gene Symbol	Fold change	Probe Set ID
Wnt receptor signaling pathway	catenin, beta interacting protein 1	CTNNBIP1	***1.6***	Bt.14644.1.A1_at
			1.6	Bt.14644.2.S1_at
urea cycle and arginine metabolic process	arginase, type II	ARG2	***2.9***	Bt.8624.1.S1_at
trophectodermal cell differentiation and cell adhesion	cadherin 1, type 1, E-cadherin (epithelial)	CDH1	***1.7***	Bt.11241.1.S1_at
small GTPase mediated signal transduction and protein transport	RAB3B, member RAS oncogene family	RAB3B	***2.1***	Bt.388.1.S1_at
organic anion transport and transmembrane transport	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	SLC16A1	1.7	Bt.13537.1.A1_at
ion transport	sodium channel, voltage-gated, type IV, beta	SCN4B	***1.8***	Bt.8809.1.S1_at
	potassium inwardly-rectifying channel, subfamily J, member 8	KCNJ8	1.7	Bt.13065.1.S1_at
	potassium channel, subfamily K, member 17	KCNK17	***1.7***	Bt.12534.1.S1_at
	scavenger receptor class A, member 5 (putative)	SCARA5	***2.6***	Bt.12715.1.S1_at
cell communication and protein transport	sorting nexin 10	SNX10	1.6	Bt.27822.2.S1_at
			1.6	Bt.27822.1.A1_at
carbon dioxide transport and ammonium transport	aquaporin 1 (Colton blood group)	AQP1	2.1	Bt.1525.1.S2_at
carbohydrate transport and transmembrane transport	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	SLC2A5	***2.2***	Bt.19805.1.A1_at
transport	lysosomal protein transmembrane 5	LAPTM5	***2.4***	Bt.16049.1.S1_at
transcription	transcription elongation factor A (SII), 3	TCEA3	***1.6***	Bt.3026.1.A1_at
	nuclear receptor subfamily 1, group H, member 3	NR1H3	***1.9***	Bt.17265.1.A1_a_at
	cell division cycle associated 7	CDCA7	***1.7***	Bt.6441.1.S1_at
tissue homeostasis and chondrocyte differentiation	collagen, type XI, alpha 2	COL11A2	***3.5***	Bt.2303.1.S1_at
tetrahydrobiopterin biosynthetic process	pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha	PCBD1	***1.6***	Bt.23171.2.S1_at
T cell costimulation	CD80 molecule	CD80	***1.6***	Bt.27986.1.S1_at
steroid biosynthetic process	cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51	1.6	Bt.621.1.S1_at
	Isopentenyl-diphosphate delta isomerase 1	IDI1	1.6	Bt.3764.1.S1_at
	farnesyl-diphosphate farnesyltransferase 1	FDFT1	1.8	Bt.21724.2.S1_a_at
small GTPase mediated signal transduction	RAB38, member RAS oncogene family	RAB38	***1.7***	Bt.27433.1.S1_at

skeletal system development	C-type lectin domain family 3, member B	CLEC3B	2.0	Bt.1366.1.S1_at
signal transduction	Similar to IQ motif containing GTPase activating protein 2	LOC100140708	***1.7***	Bt.20947.1.S1_at
	frizzled homolog 1 (Drosophila)	FZD1	1.5	Bt.26635.1.S1_at
	Ankyrin 3, node of Ranvier (ankyrin G)	ANK3	***2.7***	Bt.6086.1.S1_at
	phosphodiesterase 1A, calmodulin-dependent	PDE1A	***2.0***	Bt.512.1.S1_at
	protein phosphatase 1, regulatory (inhibitor) subunit 1A	PPP1R1A	***1.9***	Bt.8964.2.S1_a_at
	fibrinogen-like 1	FGL1	***1.7***	Bt.13204.1.S1_at
	angiopoietin-like 7	ANGPTL7	***1.7***	Bt.24342.1.A1_at
	tenascin C	TNC	1.5	Bt.11061.1.S1_at
response to hypoxia	plasminogen activator, urokinase	PLAU	2.6	Bt.2519.1.S1_at
	hypothetical protein MGC127538	MGC127538	***3.9***	Bt.2577.1.S1_at
	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	1.7	Bt.5313.1.S1_at
response to estrogen stimulus	cytokeratin 19	LOC514812	***2.2***	Bt.12663.1.S1_at
regulation of translation and insulin receptor signaling pathway	eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1	1.8	Bt.5389.1.S1_at
regulation of transcription, DNA-dependent	distal-less homeobox 4	DLX4	***1.7***	Bt.27619.1.A1_at
	zinc finger protein 75 (D8C6)	ZNF75	***1.5***	Bt.16761.1.S1_at
	nuclear receptor subfamily 3, group C, member 2	NR3C2	***1.5***	Bt.17332.1.S1_at
regulation of the force of heart contraction	myosin, light chain 3, alkali; ventricular, skeletal, slow	MYL3	1.5	Bt.23497.1.S1_at
regulation of I-kappaB kinase/NF-kappaB cascade	sequestosome 1	SQSTM1	1.7	Bt.28031.1.S1_s_at
regulation of cell growth	discoidin domain receptor tyrosine kinase 1	DDR1	***2.0***	Bt.7540.3.S1_at
	insulin-like growth factor binding protein 7	IGFBP7	***1.9***	Bt.2214.1.S1_at
	insulin-like growth factor binding protein 4	IGFBP4	2.2	Bt.5237.1.S1_at
proteolysis and metabolic process	Matrix metallopeptidase 13 (collagenase 3)	MMP13	4.5	Bt.26268.1.A1_at
			3.7	Bt.39.1.S1_at
	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	MMP3	1.7	Bt.18504.2.A1_at
			1.6	Bt.18504.1.S1_at
	aminoacylase 1 /// similar to aminoacylase 1	ACY1 /// LOC790609	1.5	Bt.12885.1.S1_at
	matrix metallopeptidase 11 (stromelysin 3)	MMP11	***1.9***	Bt.13703.1.S1_at
	carboxypeptidase A1 (pancreatic) /// carboxypeptidase A5	CPA1 /// CPA5	***1.9***	Bt.21706.2.A1_a_at
	plasma glutamate carboxypeptidase	PGCP	***1.7***	Bt.2120.2.S1_at
	ADAM metallopeptidase domain 33	ADAM33	***1.6***	Bt.2566.1.S1_at
	plasma glutamate carboxypeptidase	PGCP	***1.5***	Bt.2120.1.S1_at
	complement factor I	CFI	***1.5***	Bt.13387.1.S1_at
	fibroblast activation protein, alpha	FAP	1.8	Bt.2717.1.A1_at
	legumain	LGMN	1.6	Bt.8947.1.S1_at
	cathepsin A	CTSA	1.6	Bt.21410.1.S1_at
protein targeting and signal transduction	A kinase (PRKA) anchor protein 12	AKAP12	1.5	Bt.10458.2.S1_at

protein modification process	WW domain containing E3 ubiquitin protein ligase 2	WWP2	***1.9***	Bt.23266.1.S1_at
	tubulin tyrosine ligase-like family, member 12	TTLL12	***1.6***	Bt.12406.1.S1_at
protein import into nucleus, docking	exportin 1 (CRM1 homolog, yeast)	XPO1	1.6	Bt.9586.1.A1_at
protein folding	FK506 binding protein 14, 22 kDa	FKBP14	***1.5***	Bt.28429.1.S1_at
protein amino acid phosphorylation	G protein-coupled receptor kinase 4	GRK4	***1.6***	Bt.2996.2.S1_a_at
	mitogen-activated protein kinase 13	MAPK13	1.8	Bt.800.1.S1_at
	ribosomal protein S6 kinase, 90kDa, polypeptide 1	RPS6KA1	***1.8***	Bt.27200.1.S1_at
	p21 protein (Cdc42/Rac)-activated kinase 1	PAK1	***2.4***	Bt.5858.1.S1_at
			1.9	Bt.22106.2.S1_at
	doublecortin-like kinase 1	DCLK1	2.1	Bt.14572.1.A1_at
	TEK tyrosine kinase, endothelial	TEK	***1.8***	Bt.4390.1.S1_at
	protein kinase-like protein SgK196	SGK196	***1.6***	Bt.20140.1.S1_at
	mitogen-activated protein kinase 6	MAPK6	1.5	Bt.18165.1.A1_at
protein amino acid glycosylation	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	B3GALT2	3.3	Bt.7251.1.S1_at
			2.9	Bt.7447.1.A1_at
			2.5	Bt.19796.1.S1_at
			1.6	Bt.19796.2.A1_at
prostaglandin biosynthetic process	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	***1.6***	Bt.2151.2.A1_at
post-translational protein modification	ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	UBE2E2	***1.6***	Bt.972.1.S1_at
positive regulation of natural killer cell mediated cytotoxicity directed against tumor cell target	poliovirus receptor-related 2 (herpesvirus entry mediator B)	PVRL2	1.6	Bt.4943.3.S1_a_at
positive regulation of I-kappaB kinase/NF-kappaB cascade	lectin, galactoside-binding, soluble, 1	LGALS1	2.2	Bt.5472.1.S1_at
positive regulation of endothelial cell proliferation	cadherin 13, H-cadherin (heart)	CDH13	***2.6***	Bt.6592.1.S1_at
polyamine biosynthetic process	ornithine decarboxylase 1	ODC1	1.9	Bt.7133.1.S1_at
phosphatidylinositol metabolic process	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	***1.8***	Bt.20732.1.A1_at
pattern specification process	aspartate beta-hydroxylase	ASPH	1.5	Bt.14648.1.A1_at
	sclerostin domain containing 1	SOSTDC1	***2.4***	Bt.27202.1.A1_at
oxidation reduction	lysyl oxidase-like 4	LOXL4	***1.8***	Bt.12297.1.S1_at
	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	CYP3A4	***1.8***	Bt.82.1.A1_at
ovarian follicle rupture	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	AGT	***2.0***	Bt.14715.1.S1_at
ovarian follicle development	kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	***1.6***	Bt.13860.1.S1_at
organ morphogenesis	transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)	TGM1	***2.6***	Bt.736.1.A1_at

one-carbon metabolic process	carbonic anhydrase III, muscle specific	CA3	***2.7***	Bt.2737.1.S1_at
	carbonic anhydrase IV	CA4	***2.7***	Bt.555.1.S1_at
nucleosome assembly	histone cluster 1, H1c	HIST1H1C	***1.9***	Bt.2728.1.S1_at
	histone H4-like	LOC617875	3.1	Bt.9531.1.S1_at
	H1 histone family, member 0	H1F0	***1.6***	Bt.20929.2.A1_at
neuropeptide signaling pathway	neuromedin B	NMB	***1.6***	Bt.10630.1.S1_at
negative regulation of transcription from RNA polymerase II promoter	early growth response 1	EGR1	1.5	Bt.22265.1.S1_at
	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	***2.4***	Bt.5230.1.S1_at
	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	***3.6***	Bt.1730.1.A1_at
	LIM and cysteine-rich domains 1	LMCD1	***1.6***	Bt.624.1.S1_at
negative regulation of signal transduction	DNA-damage-inducible transcript 4-like	DDIT4L	***2.8***	Bt.22199.1.S1_at
	Regulator of G-protein signaling 5	RGS5	1.6	Bt.28182.1.A1_at
negative regulation of membrane protein ectodomain proteolysis	TIMP metallopeptidase inhibitor 3	TIMP3	***1.9***	Bt.5136.1.S1_at
negative regulation of endocytosis	protein kinase C and casein kinase substrate in neurons 2	PACSIN2	***1.6***	Bt.20341.1.S1_at
	scinderin	SCIN	***1.9***	Bt.3980.1.S1_at
	Kruppel-like factor 4 (gut)	KLF4	***1.7***	Bt.1424.1.S1_at
negative regulation of angiogenesis	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	***1.6***	Bt.2638.1.S1_at
muscle thin filament assembly	actin, alpha 2, smooth muscle, aorta	ACTA2	***2.8***	Bt.12825.1.S1_at
multicellular organismal development	frizzled-related protein	FRZB	***2.8***	Bt.121.1.S1_at
	leukocyte cell derived chemotaxin 1	LECT1	***3.3***	Bt.1509.1.S1_at
	chromosome 20 open reading frame 149 ortholog	C13H20orf149	1.6	Bt.23173.1.S1_at
	shisa homolog 2 (Xenopus laevis)	SHISA2	***2.1***	Bt.22389.1.S1_at
microtubule bundle formation	Microtubule-associated protein 2	MAP2	***1.6***	Bt.13769.1.S1_at
metabolic process	fatty acyl CoA reductase 1	FAR1	1.7	Bt.10230.1.A1_at
			1.7	Bt.20738.1.S1_at
	lysozyme (renal amyloidosis)	LYZ	***4.2***	Bt.209.3.S1_at
	NEDD8 activating enzyme E1 subunit 1	NAE1	1.5	Bt.15600.2.S1_at
L-serine biosynthetic process	phosphoserine aminotransferase 1	PSAT1	***1.9***	Bt.13588.1.A1_at
liver development	lipolysis stimulated lipoprotein receptor	LSR	***1.7***	Bt.12462.1.S1_at
lipid metabolic process	low density lipoprotein receptor	LDLR	1.6	Bt.3562.1.S1_at
	trans-2,3-enoyl-CoA reductase	TECR	1.5	Bt.7701.1.S1_at
	phospholipase C, beta 4	PLCB4	***1.6***	Bt.4552.1.S1_a_at
	insulin induced gene 1	INSIG1	1.7	Bt.24614.1.S1_at
	tenascin XB	TNXB	***2.0***	Bt.5398.1.S1_at
	stearoyl-CoA desaturase 5	SCD5	***1.7***	Bt.13249.1.A1_at
intracellular signaling cascade	Rho guanine nucleotide exchange factor (GEF) 5	ARHGEF5	***1.8***	Bt.18132.1.A1_at
	adenylate cyclase 2 (brain)	ADCY2	***1.6***	Bt.18840.1.S1_at
	pleckstrin	PLEK	***2.6***	Bt.15713.1.A1_at

	pleckstrin 2	PLEK2	***1.6***	Bt.28534.1.A1_at
intracellular protein transport	similar to AP-3 complex subunit beta-2 (Adapter-related protein complex 3 beta-2 subunit) (Beta3B-adaptin) (Adaptor protein complex AP-3 beta-2 subunit) (AP-3 complex beta-2 subunit) (Clathrin assembly protein complex 3 beta-2 large chain)	LOC790439	***2.0***	Bt.10002.1.S1_at
	secretogranin V (7B2 protein)	SCG5	***1.6***	Bt.1580.1.S1_at
inflammatory response	chemokine (C-X-C motif) ligand 2	CXCL2	***2.1***	Bt.610.1.A1_at
induction of apoptosis	prune homolog 2 (Drosophila)	PRUNE2	***2.2***	Bt.18615.1.A1_at
in utero embryonic development	GATA-6	LOC654400	1.5	Bt.24023.1.A1_at
immune response	Similar to Interferon-induced guanylate-binding protein 1 (GTP-binding protein 1) (Guanine nucleotide-binding protein 1) (HuGBP-1)	LOC512486	***3.5***	Bt.9296.1.A1_at
homophilic cell adhesion	desmoglein 2	DSG2	***1.8***	Bt.16441.1.A1_at
heart development	plakophilin 2	PKP2	***3.5***	Bt.21547.1.A1_at
			2.6	Bt.21547.2.S1_at
G-protein coupled receptor protein signaling pathway	G protein-coupled receptor 1	GPR1	***2.5***	Bt.27053.1.A1_at
glycine catabolic process	glycine cleavage system protein H (aminomethyl carrier)	GCSH	2.1	Bt.4195.1.S1_at
glycerol metabolic process	Glycerophosphodiester phosphodiesterase domain containing 1	GDPD1	***3.3***	Bt.26100.1.S1_at
			3.1	Bt.26100.2.A1_at
G2/M transition of mitotic cell cycle	tumor protein D52-like 1	TPD52L1	***2.3***	Bt.15740.1.A1_at
			2.2	Bt.15740.2.S1_at
female pregnancy	thrombomodulin	THBD	***2.9***	Bt.471.1.S1_at
	uterine milk protein	LOC286871	2.2	Bt.594.1.S1_at
			1.8	Bt.24199.1.A1_s_at
fatty acid biosynthetic process	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	ELOVL5	***1.5***	Bt.7281.1.S1_at
estrogen biosynthetic process	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	1.6	Bt.22879.1.S1_at
epidermis development	cystatin E/M	CST6	***1.6***	Bt.5468.1.S1_at
DNA repair	nei like 2 (E. coli)	NEIL2	***1.8***	Bt.26303.1.A1_at
cytokine production	fatty acid binding protein 4, adipocyte	FABP4	2.4	Bt.97.1.S1_at
cysteine metabolic process	Glutamate-cysteine ligase, catalytic subunit	GCLC	1.6	Bt.19499.1.S1_at
copulation	peptidase inhibitor 3, skin-derived (SKALP)	PI3	***2.1***	Bt.28010.1.S1_at
chromatin assembly or disassembly	Chromobox homolog 6	CBX6	***1.7***	Bt.13458.1.S1_at
cholesterol biosynthetic process	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	1.6	Bt.11465.1.S1_at
	24-dehydrocholesterol reductase	DHCR24	1.6	Bt.9996.1.S1_at
cellular glucose homeostasis	forkhead box A3	FOXA3	***1.6***	Bt.27526.1.A1_at
cellular component organization	dishevelled associated activator of morphogenesis 1	DAAM1	***1.6***	Bt.28836.1.S1_at
cellular aromatic compound metabolic process	epoxide hydrolase 1, microsomal (xenobiotic)	EPHX1	***1.6***	Bt.23354.1.S1_at
cell-matrix adhesion	melanoma inhibitory activity	MIA	***1.8***	Bt.4267.1.S1_at

	nidogen 1	NID1	2.6	Bt.8106.1.S1_at
	ependymin related protein 1 (zebrafish)	EPDR1	***1.9***	Bt.21962.1.S1_at
cell surface receptor linked signal transduction	G protein-coupled receptor 133	GPR133	***2.6***	Bt.22380.1.S1_at
	frizzled homolog 2 (Drosophila)	FZD2	***2.1***	Bt.16951.1.A1_at
cell motion	myosin regulatory light chain interacting protein	MYLIP	***2.2***	Bt.6814.1.S1_at
cell cycle	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	SPC24	1.8	Bt.9517.1.S1_a_at
	Mitochondrial tumor suppressor 1	MTUS1	1.6	Bt.8319.3.S1_a_at
cell communication	gap junction protein, beta 2, 26kDa	GJB2	2.5	Bt.9760.1.S1_at
cell aging	proline/arginine-rich end leucine-rich repeat protein	PRELP	***2.3***	Bt.3202.1.A1_at
cell adhesion	filamin binding LIM protein 1	FBLIM1	***2.5***	Bt.2860.1.S1_at
	cadherin 2, type 1, N-cadherin (neuronal)	CDH2	***1.9***	Bt.4417.1.S1_at
	protocadherin 7	PCDH7	***1.7***	Bt.12063.1.S1_at
	desmocollin 2	DSC2	***1.6***	Bt.4107.1.S1_at
	cadherin 11, type 2, OB-cadherin (osteoblast) /// similar to Cadherin-11 precursor (Osteoblast cadherin) (OB-cadherin) (OSF-4)	CDH11 /// LOC790083	1.6	Bt.2696.1.A1_at
			1.6	Bt.2696.2.S1_at
	similar to Transforming growth factor-beta induced protein IG-H3 precursor (Beta IG-H3) (Kerato-epithelin) (RGD-containing collagen associated protein) (RGD-CAP)	LOC539596	1.7	Bt.4670.1.S1_at
	junction plakoglobin	JUP	***1.7***	Bt.20230.1.S1_at
	CD96 molecule	CD96	***5.5***	Bt.20940.1.S1_at
	glycoprotein (transmembrane) nmb	GPNMB	3.0	Bt.9807.3.S1_at
			3.0	Bt.9807.2.S1_at
			2.8	Bt.9807.1.S1_at
	spondin 1, extracellular matrix protein	SPON1	2.0	Bt.7176.1.S1_at
			1.6	Bt.9640.1.A1_at
cartilage condensation	collagen, type II, alpha 1	COL2A1	***1.8***	Bt.21390.1.S1_at
	aggrecan	ACAN	***2.1***	Bt.4953.1.S1_at
			1.6	Bt.29859.1.S1_at
carbohydrate metabolic process	similar to Putative hexokinase HKDC1 (Hexokinase domain-containing protein 1)	LOC614824	***1.5***	Bt.1522.1.A1_at
	similar to Putative glycerophosphodiester phosphodiesterase 5	LOC518469	1.8	Bt.12179.1.S1_at
	glutamine-fructose-6-phosphate transaminase 2	GFPT2	1.5	Bt.8781.1.S1_at
	Alpha-galactosyltransferase 1 (glycoprotein)	GGTA1	1.9	Bt.22564.2.S1_at
brown fat cell differentiation	laminin, beta 3	LAMB3	***1.6***	Bt.16740.1.A1_at
branching involved in ureteric bud morphogenesis	glypican 3	GPC3	***1.7***	Bt.11178.1.S1_at
base-excision repair, DNA ligation	High-mobility group box 2	HMGB2	1.7	Bt.6397.1.S1_at
			1.7	Bt.6397.2.S1_at
apoptosis	keratin 8	KRT8	***3.0***	Bt.23608.1.S1_s_at
	keratin 18	KRT18	***2.4***	Bt.1745.1.S1_at
	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	1.7	Bt.26692.1.S1_a_at

antigen processing and presentation of peptide antigen via MHC class I	MHC class I antigen /// MHC Class I JSP.1	BOLA-N /// JSP.1	1.6	Bt.3805.1.S1_at
antigen processing and presentation of exogenous peptide antigen via MHC class II	gamma-inducible protein 30	IFI30	***1.5***	Bt.1548.1.S1_at
angiogenesis	angiopoietin-like 4	ANGPTL4	1.5	Bt.4816.1.S1_at
angiogenesis /// regulation of transcription, DNA-dependent /// microvillus assembly /// positive regulation of transcription	Kruppel-like factor 5 (intestinal)	KLF5	***2.1***	Bt.15802.2.S1_at
	angiogenin, ribonuclease, RNase A family, 5 /// ribonuclease, RNase A family, 4	ANG /// RNASE4	***1.7***	Bt.12722.1.A1_at
			1.6	Bt.20883.1.S1_at
adult walking behavior	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1	1.9	Bt.22763.2.S1_a_at
acute inflammatory response /// chronic inflammatory response	vanin 1	VNN1	***1.5***	Bt.28243.1.S1_a_at
Promotes attachment of chondrocytes, fibroblasts, and osteoblasts.	chondroadherin	CHAD	***2.9***	Bt.347.1.S1_at
May play a role in a signaling cascade by enhancing the kinase activity of SRC. Contributes to SRC-regulated transcription activation	actin filament associated protein 1-like 2	AFAP1L2	***2.8***	Bt.22165.1.S1_at
Inhibitor of phospholipase A2, also possesses anti-coagulant properties	annexin A3	ANXA3	***2.4***	Bt.6775.1.S1_at
inhibitor of osteoblast differentiation	Dickkopf homolog 1 (Xenopus laevis)	DKK1	***2.4***	Bt.13880.1.S1_at
Interacting selectively and non-covalently with any nucleic acid/nucleotide	RNA binding protein with multiple splicing	RBPMS	***2.1***	Bt.11525.2.A1_at
Interacting selectively and non-covalently with calcium ions	S100 calcium binding protein A4	S100A4	***2.0***	Bt.5493.1.S1_at
Catalysis of facilitated diffusion of an ion	hydrogen voltage-gated channel 1	HVCN1	***2.0***	Bt.797.1.S1_at
a regulator of protein phosphorylation in that the ANXA2 monomer is the preferred target (in vitro) of tyrosine-specific kinase	S100 calcium-binding protein A10	S100A10	***2.0***	Bt.5037.1.S1_at
chloride channel modulator and cell adhesion molecule	chloride channel accessory 2	CLCA2	***1.8***	Bt.15970.1.A1_at
May be involved in the development and regeneration of the musculoskeletal system	Musculoskeletal, embryonic nuclear protein 1	MUSTN1	***1.7***	Bt.9832.1.S1_at
May be involved in the development and regeneration of the musculoskeletal system	musculoskeletal, embryonic nuclear protein 1	MUSTN1	***1.7***	Bt.9832.1.S1_a_at

Interacting selectively and non-covalently with any nucleic acid/nucleotide	RNA binding protein with multiple splicing	RBPMS	***1.7***	Bt.11525.3.S1_at
Regulator of cell surface receptor signal transduction	tetraspanin 12	TSPAN12	***1.6***	Bt.29152.1.S1_at
Regulator of cell surface receptor signal transduction.	Tetraspanin 12	TSPAN12	***1.5***	Bt.29152.2.A1_at
growth factor activity	fibroblast growth factor 12	FGF12	***1.5***	Bt.25062.1.A1_at
Interacting selectively and non-covalently with zinc (Zn) ions	prickle homolog 1 (Drosophila)	PRICKLE1	1.7	Bt.18126.2.S1_at
Interacting selectively and non-covalently with zinc (Zn) ions	WD repeat and FYVE domain containing 1	WDFY1	1.7	Bt.27929.1.S1_at
Phosphodiesterase capable of cleaving at phosphodiester internal bonds within a DNA or RNA substrate	heat-responsive protein 12	HRSP12	1.6	Bt.1556.1.S1_at
one of the nuclear-coded polypeptide chains of cytochrome c oxidase	cytochrome c oxidase subunit VIa polypeptide 1	COX6A1	1.5	Bt.23002.1.S1_at
the most prominent cellular substrate for protein kinase C	myristoylated alanine-rich protein kinase C substrate	MARCKS	1.5	Bt.4067.1.S1_at
Enhances Ca^{2+}-mediated inhibition of inositol-1,4,5-triphosphate receptor (ITPR) Ca^{2+} release	inositol 1,4,5-triphosphate receptor interacting protein	ITPRIP	1.5	Bt.26784.1.S1_at
Unknown function	thyroid hormone responsive (SPOT14 homolog, rat)	THRSP	***4.2***	Bt.15842.1.S1_at
	Plexin C1	PLXNC1	***3.9***	Bt.26968.1.S1_at
	collagen, type IX, alpha 2	COL9A2	***3.1***	Bt.4235.1.S1_at
	monooxygenase, DBH-like 1	MOXD1	***2.9***	Bt.20519.1.A1_at
	SH3 domain binding glutamic acid-rich protein like 2	SH3BGRL2	***2.7***	Bt.16036.1.S1_at
	mal, T-cell differentiation protein-like	MALL	***2.6***	Bt.13290.1.S1_at
	occludin	OCLN	***2.5***	Bt.13868.1.S1_at
	Transmembrane protein 163	TMEM163	***2.5***	Bt.20951.1.A1_at
	similar to Mal, T-cell differentiation protein /// mal, T-cell differentiation protein	LOC782642 /// MAL	***2.5***	Bt.4060.1.S1_at
	hypothetical LOC513129	LOC513129	***2.4***	Bt.10124.1.S1_at
	Plexin C1	PLXNC1	***2.4***	Bt.6608.1.S1_at
	Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	CELSR2	***2.3***	Bt.13493.1.A1_at
	similar to F47B8.14	LOC525174	***2.3***	Bt.8637.1.S1_at
	proline-serine-threonine phosphatase interacting protein 2	PSTPIP2	***2.3***	Bt.28073.1.S1_at
	ash1 (absent, small, or homeotic)-like (Drosophila)	ASH1L	***2.2***	Bt.5037.1.S2_at
	SH3 domain and tetratricopeptide repeats 2	SH3TC2	***2.1***	Bt.711.1.A1_at

	hypothetical LOC507695	LOC507695	***2.1***	Bt.28344.1.S1_at
	similar to RNA-binding protein 35A (RNA-binding motif protein 35A)	LOC788414	***2.1***	Bt.27030.1.S1_at
	hemicentin 1	HMCN1	***2.1***	Bt.26051.1.A1_at
	transmembrane protein 163	TMEM163	***2.1***	Bt.20951.2.S1_at
	similar to amine oxidase, copper containing 2	LOC100140673	***2.1***	Bt.12704.1.S1_at
	RAB38, member RAS oncogene family	RAB38	***2.0***	Bt.27433.2.A1_at
	Similar to guanylate binding protein 1	LOC511531	***2.0***	Bt.24012.1.A1_at
	Raftlin family member 2	RFTN2	***2.0***	Bt.24894.1.S1_at
	fin bud initiation factor homolog (zebrafish)	FIBIN	***2.0***	Bt.3435.1.A1_at
	sushi-repeat-containing protein, X-linked 2	SRPX2	***2.0***	Bt.8109.2.A1_at
	zinc finger, DHHC-type containing 14	ZDHHC14	***2.0***	Bt.22230.1.S1_at
	Trophoblast glycoprotein	TPBG	***2.0***	Bt.24902.1.S1_at
	AMME chromosomal region gene 1-like	AMMECR1L	***2.0***	Bt.17508.1.S1_at
	Similar to cell adhesion molecule JCAM	LOC511594	***1.9***	Bt.3194.1.S1_at
	shisa homolog 3 (Xenopus laevis)	SHISA3	***1.9***	Bt.11057.1.S1_at
	Platelet derived growth factor C	PDGFC	***1.9***	Bt.3014.1.A1_at
	desmoplakin	DSP	***1.9***	Bt.1818.1.S1_at
	acid phosphatase 5, tartrate resistant	ACP5	***1.9***	Bt.5193.2.S1_a_at
	pirin (iron-binding nuclear protein)	PIR	***1.9***	Bt.21680.1.S1_a_at
	transmembrane protein 98	TMEM98	***1.8***	Bt.23548.2.S1_at
	furry homolog (Drosophila)	FRY	***1.8***	Bt.8327.1.S1_at
	Integrin, alpha 6	ITGA6	***1.8***	Bt.4544.1.S1_at
	tensin 3	TNS3	***1.8***	Bt.2399.1.S1_at
	cytoplasmic FMR1 interacting protein 2	CYFIP2	***1.7***	Bt.7948.1.S1_at
	Exostoses (multiple)-like 1	EXTL1	***1.7***	Bt.25895.2.A1_at
	leucine rich repeat neuronal 1	LRRN1	***1.7***	Bt.17579.1.A1_at
	monooxygenase, DBH-like 1	MOXD1	***1.7***	Bt.20519.2.S1_at
	similar to beta-1,4-N-acetyl-galactosaminyl transferase 4	LOC100137759	***1.7***	Bt.14237.1.A1_at
	similar to inositol polyphosphate-5-phosphatase F	LOC539889	***1.7***	Bt.15840.1.A1_at
	pleckstrin homology domain containing, family B (evectins) member 1	PLEKHB1	***1.7***	Bt.20038.1.S1_at
	AMME chromosomal region gene 1-like	AMMECR1L	***1.7***	Bt.17508.2.A1_at
	pirin (iron-binding nuclear protein)	PIR	***1.7***	Bt.21680.1.S1_at
	pigeon homolog (Drosophila)	PION	***1.7***	Bt.16521.2.A1_at
	hypothetical LOC100174923	LOC100174923	***1.7***	Bt.2521.1.S1_at
	Dedicator of cytokinesis 5	DOCK5	***1.7***	Bt.20310.1.S1_at
	contactin associated protein-like 2	CNTNAP2	***1.7***	Bt.5618.1.A1_at
	allograft inflammatory factor 1-like	AIF1L	***1.7***	Bt.10983.1.S1_at
	similar to Protein FAM101B	LOC618459	***1.7***	Bt.3701.1.S1_at
	Minichromosome maintenance complex component 10	MCM10	***1.6***	Bt.16556.1.A1_at
	midline 1 (Opitz/BBB syndrome)	MID1	***1.6***	Bt.16611.1.A1_at
	Golgi-localized protein	GOLSYN	***1.6***	Bt.28562.1.S1_at

	neural proliferation, differentiation and control, 1	NPDC1	***1.6***	Bt.1791.1.A1_at
	Tight junction protein 2 (zona occludens 2)	TJP2	***1.6***	Bt.20601.2.S1_at
	src kinase associated phosphoprotein 1	SKAP1	***1.6***	Bt.20853.1.S1_a_at
	hypothetical LOC100196901	LOC100196901	***1.6***	Bt.21688.1.S1_at
	tight junction protein 2 (zona occludens 2)	TJP2	***1.6***	Bt.20601.1.A1_at
	GDNF family receptor alpha 4	GFRA4	***1.6***	Bt.21175.1.S1_at
	similar to chromosome 9 open reading frame 61	LOC509420	***1.6***	Bt.26538.1.S1_at
	chondroitin sulfate proteoglycan 4	CSPG4	***1.6***	Bt.28247.1.A1_s_at
	serpin peptidase inhibitor, clade I (neuroserpin), member 1	SERPINI1	***1.5***	Bt.19231.1.S1_at
	similar to nucleolar protein No55	LOC788823	***1.5***	Bt.1720.1.S1_at
	indoleamine 2,3-dioxygenase 2	IDO2	***1.5***	Bt.18821.1.A1_at
	similar to LPS-responsive vesicle trafficking, beach and anchor containing	LOC524279	***1.5***	Bt.13411.1.S1_at
	filamin B, beta	FLNB	***1.5***	Bt.7646.1.S1_at
	transmembrane protein 30B	TMEM30B	***1.5***	Bt.22042.1.S1_at
	transmembrane protein 165	TMEM165	***1.5***	Bt.4994.1.S1_at
	Hypothetical LOC505226	MGC134574	3.8	Bt.17517.1.S1_at
	leucine rich repeat containing 17	LRRC17	2.4	Bt.4886.1.A1_at
	transmembrane protein 220	TMEM220	2.0	Bt.28251.1.S1_at
	CD68 molecule	CD68	1.9	Bt.2334.1.S1_at
	Complement factor H-related 5	CFHR5	1.8	Bt.17612.2.S1_at
	complement factor H-related 4	CFHR4	1.8	Bt.17612.1.A1_at
	Insulin-like growth factor binding protein, acid labile subunit	IGFALS	1.6	Bt.24635.2.A1_at
	similar to meningioma expressed antigen 5 (hyaluronidase)	LOC538561	1.6	Bt.28653.1.S1_at
	Similar to nucleoporin 62	LOC516074	1.6	Bt.19355.1.A1_at
	LIM domain and actin binding 1	LIMA1	1.5	Bt.10730.1.S1_at
	Ubiquitin domain containing 2	UBTD2	1.5	Bt.27591.1.S1_at

Fold change in bold: up-regulation; not in bold: down-regulation

Appendix 5 Genes from 399 probes that are altered in 461mOsm compared to 368mOsm that change in signal with increasing osmolarity (145 unnamed probes deleted)

Cluster Name	Gene	Gene Symbol	Fold change	Probe Set ID
proteolysis /// metabolic process /// collagen catabolic process	matrix metallopeptidase 1 (interstitial collagenase)	MMP1	***11.6***	Bt.72.1.S1_at
chloride channel modulator and cell adhesion molecule	chloride channel accessory 2	CLCA2	***5.8***	Bt.15970.1.A1_at
inhibitor of osteoblast differentiation	Dickkopf homolog 1 (Xenopus laevis)	DKK1	***5.1***	Bt.13880.1.S1_at
acute inflammatory response and chronic inflammatory response process	vanin 1	VNN1	3.0	Bt.28243.1.S1_a_at
			2.2	Bt.28243.2.S1_at
	nuclear protein, transcriptional regulator, 1	NUPR1	***1.7***	Bt.23597.1.S1_at
AMP catabolic process and nucleotide catabolic process	5'-nucleotidase, ecto (CD73)	NT5E	***1.6***	Bt.2367.1.A1_at
angiogenesis	angiogenin, ribonuclease, RNase A family, 5	ANG /// RNASE4	***2.6***	Bt.20883.1.S1_at
			2.4	Bt.12722.1.A1_at
			1.8	Bt.29009.1.A1_at
	ras homolog gene family, member B	RHOB	1.7	Bt.8382.2.S1_at
antigen processing and presentation of peptide antigen via MHC class I	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A	BoLA /// HLA-A	1.9	Bt.29814.1.S1_at
	MHC class I antigen /// MHC Class I JSP.1	BOLA-N /// JSP.1	1.6	Bt.3805.1.S1_at
apoptosis	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	2.2	Bt.26692.1.S1_a_at
			1.6	Bt.6958.3.A1_at
arginine catabolic process	fumarylacetoacetate hydrolase (fumarylacetoacetase)	FAH	1.6	Bt.16412.1.S1_a_at
aromatic compound catabolic process	paraoxonase 3	PON3	1.6	Bt.20372.1.S1_at
ATP biosynthetic process	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	***1.5***	Bt.6993.1.S1_at
B cell homeostasis	tumor necrosis factor (ligand) superfamily, member 13b	TNFSF13B	***1.7***	Bt.24855.2.S1_at
blood coagulation	tissue factor pathway inhibitor 2	TFPI2	***1.9***	Bt.2017.1.S1_at
blood vessel development	collagen, type I, alpha 1	COL1A1	1.6	Bt.28583.1.S1_s_at
	Laminin, alpha 4	LAMA4	1.6	Bt.7984.1.S1_at
calcium-mediated signaling	regulator of calcineurin 1	RCAN1	***1.8***	Bt.6377.1.S1_at
carbohydrate metabolic process	mannose phosphate isomerase	MPI	1.6	Bt.27806.1.S1_at
	Phosphorylase, glycogen, liver	PYGL	1.6	Bt.17714.1.A1_at
			1.5	Bt.28093.1.S1_at
	Glucokinase (hexokinase 4)	GCK	1.6	Bt.27524.1.A1_at

carbohydrate transport	hypothetical protein LOC613571	MGC152007	1.6	Bt.9320.1.S1_a_at
cell adhesion	CD96 molecule	CD96	***1.9***	Bt.20940.1.S1_at
	collagen, type XV, alpha 1	COL15A1	***1.7***	Bt.20512.1.S1_at
	neuropilin 1	NRP1	***1.5***	Bt.20373.1.S1_at
	glycoprotein (transmembrane) nmb	GPNMB	7.1	Bt.9807.1.S1_at
			2.3	Bt.9807.2.S1_at
			2.2	Bt.9807.3.S1_at
	collagen, type XVI, alpha 1	COL16A1	2.2	Bt.12697.1.S1_a_at
			2.1	Bt.12697.1.S1_at
	collagen, type XII, alpha 1	COL12A1	1.6	Bt.10442.2.S1_a_at
	similar to Transforming growth factor-beta induced protein IG-H3 precursor (Beta IG-H3) (Kerato-epithelin) (RGD-containing collagen associated protein) (RGD-CAP)	LOC539596	***1.6***	Bt.4670.1.S1_at
	cadherin 11, type 2, OB-cadherin (osteoblast) /// similar to Cadherin-11 precursor (Osteoblast cadherin) (OB-cadherin) (OSF-4)	CDH11 /// LOC790083	1.8	Bt.2696.2.S1_at
			1.7	Bt.2696.1.A1_at
	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	ITGB2	2.4	Bt.4615.1.S1_at
cell cycle and mitosis	ubiquitin-conjugating enzyme E2C	UBE2C	1.8	Bt.2725.1.S1_at
cell differentiation	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	GNPTAB	1.5	Bt.8513.1.A1_at
cell morphogenesis involved in differentiation	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	DAB2	1.5	Bt.3814.1.S1_at
cell proliferation	vascular endothelial growth factor C	VEGFC	***1.9***	Bt.3913.1.S1_at
cellular amino acid and derivative metabolic process	histidine decarboxylase	HDC	***1.6***	Bt.18146.1.S1_at
cellular component organization	formin homology 2 domain containing 3	FHOD3	3.4	Bt.24819.1.S1_at
chemotaxis and G-protein coupled receptor protein signaling pathway	G protein-coupled receptor 77	GPR77	***2.1***	Bt.26157.1.A1_at
chronological cell aging	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	***1.8***	Bt.5229.1.S1_at
creatine biosynthetic process	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	GATM	1.6	Bt.23250.2.S1_at
cysteine metabolic process	Glutamate-cysteine ligase, catalytic subunit	GCLC	2.3	Bt.19499.1.S1_at
			1.8	Bt.16068.1.A1_at
deoxyribonucleoside diphosphate metabolic process	ribonucleotide reductase M2 polypeptide	RRM2	2.2	Bt.10648.1.S1_at
DNA topological change	Topoisomerase (DNA) II alpha 170kDa	TOP2A	1.9	Bt.20277.1.S1_at
dUTP metabolic process	deoxyuridine triphosphatase	DUT	1.7	Bt.11936.1.S1_at
			1.7	Bt.11936.1.S1_a_at
erythrocyte maturation	TIMP metallopeptidase inhibitor 1	TIMP1	***1.7***	Bt.632.1.S1_s_at
			1.7	Bt.632.1.S1_at
estrogen biosynthetic process	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	2.4	Bt.22879.1.S1_at

fatty acid beta-oxidation	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	DCI	1.6	Bt.13530.1.S1_at
	2,4-dienoyl CoA reductase 1, mitochondrial	DECR1	1.5	Bt.13390.2.S1_a_at
fatty acid biosynthetic process	fatty acid synthase	FASN	***1.7***	Bt.22854.2.S1_at
female pregnancy	uterine milk protein	LOC286871	***7.0***	Bt.594.1.S1_at
			6.7	Bt.24199.1.A1_s_at
	thrombomodulin	THBD	***2.3***	Bt.471.1.S1_at
formation of translation initiation complex	eukaryotic translation initiation factor 3, subunit D	EIF3D	1.6	Bt.23244.1.S1_at
G1/S transition of mitotic cell cycle	inhibin, beta A	INHBA	***2.0***	Bt.12760.1.S1_at
G2 phase of mitotic cell cycle	amyloid beta (A4) precursor protein	APP	1.5	Bt.13152.1.S1_s_at
glycine catabolic process	glycine cleavage system protein H (aminomethyl carrier)	GCSH	2.2	Bt.4195.1.S1_at
G-protein coupled receptor protein signaling pathway	G protein-coupled receptor 88	GPR88	***1.7***	Bt.9893.1.S1_at
healing during inflammatory response	heme oxygenase (decycling) 1	HMOX1	2.0	Bt.4001.1.S1_at
heart development	adrenomedullin	ADM	1.5	Bt.2047.1.S1_at
immune response	proteoglycan 4	PRG4	***2.3***	Bt.22987.1.S1_at
			2.3	Bt.22987.2.A1_at
			1.9	Bt.22987.1.A1_at
	chemokine (C-X-C motif) ligand 14	CXCL14	***1.6***	Bt.20397.1.S1_at
	similar to fractalkine	LOC517354	2.0	Bt.1275.1.S1_at
	chemokine (C-X-C motif) ligand 2	CXCL2	1.8	Bt.23093.1.S1_at
	sushi domain containing 2	SUSD2	1.5	Bt.3206.1.A1_at
	complement component 7	C7	1.8	Bt.18557.1.S1_at
in utero embryonic development	endonuclease G	ENDOG	1.6	Bt.3857.1.S1_at
	complement component 6	C6	2.3	Bt.23722.1.A1_at
	complement component 5	C5	***4.7***	Bt.5622.1.S1_at
induction of apoptosis	pleckstrin homology domain containing, family F (with FYVE domain) member 1	PLEKHF1	1.6	Bt.2421.1.A1_at
	hypothetical protein LOC617441	C14orf153	1.6	Bt.13847.1.S1_at
inflammatory response	interleukin 17D	IL17D	1.6	Bt.20223.2.S1_at
	chemokine (C-X-C motif) ligand 2	CXCL2	2.3	Bt.610.1.A1_at
intermediate filament-based process	vimentin	VIM	1.7	Bt.11149.1.S1_at
intracellular protein transport	mitochondrial ribosomal protein L45	MRPL45	1.6	Bt.853.1.S1_at
intracellular signaling cascade	adenylate cyclase 2 (brain)	ADCY2	1.6	Bt.18840.1.S1_at
	stathmin 1	STMN1	1.9	Bt.3137.1.S1_at
	suppressor of cytokine signaling 2	SOCS2	***2.6***	Bt.13273.1.S1_at
	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	***1.7***	Bt.10831.1.S1_at
	epithelial cell transforming sequence 2 oncogene	ECT2	***1.5***	Bt.28366.2.S1_at
			1.5	Bt.28366.1.A1_at
ion transport	Mucolipin 2	MCOLN2	***1.6***	Bt.25669.1.S1_at

learning or memory and dendrite development	abl-interactor 2	ABI2	1.6	Bt.26877.1.A1_at
lipid metabolic process	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	SRD5A1	1.9	Bt.27694.1.S1_at
	stearoyl-CoA desaturase (delta-9-desaturase)	SCD	***1.8***	Bt.4798.1.S2_at
	phospholipase A1 member A	PLA1A	***2.3***	Bt.19884.1.A1_at
			2.1	Bt.19884.2.S1_at
	low density lipoprotein receptor	LDLR	***1.6***	Bt.3562.1.S1_at
malate metabolic process	malic enzyme 1, NADP(+)-dependent, cytosolic	ME1	1.6	Bt.26829.1.S1_at
MAPKKK cascade and activation of MAPK activity	mitogen-activated protein kinase kinase 6	MAP2K6	1.7	Bt.561.1.S1_at
metabolic process	arylsulfatase B	ARSB	***2.1***	Bt.18689.1.S1_at
			1.9	Bt.18689.2.A1_at
	solute carrier family 27 (fatty acid transporter), member 3	SLC27A3	***1.8***	Bt.1828.1.A1_at
	Similar to Long-chain-fatty-acid--CoA ligase 3 (Long-chain acyl-CoA synthetase 3) (LACS 3)	LOC100138312	***1.5***	Bt.17121.1.S1_at
	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	AGPAT5	1.9	Bt.22170.1.S1_a_at
			1.9	Bt.16140.1.A1_at
			1.7	Bt.22170.2.A1_at
			1.7	Bt.22170.1.S1_at
			1.7	Bt.23626.1.S1_at
	lysozyme (renal amyloidosis)	LYZ	***1.8***	Bt.209.3.S1_at
	branched chain aminotransferase 1, cytosolic	BCAT1	1.9	Bt.17278.3.S1_a_at
			1.7	Bt.17278.3.S1_at
			1.7	Bt.17278.1.A1_at
	enolase superfamily member 1	ENOSF1	1.5	Bt.3276.1.A1_at
	sorbitol dehydrogenase	SORD	1.7	Bt.16122.1.S1_at
	DPH5 homolog (S. cerevisiae)	DPH5	1.6	Bt.21419.1.S1_at
			1.5	Bt.24263.1.S1_at
monocyte chemotaxis	chemokine (C-C motif) ligand 2	CCL2	2.4	Bt.2408.1.S1_s_at
			2.1	Bt.2408.1.S1_at
morphogenesis of an epithelium	carbonic anhydrase II	CA2	***4.0***	Bt.22854.1.S1_at
multicellular organismal development	cysteine and glycine-rich protein 2	CSRP2	***1.7***	Bt.2262.1.S1_at
	leukocyte cell derived chemotaxin 1	LECT1	1.8	Bt.1509.1.S1_at
	dickkopf homolog 1 (Xenopus laevis)	DKK1	***2.0***	Bt.13880.2.S1_at
	mab-21-like 1 (C. elegans)	MAB21L1	1.6	Bt.19449.1.A1_at
muscle organ development	transgelin 2	TAGLN2	***1.5***	Bt.23135.1.S1_at
myeloid progenitor cell differentiation	myeloid leukemia factor 1	MLF1	2.2	Bt.21766.3.S1_at
			2.0	Bt.21766.1.A1_at
			1.9	Bt.21766.2.S1_at
negative regulation of angiogenesis	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	1.5	Bt.2638.1.S1_at

negative regulation of endothelial cell proliferation	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	AIMP1	1.5	Bt.9072.1.S1_at
negative regulation of protein amino acid phosphorylation caffeine	FK506 binding protein 1A, 12kDa	FKBP1A	1.5	Bt.22329.1.S1_at
negative regulation of signal transduction	Regulator of G-protein signaling 5	RGS5	2.2	Bt.28182.1.A1_at
negative regulation of T-helper 1 cell differentiation	interleukin 4 receptor	IL4R	***1.7***	Bt.21831.1.S1_at
negative regulation of transcription from RNA polymerase II promoter	LIM and cysteine-rich domains 1	LMCD1	1.7	Bt.624.1.S1_at
	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	***2.3***	Bt.1730.1.A1_at
	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	***1.5***	Bt.5230.1.S1_at
	early growth response 1	EGR1	2.5	Bt.22265.1.S1_at
neuron migration	FYN oncogene related to SRC, FGR, YES	FYN	***1.5***	Bt.2359.1.A1_at
neuron remodeling	netrin 4	NTN4	***1.8***	Bt.26919.1.S1_at
neurotransmitter secretion	forty-two-three domain containing 1 /// synaptosomal-associated protein, 25kDa	FYTTD1 /// SNAP25	1.6	Bt.20032.1.A1_at
nucleosome assembly	histone H4-like	LOC617875	2.4	Bt.9531.1.S1_at
one-carbon metabolic process	carbonic anhydrase IV	CA4	***2.0***	Bt.555.1.S1_at
	carbonic anhydrase IX	CA9	2.9	Bt.27842.1.S1_at
	Carbonic anhydrase VIII	CA8	1.5	Bt.22726.1.A1_at
ossification	pleiotrophin	PTN	1.7	Bt.4844.1.S1_at
ovarian follicle development	kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	***1.7***	Bt.13860.1.S1_at
			1.6	Bt.13860.2.A1_at
ovulation from ovarian follicle	ADAM metallopeptidase with thrombospondin type 1 motif, 1	ADAMTS1	***3.4***	Bt.12010.2.S1_at
			3.2	Bt.12010.1.S1_at
patterning of blood vessels	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	1.6	Bt.2524.1.S1_at
peptide secretion and protein amino acid lipidation	ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	***1.7***	Bt.19423.1.S1_at
phagocytosis, recognition	collectin sub-family member 12	COLEC12	1.6	Bt.4492.1.S1_at
phosphatidylinositol metabolic process	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	***1.6***	Bt.20732.1.A1_at
phospholipid biosynthetic process	lysophosphatidylcholine acyltransferase 3	LPCAT3	***1.8***	Bt.27204.1.S1_at
placenta development and Wnt receptor signaling pathway	Norrie disease (pseudoglioma)	NDP	2.5	Bt.3307.1.A1_at
polyamine biosynthetic process	ornithine decarboxylase 1	ODC1	1.5	Bt.7133.1.S1_at
positive regulation of I-kappaB kinase	lectin, galactoside-binding, soluble, 1	LGALS1	2.1	Bt.5472.1.S1_at
positive regulation of leukocyte chemotaxis	Coagulation factor II (thrombin) receptor-like 1	F2RL1	***1.6***	Bt.28745.1.S1_at
postsynaptic membrane organization	cholinergic receptor, nicotinic, beta 1 (muscle)	CHRNB1	1.5	Bt.5107.1.S1_at

prostaglandin biosynthetic process	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	***2.5***	Bt.2151.2.A1_at
	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	1.7	Bt.15528.1.S1_at
	prostaglandin D2 synthase 21kDa (brain)	PTGDS	2.9	Bt.1645.1.S1_at
protein amino acid dephosphorylation	protein tyrosine phosphatase, receptor type, S	PTPRS	***1.7***	Bt.21399.3.S1_a_at
	dual specificity phosphatase 10	DUSP10	1.5	Bt.2070.1.A1_at
protein amino acid phosphorylation	renal tumor antigen	RAGE	***1.6***	Bt.18292.1.A1_at
	WNK lysine deficient protein kinase 2	WNK2	1.6	Bt.21050.1.S1_at
	similar to Serine/threonine-protein kinase Sgk3 (Serum/glucocorticoid-regulated kinase 3) (Serum/glucocorticoid-regulated kinase-like)	SGK3	***1.9***	Bt.16701.1.S1_at
	TEK tyrosine kinase, endothelial	TEK	***2.3***	Bt.4390.1.S1_at
	protein kinase C, epsilon	PRKCE	***1.6***	Bt.13959.1.S1_at
	doublecortin-like kinase 1	DCLK1	1.8	Bt.14572.1.A1_at
	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	***1.6***	Bt.19517.1.S1_at
protein folding	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	PTPLA	1.6	Bt.23355.1.S1_at
protein modification process	serine/threonine kinase 38	STK38	***1.8***	Bt.7505.1.S1_at
protein secretion	endoplasmic reticulum protein 29	ERP29	1.5	Bt.3152.1.S1_at
protein targeting to mitochondrion	cysteine-rich secretory protein LCCL domain containing 2 /// translocase of inner mitochondrial membrane 13 homolog (yeast)	CRISPLD2 /// TIMM13	***1.7***	Bt.1927.1.S1_at
proteolysis	cathepsin L1	CTSL1	***2.2***	Bt.10200.1.S1_at
	ADAM metallopeptidase with thrombospondin type 1 motif, 5	ADAMTS5	***2.0***	Bt.13108.1.S1_at
			1.5	Bt.13108.1.A1_at
	plasma glutamate carboxypeptidase	PGCP	1.8	Bt.2120.2.S1_at
	similar to Cathepsin O	CTSO	1.6	Bt.28045.1.S1_at
	peptidase (mitochondrial processing) beta	PMPCB	1.6	Bt.9797.1.S1_at
	HtrA serine peptidase 1	HTRA1	***1.6***	Bt.1613.1.S1_at
pseudouridine synthesis and tRNA processing	pseudouridylate synthase 7 homolog (S. cerevisiae)	PUS7	1.7	Bt.28327.1.A1_at
pyrimidine nucleotide metabolic process	uridine phosphorylase 1	UPP1	1.7	Bt.20327.1.S1_a_at
regulation of cell growth	nephroblastoma overexpressed	NOV	***2.0***	Bt.13482.1.S1_at
	WNT1 inducible signaling pathway protein 2	WISP2	2.3	Bt.27689.1.A1_at
	insulin-like growth factor binding protein 6	IGFBP6	1.6	Bt.9958.1.S1_at
regulation of cell shape	CDC42 effector protein (Rho GTPase binding) 2	CDC42EP2	***1.7***	Bt.20259.1.S1_at
regulation of the force of heart contraction	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	***1.8***	Bt.21223.1.S1_at
regulation of transcription, DNA-dependent	zinc finger protein 75 (D8C6)	ZNF75	1.6	Bt.16761.1.S1_at
	paternally expressed 3	PEG3	***1.7***	Bt.5027.1.A1_at

	homeobox A4	HOXA4	1.5	Bt.28155.1.S1_at
	transcription factor 7 (T-cell specific, HMG-box)	TCF7	***1.8***	Bt.21037.1.S1_at
			1.7	Bt.21037.2.S1_at
regulation of translation	S100 calcium binding protein B	S100B	1.5	Bt.4775.1.S1_at
response to hypoxia	hypothetical protein MGC127538	MGC127538	***10.2***	Bt.2577.1.S1_at
response to molecule of bacterial origin	CD14 molecule	CD14	***1.5***	Bt.13789.1.A1_at
response to oxidative stress	Peroxidasin homolog (Drosophila)	PXDN	***1.5***	Bt.24695.1.S1_at
	thioredoxin interacting protein	TXNIP	2.0	Bt.12327.1.S1_at
response to yeast and opsonization	pentraxin-related gene, rapidly induced by IL-1 beta	PTX3	1.9	Bt.10398.1.S1_at
rRNA processing	UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	UTP14A	1.5	Bt.2589.1.S1_at
selenium metabolic process	selenoprotein P, plasma, 1	SEPP1	2.5	Bt.2220.2.A1_a_at
signal transduction	fibrinogen-like 2	FGL2	3.9	Bt.9202.1.S1_at
	SPARC-like 1 (hevin)	SPARCL1	3.1	Bt.5153.1.S1_at
	angiopoietin-like 7	ANGPTL7	1.5	Bt.24342.1.A1_at
	endothelin receptor type A	EDNRA	***1.6***	Bt.4719.1.S1_at
	prostaglandin E receptor 2 (subtype EP2), 53kDa	PTGER2	2.1	Bt.12301.1.S1_at
	prostaglandin F receptor (FP)	PTGFR	1.6	Bt.352.2.S1_at
	coagulation factor II (thrombin) receptor-like 2	F2RL2	***1.7***	Bt.1345.1.S1_at
			1.6	Bt.24447.2.S1_at
skeletal system development	C-type lectin domain family 3, member B	CLEC3B	1.6	Bt.1366.1.S1_at
	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	***2.6***	Bt.4714.1.S1_at
small GTPase mediated signal transduction	RAB7, member RAS oncogene family-like 1	RAB7L1	1.6	Bt.28513.1.S1_at
spermine biosynthetic process	spermine synthase	SMS	1.9	Bt.14326.1.S1_at
steroid biosynthetic process	cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51	***1.5***	Bt.621.1.S1_at
striated muscle cell development	Syndecan 1	SDC1	1.6	Bt.21944.1.S1_at
T cell costimulation	CD80 molecule	CD80	***1.5***	Bt.27986.1.S1_at
thyroid hormone generation	thyroglobulin	TG	***2.3***	Bt.331.1.S1_at
transcription	TWIST neighbor	TWISTNB	***1.8***	Bt.13392.1.S1_at
	polymerase (RNA) I polypeptide D, 16kDa	POLR1D	1.6	Bt.23590.1.S1_at
	glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	GRINL1A	1.6	Bt.25922.1.A1_at
	polymerase (RNA) III (DNA directed) polypeptide C (62kD)	POLR3C	1.7	Bt.14013.2.S1_at
	limb bud and heart development homolog (mouse)	LBH	***1.7***	Bt.17942.1.S1_at
	interferon regulatory factor 1	IRF1	1.7	Bt.10077.1.S2_at
	polymerase (RNA) I polypeptide E, 53kDa	POLR1E	1.7	Bt.10981.2.S1_at
	protein phosphatase 1, regulatory (inhibitor) subunit 1B	PPP1R1B	1.6	Bt.4706.1.S1_at
translation	mitochondrial ribosomal protein S6	MRPS6	***1.8***	Bt.9428.1.S1_at
	mitochondrial ribosomal protein L3	MRPL3	1.7	Bt.5225.1.S1_at
	mitochondrial ribosomal protein L10	MRPL10	1.5	Bt.21070.1.S1_at
	RNA binding motif (RNP1, RRM) protein 3	RBM3	1.7	Bt.9571.2.S1_a_at

	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	EIF2S3	1.6	Bt.21351.1.S1_a_at
			1.6	Bt.21351.1.S1_at
	tryptophanyl-tRNA synthetase	WARS	1.6	Bt.2841.1.S1_at
			1.5	Bt.2841.2.S1_a_at
transmembrane receptor protein serine/threonine kinase signaling pathway	A kinase (PRKA) anchor protein 4	AKAP4	***2.3***	Bt.218.1.S1_at
transport	phosphatidylinositol transfer protein, membrane-associated 1	PITPNM1	***1.8***	Bt.18627.1.S1_at
	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	ABCC10	***1.5***	Bt.11804.1.S1_at
	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	***1.5***	Bt.9593.1.S1_at
	ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	1.6	Bt.27938.1.A1_at
	aquaporin 1 (Colton blood group)	AQP1	***1.6***	Bt.1525.1.S2_at
	cytochrome b-561	CYB561	***1.7***	Bt.4360.1.S1_at
	FXYD domain containing ion transport regulator 6	FXYD6	***1.5***	Bt.5369.1.S1_at
	inositol 1,4,5-triphosphate receptor, type 2	ITPR2	***1.6***	Bt.9026.1.S1_at
	transferrin	TF	***1.8***	Bt.5336.1.A1_a_at
	potassium channel, subfamily K, member 1	KCNK1	***2.1***	Bt.8363.1.A1_at
	potassium voltage-gated channel, Isk-related family, member 4	KCNE4	3.1	Bt.22411.1.A1_at
	potassium inwardly-rectifying channel, subfamily J, member 15	KCNJ15	2.0	Bt.27605.2.S1_at
			1.9	Bt.27605.1.A1_at
	potassium channel, subfamily K, member 17	KCNK17	1.9	Bt.12534.1.S1_at
	sodium channel, voltage-gated, type IV, beta	SCN4B	2.6	Bt.8809.1.S1_at
	Solute carrier family 6 (proline IMINO transporter), member 20	SLC6A20	***1.9***	Bt.28747.1.S1_at
	hemoglobin, mu	HBM	1.7	Bt.5773.1.S1_a_at
	janus kinase and microtubule interacting protein 1	JAKMIP1	***4.1***	Bt.19810.1.A1_at
	retinol binding protein 1, cellular	RBP1	***2.5***	Bt.17810.1.S1_a_at
	RAB3B, member RAS oncogene family	RAB3B	***1.7***	Bt.388.1.S1_at
	ERGIC and golgi 3	ERGIC3	1.5	Bt.18891.1.S1_at
ubiquitin-dependent protein catabolic process	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	***1.7***	Bt.5408.1.A1_at
urea cycle and arginine metabolic process	arginase, type II	ARG2	***1.9***	Bt.8624.1.S1_at
vasculogenesis	tryptophanyl tRNA synthetase 2, mitochondrial	WARS2	1.8	Bt.1708.1.S1_at
viral reproduction	uveal autoantigen with coiled-coil domains and ankyrin repeats	UACA	1.6	Bt.12811.1.S1_a_at
			1.5	Bt.12811.1.S1_at
xenobiotic metabolic process	S100 calcium binding protein A12 (calgranulin C)	S100A12	***1.6***	Bt.357.1.S1_at
Unknown function	similar to RNA-binding protein 35A (RNA-binding motif protein 35A)	LOC788414	***3.9***	Bt.27030.1.S1_at
	similar to C4b-binding protein alpha chain precursor (C4bp) (Proline-rich protein) (PRP)	LOC515150	***3.0***	Bt.12136.1.S1_at

	latent transforming growth factor beta binding protein 1	LTBP1	***2.9***	Bt.20820.1.A1_at
	heparanase	HPSE	***2.6***	Bt.8127.1.S2_at
	Uncharacterized protein C13orf33 homolog	MGC155285	***2.3***	Bt.22080.1.S1_at
	CREB regulated transcription coactivator 1	CRTC1	***2.2***	Bt.12510.3.S1_at
	similar to Mal, T-cell differentiation protein /// mal, T-cell differentiation protein	LOC782642 /// MAL	***2.2***	Bt.4060.1.S1_at
	olfactomedin 1	OLFM1	***2.1***	Bt.18101.1.A1_at
	retinoic acid receptor responder (tazarotene induced) 1	RARRES1	***2.0***	Bt.24933.1.S1_at
	S100 calcium binding protein A9	S100A9	***1.9***	Bt.16201.1.S1_at
			1.9	Bt.16201.2.A1_at
	lumican	LUM	***1.9***	Bt.2452.1.S1_at
	heparanase	HPSE	***1.8***	Bt.8127.1.S1_at
	similar to inositol polyphosphate-5-phosphatase F	LOC539889	***1.8***	Bt.15840.1.A1_at
	midline 1 (Opitz/BBB syndrome)	MID1	***1.8***	Bt.16611.1.A1_at
	solute carrier family 35, member D2	SLC35D2	***1.8***	Bt.28522.1.S1_at
	S100 calcium binding protein A4	S100A4	***1.8***	Bt.5493.1.S1_at
	tandem C2 domains, nuclear	TC2N	***1.8***	Bt.25489.1.A1_at
	interleukin 13 receptor, alpha 1	IL13RA1	***1.8***	Bt.28750.1.S1_at
	monooxygenase, DBH-like 1	MOXD1	***1.7***	Bt.20519.1.A1_at
	similar to C27H2.2a	LOC516658	***1.7***	Bt.3291.1.A1_at
	Olfactomedin 1	OLFM1	***1.7***	Bt.876.1.S1_at
	tetraspanin 33	TSPAN33	***1.7***	Bt.7808.1.A1_at
	solute carrier family 44, member 4	SLC44A4	***1.7***	Bt.24374.1.S1_at
	IQ motif containing with AAA domain 1	IQCA1	***1.7***	Bt.26706.1.S1_at
	leucine rich repeat neuronal 1	LRRN1	***1.7***	Bt.17579.1.A1_at
	Phosphodiesterase 4D interacting protein	PDE4DIP	***1.7***	Bt.3736.1.A1_at
	Transducin (beta)-like 1X-linked	TBL1X	***1.7***	Bt.9988.1.S1_at
	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	SERPINA1	***1.6***	Bt.982.1.S1_at
	tweety homolog 2 (Drosophila)	TTYH2	***1.6***	Bt.4194.1.S1_at
	transmembrane protein 176A	TMEM176A	***1.6***	Bt.15565.1.S1_at
	Coiled-coil domain containing 104	CCDC104	***1.6***	Bt.27498.1.S1_at
	chromosome 14 open reading frame 38 ortholog	C10H14orf38	***1.6***	Bt.5630.1.S1_at
	annexin A3	ANXA3	***1.6***	Bt.6775.1.S1_at
	Kelch-like 8 (Drosophila)	KLHL8	***1.6***	Bt.16467.2.A1_at
	Hypothetical protein LOC614841	LOC614841	***1.6***	Bt.22149.1.S1_at
	six transmembrane epithelial antigen of the prostate 1	STEAP1	***1.6***	Bt.16925.1.A1_at
	Hypothetical protein LOC614841	LOC614841	***1.6***	Bt.27272.1.A1_at
	tetraspanin 5	TSPAN5	***1.6***	Bt.22126.1.S1_at
	Adhesion molecule with Ig-like domain 2	AMIGO2	***1.6***	Bt.12294.2.A1_at
	nuclear receptor coactivator 7	NCOA7	***1.6***	Bt.28087.1.S1_at
	leucine rich repeat containing 16A	LRRC16A	***1.6***	Bt.26708.1.A1_at
	integrin, alpha 10	ITGA10	***1.6***	Bt.6610.1.S1_at

	Cerebellin 4 precursor	CBLN4	***1.5***	Bt.24465.1.A1_at
	interleukin 17 receptor B	IL17RB	***1.5***	Bt.24532.1.A1_at
	immediate early response 3	IER3	***1.5***	Bt.2153.1.S1_at
	Jumonji, AT rich interactive domain 2	JARID2	***1.5***	Bt.7008.1.S1_at
	Solute carrier organic anion transporter family, member 4A1	SLCO4A1	***1.5***	Bt.27240.1.A1_at
	furry homolog (Drosophila)	FRY	***1.5***	Bt.8327.1.S1_at
	similar to hCG1980844	LOC789183	***1.5***	Bt.21561.1.S1_at
	BEN domain containing 6	BEND6	***1.5***	Bt.3099.1.S1_at
	dual adaptor of phosphotyrosine and 3-phosphoinositides	DAPP1	***1.5***	Bt.24335.1.S1_at
	immunoglobulin superfamily, member 10	IGSF10	3.4	Bt.26774.1.S1_at
	leucine rich repeat containing 17	LRRC17	3.2	Bt.4886.1.A1_at
	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	NDUFA4L2	2.5	Bt.1746.1.S1_at
	similar to CG33196-PB	LOC515676	2.4	Bt.2987.1.A1_at
	myocilin, trabecular meshwork inducible glucocorticoid response	MYOC	2.4	Bt.4703.1.S1_at
	immunoglobulin superfamily containing leucine-rich repeat	ISLR	2.3	Bt.1899.1.A1_at
	phosphatidylethanolamine-binding protein 4	PEBP4	2.3	Bt.14398.1.S1_at
	pirin (iron-binding nuclear protein)	PIR	2.1	Bt.21680.1.S1_a_at
	growth and transformation-dependent protein	LOC617104	2.1	Bt.9210.1.S1_at
	carboxymethylenebutenolidase homolog (Pseudomonas)	CMBL	2.1	Bt.3203.1.S1_at
	multiple EGF-like-domains 10	MEGF10	2.0	Bt.3996.1.S1_at
	transmembrane protein 220	TMEM220	2.0	Bt.28251.1.S1_at
	similar to dapper 1	LOC538778	1.9	Bt.2726.2.S1_at
	histamine N-methyltransferase	HNMT	1.9	Bt.26700.1.S1_at
	pirin (iron-binding nuclear protein)	PIR	1.9	Bt.21680.1.S1_at
	Similar to uronyl-2-sulfotransferase	LOC535975	1.9	Bt.21924.2.S1_at
	Coiled-coil domain containing 39	CCDC39	1.9	Bt.27560.1.A1_at
	pirin (iron-binding nuclear protein)	PIR	1.9	Bt.21680.2.S1_at
	placenta-specific 9	PLAC9	1.9	Bt.11521.2.A1_a_at
	WD repeat, sterile alpha motif and U-box domain containing 1	WDSUB1	1.8	Bt.8290.1.S1_at
	Nephronectin	NPNT	1.8	Bt.7393.1.S1_at
	similar to dapper 1	LOC538778	1.8	Bt.2726.1.S1_at
	similar to ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative function)	LOC512304	1.8	Bt.22797.1.A1_at
	BMP binding endothelial regulator	BMPER	1.8	Bt.27778.1.A1_at
	similar to hCG1777421	LOC787439	1.8	Bt.17329.1.A1_at
	heme binding protein 1	HEBP1	1.8	Bt.5047.1.S1_at
	Membrane bound O-acyltransferase domain containing 2	MBOAT2	1.8	Bt.8669.2.S1_at
	prickle homolog 1 (Drosophila)	PRICKLE1	1.8	Bt.12688.1.A1_at
			1.7	Bt.18126.1.A1_at
	synaptosomal-associated protein, 23kDa	SNAP23	1.7	Bt.23682.1.S1_at

	similar to C1orf51 protein	LOC539246	1.7	Bt.14114.1.A1_at
	MARCKS-like 1	MARCKSL1	1.7	Bt.3880.1.S1_at
	CAP-GLY domain containing linker protein family, member 4	CLIP4	1.7	Bt.13892.1.S1_at
	similar to Hook homolog 1 (h-hook1) (hHK1)	LOC505399	1.7	Bt.23367.1.S1_at
	placenta-specific 9	PLAC9	1.7	Bt.11521.1.A1_at
	ankyrin repeat domain 29	ANKRD29	1.7	Bt.20382.1.S1_at
	myozenin 1	MYOZ1	1.7	Bt.11199.1.S1_at
	RAB3A interacting protein (rabin3)	RAB3IP	1.7	Bt.14853.1.A1_at
	torsin A interacting protein 1	TOR1AIP1	1.7	Bt.19402.1.A1_at
	hypothetical protein LOC535260	FAM151B	1.6	Bt.27442.1.S1_at
	zinc finger protein 12	ZNF12	1.6	Bt.23663.1.S1_at
	dystrobrevin, beta	DTNB	1.6	Bt.7860.2.S1_a_at
	Similar to Hook homolog 1 (h-hook1) (hHK1)	LOC505399	1.6	Bt.23367.2.S1_at
	BEX family member 5	BEX5	1.6	Bt.20617.1.S1_at
	transmembrane protein 140	TMEM140	1.6	Bt.16326.2.S1_at
			1.6	Bt.16326.1.S1_at
	prickle homolog 1 (Drosophila)	PRICKLE1	1.6	Bt.18126.2.S1_at
	sarcalumenin	SRL	1.6	Bt.11687.1.S1_a_at
	FERM domain containing 6	FRMD6	1.6	Bt.28695.1.S1_at
	UBX domain protein 8	UBXN8	1.6	Bt.28321.1.A1_at
	cartilage acidic protein 1	CRTAC1	1.6	Bt.27196.1.A1_at
	similar to zinc finger protein 239	ZNF239	1.6	Bt.26640.1.S1_at
	WD repeat domain 41	WDR41	1.6	Bt.16334.2.S1_a_at
	CD109 molecule	CD109	1.6	Bt.14520.1.A1_at
	similar to ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative function)	LOC512304	1.6	Bt.14001.1.A1_at
	transferrin receptor 2	TFR2	1.6	Bt.6186.1.S1_at
	ribosomal RNA processing 9, small subunit (SSU) processome component, homolog (yeast)	RRP9	1.6	Bt.9939.2.S1_at
	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	1.6	Bt.5056.3.S1_at
	uronyl-2-sulfotransferase	UST	1.6	Bt.21924.1.A1_at
	LIM domain and actin binding 1	LIMA1	1.6	Bt.10730.1.S1_at
	chromosome 18 open reading frame 21 ortholog	C24H18orf21	1.5	Bt.13875.1.S1_at
	Membrane associated guanylate kinase, WW and PDZ domain containing 2	MAGI2	1.5	Bt.17191.1.A1_at
	similar to G protein-coupled receptor	LOC782069	1.5	Bt.25616.1.S1_at
	dopey family member 2	DOPEY2	1.5	Bt.28524.1.S1_at
	DDB1 and CUL4 associated factor 4	DCAF4	1.5	Bt.29346.1.S1_at
	high-mobility group nucleosomal binding domain 2	HMGN2	1.5	Bt.1758.1.S1_at
	LIM and senescent cell antigen-like domains 2	LIMS2	1.5	Bt.21349.1.S1_at
	synaptosomal-associated protein, 23kDa	SNAP23	1.5	Bt.10994.1.S1_at
	hypothetical protein LOC613274	LOC613274	1.5	Bt.13584.2.S1_at
	nudix (nucleoside diphosphate linked moiety X)-type motif 16	NUDT16	1.5	Bt.20950.1.S1_at

	poly(A) binding protein, cytoplasmic 4 (inducible form)	PABPC4	1.5	Bt.11192.1.S1_at
	N-myc (and STAT) interactor	NMI	1.5	Bt.12285.2.S1_a_at
	Prickle homolog 1	PRICKLE1	1.5	Bt.12688.2.A1_at
	Mitochondrial ribosomal protein S35	MRPS35	1.5	Bt.13205.1.A1_at
	Complement factor H-related 5	CFHR5	1.5	Bt.17612.2.S1_at
	ring finger protein 157	RNF157	1.5	Bt.7390.1.S1_at
	N-myc (and STAT) interactor	NMI	1.5	Bt.12285.3.S1_a_at
	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	1.5	Bt.5056.2.A1_at
	glycophorin C (Gerbich blood group)	GYPC	1.5	Bt.11221.1.S1_at
	hypothetical LOC540253	LOC540253	1.5	Bt.2859.1.A1_at
	osteoglycin	OGN	1.5	Bt.5341.1.S1_at

Fold change in bold: up-regulation; not in bold: down-regulation

Appendix 6 Genes from 999 probes that are altered in 461mOsm compared to 268mOsm that change in signal with increasing osmolarity (376 unnamed probes deleted)

Cluster Name	Gene	Gene Symbol	Fold change	Probe Set ID
acetyl-CoA biosynthetic process	acyl-CoA synthetase short-chain family member 2	ACSS2	1.7	Bt.12640.1.S1_at
actin filament-based movement	WAS/WASL interacting protein family, member 1	WIPF1	2.0	Bt.9124.1.A1_at
acute inflammatory response	vanin 1	VNN1	2.0	Bt.28243.1.S1_a_at
	nuclear protein, transcriptional regulator, 1	NUPR1	***2.3***	Bt.23597.1.S1_at
adult somatic muscle development	interferon-related developmental regulator 1	IFRD1	2.3	Bt.14054.2.S1_at
			1.8	Bt.14054.1.A1_at
AMP catabolic process and nucleotide catabolic process	5'-nucleotidase, ecto (CD73)	NT5E	***1.9***	Bt.2367.1.A1_at
			1.6	Bt.5515.1.S1_at
angiogenesis	angiogenin, ribonuclease, RNase A family, 5	ANG	***4.1***	Bt.20883.1.S1_at
			4.1	Bt.12722.1.A1_at
			3.0	Bt.29009.1.A1_at
	placental growth factor	PGF	1.9	Bt.4216.1.S1_at
	interleukin 18 (interferon-gamma-inducing factor)	IL18	***1.7***	Bt.234.1.S1_at
	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT1	***1.7***	Bt.13148.1.S1_at
	Kruppel-like factor 5 (intestinal)	KLF5	***2.6***	Bt.15802.1.S1_at
			2.3	Bt.15802.2.S1_at
	angiopoietin-like 4	ANGPTL4	1.6	Bt.4816.1.S1_at
	Angiopoietin 1	ANGPT1	1.5	Bt.1246.1.S1_at
	ras homolog gene family, member B	RHOB	2.0	Bt.8382.2.S1_at
antigen processing and presentation of exogenous peptide antigen via MHC class II	gamma-inducible protein 30	IFI30	***2.2***	Bt.1548.1.S1_at
	MHC class II antigen	BLA-DQB	1.9	Bt.350.1.S1_s_at
antigen processing and presentation of peptide antigen via MHC class I	MHC class I antigen /// MHC Class I JSP.1	BOLA-N /// JSP.1	2.5	Bt.3805.1.S1_at
	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A	BoLA /// HLA-A	2.3	Bt.29814.1.S1_at
	MHC class I heavy chain	BOLA	1.5	Bt.27283.1.S1_x_at
			1.5	Bt.29823.1.S1_x_at
apoptosis	serglycin	SRGN	***1.6***	Bt.16048.1.S1_at
	DNA-damage-inducible transcript 4	DDIT4	1.8	Bt.2091.1.S1_at
	BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	3.7	Bt.26692.1.S1_a_at
			1.9	Bt.6958.3.A1_at
	keratin 18	KRT18	***3.1***	Bt.1745.1.S1_at

	keratin 8	KRT8	***3.1***	Bt.23608.1.S1_s_at
arginine catabolic process	fumarylacetoacetate hydrolase (fumarylacetoacetase)	FAH	1.7	Bt.16412.1.S1_a_at
ATP biosynthetic process	ATPase, Ca++ transporting, plasma membrane 1	ATP2B1	***1.6***	Bt.8872.1.S2_at
	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	***2.0***	Bt.6993.1.S1_at
	ATPase, Na+/K+ transporting, beta 3 polypeptide	ATP1B3	***1.7***	Bt.9728.1.S1_at
B cell homeostasis	tumor necrosis factor (ligand) superfamily, member 13b	TNFSF13B	***1.9***	Bt.24855.1.S1_at
			1.8	Bt.24855.2.S1_at
	caspase 3, apoptosis-related cysteine peptidase	CASP3	1.8	Bt.29166.1.S1_at
base-excision repair, DNA ligation	high-mobility group box 2	HMGB2	2.5	Bt.6397.2.S1_at
			2.3	Bt.6397.1.S1_at
bile acid biosynthetic process	cytochrome P450, family 39, subfamily A, polypeptide 1	CYP39A1	***1.6***	Bt.14369.1.A1_at
biomineral formation	tuftelin 1	TUFT1	1.6	Bt.4989.1.S1_at
biosynthetic process	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)	ACCS	1.6	Bt.21964.3.S1_at
blastocyst development	mediator complex subunit 21	MED21	1.5	Bt.5469.1.S1_at
blood coagulation	tissue factor pathway inhibitor 2	TFPI2	***2.4***	Bt.2017.1.S1_at
	protein S (alpha)	PROS1	***1.9***	Bt.5366.1.S1_at
	annexin A8-like 1	ANXA8L1	***1.5***	Bt.5026.1.S1_at
blood vessel development	collagen, type I, alpha 1	COL1A1	1.9	Bt.28583.1.S1_s_at
	Laminin, alpha 4	LAMA4	1.8	Bt.7984.1.S1_at
bone mineralization	Kruppel-like factor 10	KLF10	1.5	Bt.9527.2.S1_at
branching involved in ureteric bud morphogenesis	glypican 3	GPC3	***2.1***	Bt.11178.1.S1_at
brown fat cell differentiation	laminin, beta 3	LAMB3	***1.6***	Bt.16740.1.A1_at
calcium-mediated signaling	regulator of calcineurin 1	RCAN1	***2.1***	Bt.6377.1.S1_at
carbohydrate metabolic process	Alpha-galactosyltransferase 1 (glycoprotein)	GGTA1	2.6	Bt.22564.2.S1_at
	glucuronidase, beta	GUSB	1.5	Bt.15712.1.S1_at
	mannose phosphate isomerase	MPI	1.5	Bt.27806.1.S1_at
	regulating synaptic membrane exocytosis 1	RIMS1	***1.6***	Bt.27343.1.A1_at
	glutamine-fructose-6-phosphate transaminase 2	GFPT2	2.0	Bt.8781.1.S1_at
	similar to Putative glycerophosphodiester phosphodiesterase 5	LOC518469	2.0	Bt.12179.1.S1_at
	protein phosphatase 1, regulatory (inhibitor) subunit 3B	PPP1R3B	1.5	Bt.26905.2.S1_at
	Phosphorylase, glycogen, liver	PYGL	1.7	Bt.17714.1.A1_at
			1.7	Bt.28093.1.S1_at
	similar to Hexokinase-2 (Hexokinase type II) (HK II)	LOC614107	1.8	Bt.28139.1.S1_at
	Glucokinase (hexokinase 4)	GCK	1.7	Bt.27524.1.A1_at
	glucosidase, beta; acid (includes glucosylceramidase)	GBA	1.6	Bt.5413.2.S1_at
			1.5	Bt.5413.3.A1_a_at
	mannosidase, alpha, class 2B, member 1	MAN2B1	1.8	Bt.4622.1.S1_at

	hyaluronoglucosaminidase 1	HYAL1	***1.7***	Bt.4740.1.S1_at
	galactosidase, beta 1	GLB1	1.5	Bt.6127.2.S1_at
			1.5	Bt.6127.1.S1_at
carbohydrate transport	hypothetical protein LOC613571	MGC152007	1.5	Bt.9320.1.S1_a_at
cartilage condensation	aggrecan	ACAN	***2.6***	Bt.4953.1.S1_at
			2.1	Bt.29859.1.S1_at
cell adhesion	CD96 molecule	CD96	***10.3***	Bt.20940.1.S1_at
	hyaluronan and proteoglycan link protein 3	HAPLN3	***1.7***	Bt.3229.1.S1_at
	glycoprotein (transmembrane) nmb	GPNMB	20.0	Bt.9807.1.S1_at
			6.7	Bt.9807.2.S1_at
			6.7	Bt.9807.3.S1_at
	Spondin 1, extracellular matrix protein	SPON1	1.8	Bt.9640.1.A1_at
	spondin 1, extracellular matrix protein	SPON1	3.1	Bt.7176.1.S1_at
	collagen, type XVI, alpha 1	COL16A1	2.3	Bt.12697.1.S1_a_at
			2.1	Bt.12697.1.S1_at
	collagen, type XII, alpha 1	COL12A1	1.8	Bt.10442.2.S1_a_at
			2.0	Bt.11324.1.A1_s_at
	laminin, gamma 1 (formerly LAMB2)	LAMC1	2.0	Bt.23129.2.S1_at
			1.8	Bt.19181.1.A1_at
			1.6	Bt.20793.1.A1_s_at
			1.6	Bt.23129.3.S1_a_at
	F11 receptor	F11R	***1.7***	Bt.4768.1.S2_at
	desmocollin 2	DSC2	***1.6***	Bt.4107.1.S1_at
	protocadherin 7	PCDH7	***1.6***	Bt.12063.1.S1_at
	cadherin 11, type 2, OB-cadherin (osteoblast) /// similar to Cadherin-11 precursor (Osteoblast cadherin) (OB-cadherin) (OSF-4)	CDH11 /// LOC790083	2.8	Bt.2696.2.S1_at
			2.7	Bt.2696.1.A1_at
	cadherin 2, type 1, N-cadherin (neuronal)	CDH2	***2.2***	Bt.4417.1.S1_at
	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	ITGB2	3.2	Bt.4615.1.S1_at
	filamin binding LIM protein 1	FBLIM1	***3.7***	Bt.2860.1.S1_at
	retinal outer segment membrane protein 1	ROM1	***1.5***	Bt.558.1.S1_at
cell aging	proline/arginine-rich end leucine-rich repeat protein	PRELP	***2.2***	Bt.3202.1.A1_at
cell communication	neutrophil cytosolic factor 4, 40kDa	NCF4	***1.6***	Bt.7625.1.S1_a_at
	sorting nexin 7	SNX7	1.7	Bt.15717.1.A1_at
	gap junction protein, beta 2, 26kDa	GJB2	3.3	Bt.9760.1.S1_at
cell cycle	Septin 8	SEP8	1.6	Bt.22466.2.S1_at
	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	SPC24	2.4	Bt.9517.1.S1_a_at
	regulator of G-protein signaling 2, 24kDa	RGS2	***1.6***	Bt.10855.1.S1_at
cell differentiation	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	GNPTAB	1.9	Bt.8513.1.A1_at
cell morphogenesis involved in	Disabled homolog 2, mitogen-responsive	DAB2	2.3	Bt.3814.1.S1_at

differentiation	phosphoprotein (Drosophila)			
cell motion	myosin regulatory light chain interacting protein	MYLIP	***3.0***	Bt.6814.1.S1_at
cell projection assembly	capping protein (actin filament), gelsolin-like	CAPG	1.6	Bt.5499.1.S1_at
cell redox homeostasis	thioredoxin domain containing 5 (endoplasmic reticulum)	TXNDC5	1.6	Bt.7078.1.S1_at
cell surface receptor linked signal transduction	frizzled homolog 2 (Drosophila)	FZD2	***2.2***	Bt.16951.1.A1_at
cell surface receptor linked signal transduction	G protein-coupled receptor 133	GPR133	***3.1***	Bt.22380.1.S1_at
cell-matrix adhesion	ependymin related protein 1 (zebrafish)	EPDR1	***2.0***	Bt.21962.1.S1_at
	nidogen 1	NID1	6.0	Bt.8106.1.S1_at
cellular amino acid and derivative metabolic process	histidine decarboxylase	HDC	***2.3***	Bt.18146.1.S1_at
cellular aromatic compound metabolic process	epoxide hydrolase 1, microsomal (xenobiotic)	EPHX1	***1.6***	Bt.23354.1.S1_at
cellular calcium ion homeostasis	regucalcin (senescence marker protein-30)	RGN	***1.6***	Bt.502.1.A1_at
cellular component organization	Dishevelled associated activator of morphogenesis 1	DAAM1	***1.9***	Bt.8208.1.A1_at
			1.7	Bt.28836.1.S1_at
	formin homology 2 domain containing 3	FHOD3	3.0	Bt.24819.1.S1_at
cellular glucose homeostasis	forkhead box A3	FOXA3	***2.2***	Bt.27526.1.A1_at
cellular process	neuronal guanine nucleotide exchange factor	NGEF	***1.9***	Bt.23388.1.A1_at
cellular protein metabolic process	L-2-hydroxyglutarate dehydrogenase	L2HGDH	***1.5***	Bt.26150.1.A1_at
cellular response to starvation	Sterol regulatory element binding transcription factor 1	SREBF1	***1.6***	Bt.15667.1.S1_a_at
chemotaxis	G protein-coupled receptor 77	GPR77	***2.5***	Bt.26157.1.A1_at
	chemokine (C-C motif) ligand 5	CCL5	1.5	Bt.552.1.S1_at
cholesterol efflux	apolipoprotein M	APOM	1.6	Bt.9735.1.S1_at
chromatin assembly or disassembly	Chromobox homolog 6	CBX6	***1.7***	Bt.13458.1.S1_at
chromatin silencing	sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)	SIRT2	1.5	Bt.7373.3.S1_a_at
chromosome segregation	ADP-ribosylation factor-like 8B	ARL8B	1.6	Bt.28064.2.S1_at
			1.5	Bt.9929.1.A1_at
copper ion transport	similar to COX17 homolog, cytochrome c oxidase assembly protein	MGC128036	***1.5***	Bt.9457.1.S1_at
	solute carrier family 31 (copper transporters), member 2	SLC31A2	1.8	Bt.12565.1.S1_at
creatine biosynthetic process	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	GATM	2.1	Bt.23250.2.S1_at
cysteine metabolic process	Glutamate-cysteine ligase, catalytic subunit	GCLC	3.8	Bt.19499.1.S1_at
			2.7	Bt.16068.1.A1_at
cytokine production	platelet-activating factor receptor	PTAFR	***1.6***	Bt.27364.1.S1_at
	fatty acid binding protein 4, adipocyte	FABP4	2.5	Bt.97.1.S1_at
cytoskeleton organization	actin binding LIM protein 1	ABLIM1	***1.5***	Bt.9778.1.S1_at

D-amino acid catabolic process	D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae)	DTD1	1.5	Bt.694.1.A1_at
de novo' pyrimidine base biosynthetic process	dihydroorotate dehydrogenase	DHODH	1.6	Bt.7483.1.S1_at
defense response	cystatin C	CST3	***1.5***	Bt.1855.1.S1_at
DNA damage checkpoint	mitogen-activated protein kinase 14	MAPK14	1.6	Bt.9698.1.S1_at
DNA metabolic process	Fanconi anemia, complementation group M	FANCM	1.7	Bt.26857.1.A1_a_at
DNA repair	nei like 2 (E. coli)	NEIL2	***1.5***	Bt.26303.1.A1_at
	cryptochrome 1 (photolyase-like)	CRY1	1.5	Bt.8995.1.A1_at
	ring finger protein 168	RNF168	1.7	Bt.6014.2.S1_at
DNA replication	nuclear factor I/B	NFIB	1.7	Bt.21763.3.S1_at
	Primase, DNA, polypeptide 1 (49kDa)	PRIM1	***1.5***	Bt.18589.1.A1_at
dUTP metabolic process	deoxyuridine triphosphatase	DUT	1.9	Bt.11936.1.S1_at
			1.9	Bt.11936.1.S1_a_at
endocytosis	EH-domain containing 1	EHD1	***1.5***	Bt.7963.1.S1_at
erythrocyte maturation	TIMP metallopeptidase inhibitor 1	TIMP1	***1.9***	Bt.632.1.S1_s_at
			1.8	Bt.632.1.S1_at
estrogen biosynthetic process	hydroxysteroid (17-beta) dehydrogenase 1	HSD17B1	3.8	Bt.22879.1.S1_at
fatty acid beta-oxidation	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	DCI	1.8	Bt.13530.1.S1_at
	2,4-dienoyl CoA reductase 1, mitochondrial	DECR1	1.8	Bt.13390.2.S1_a_at
fatty acid biosynthetic process	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	ELOVL5	***1.9***	Bt.7281.1.S1_at
	fatty acid synthase	FASN	***2.2***	Bt.22854.2.S1_at
fatty acid metabolic process	acyl-Coenzyme A oxidase 2, branched chain	ACOX2	1.7	Bt.20384.1.S1_at
female pregnancy	uterine milk protein	LOC286871	***3.7***	Bt.24199.1.A1_s_at
			3.2	Bt.594.1.S1_at
	thrombomodulin	THBD	***6.9***	Bt.471.1.S1_at
fructose 6-phosphate metabolic process	phosphofructokinase, liver	PFKL	1.5	Bt.5061.1.S1_at
fructose metabolic process	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	PFKFB3	1.8	Bt.12314.1.S1_at
G1/S transition of mitotic cell cycle	inhibin, beta A	INHBA	***2.7***	Bt.12760.1.S1_at
	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	PPP3CA	***1.6***	Bt.3966.1.S1_at
G2 phase of mitotic cell cycle	amyloid beta (A4) precursor protein	APP	2.0	Bt.13152.1.S1_s_at
G2/M transition of mitotic cell cycle	tumor protein D52-like 1	TPD52L1	***2.8***	Bt.15740.1.A1_at
gluconeogenesis	serine dehydratase	SDS	1.6	Bt.5878.1.A1_at
	pyruvate carboxylase	PC	***1.6***	Bt.12339.1.S1_at
glucose metabolic process	triosephosphate isomerase 1	TPI1	1.7	Bt.3487.1.S1_at
	glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.7	AFFX-Bt-gapd-M_at
			1.6	Bt.6091.1.S1_at
			1.5	AFFX-Bt-gapd-5_at
	glutathione S-transferase kappa 1	GSTK1	1.7	Bt.13751.2.S1_a_at
	Glycerophosphodiester phosphodiesterase domain	GDPD1	***3.5***	Bt.26100.1.S1_at

	containing 1		***3.3***	Bt.26100.2.A1_at
glycine catabolic process	glycine cleavage system protein H (aminomethyl carrier)	GCSH	4.6	Bt.4195.1.S1_at
glycolysis	aldolase A, fructose-bisphosphate	ALDOA	1.6	Bt.22533.1.S1_at
Golgi organization and muscle cell differentiation	spectrin repeat containing, nuclear envelope 1	SYNE1	***1.8***	Bt.10565.1.A1_at
Golgi to plasma membrane transport	six transmembrane epithelial antigen of the prostate 2	STEAP2	***1.5***	Bt.22045.1.S1_at
G-protein coupled receptor protein signaling pathway	G protein-coupled receptor 1	GPR1	***3.3***	Bt.27053.1.A1_at
	G protein-coupled receptor 88	GPR88	***1.9***	Bt.9893.1.S1_at
healing during inflammatory response	heme oxygenase (decycling) 1	HMOX1	2.3	Bt.4001.1.S1_at
heart development and cell-cell adhesion	plakophilin 2	PKP2	***4.5***	Bt.21547.1.A1_at
			3.0	Bt.21547.2.S1_at
	adrenomedullin	ADM	1.9	Bt.2047.1.S1_at
immune response	Similar to Interferon-induced guanylate-binding protein 1 (GTP-binding protein 1) (Guanine nucleotide-binding protein 1) (HuGBP-1)	LOC512486	***2.6***	Bt.9296.1.A1_at
	proteoglycan 4	PRG4	***2.0***	Bt.22987.1.S1_at
	similar to fractalkine	LOC517354	2.3	Bt.1275.1.S1_at
	chemokine (C-X-C motif) ligand 2	CXCL2	1.9	Bt.23093.1.S1_at
	sushi domain containing 2	SUSD2	1.5	Bt.3206.1.A1_at
	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	PSMB9	1.6	Bt.20532.1.S1_at
in utero embryonic development	endonuclease G	ENDOG	1.8	Bt.3857.1.S1_at
	complement component 6	C6	2.0	Bt.23722.1.A1_at
	complement component 5	C5	***5.8***	Bt.5622.1.S1_at
	GATA-6	LOC654400	2.3	Bt.24023.1.A1_at
	N-myristoyltransferase 1	NMT1	1.5	Bt.250.1.S1_at
	oocyte expressed protein homolog (dog)	OOEP	***1.6***	Bt.20749.1.A1_at
induction of apoptosis	pleckstrin homology domain containing, family F (with FYVE domain) member 1	PLEKHF1	1.9	Bt.2421.1.A1_at
induction of apoptosis and G1 phase	prune homolog 2 (Drosophila)	PRUNE2	***2.5***	Bt.18615.1.A1_at
induction of apoptosis by intracellular signals	hypothetical protein LOC617441	C14orf153	2.1	Bt.13847.1.S1_at
intermediate filament-based process	vimentin	VIM	1.5	Bt.11149.1.S1_at
intracellular protein transport	mitochondrial ribosomal protein L45	MRPL45	1.7	Bt.853.1.S1_at
	receptor (G protein-coupled) activity modifying protein 2	RAMP2	1.5	Bt.23502.1.S1_at
	receptor (G protein-coupled) activity modifying protein 1	RAMP1	1.5	Bt.2331.2.S1_at

	similar to AP-3 complex subunit beta-2 (Adapter-related protein complex 3 beta-2 subunit) (Beta3B-adaptin) (Adaptor protein complex AP-3 beta-2 subunit) (AP-3 complex beta-2 subunit) (Clathrin assembly protein complex 3 beta-2 large chain) (Neuron-	LOC790439	***2.2***	Bt.10002.1.S1_at
intracellular signaling cascade	pleckstrin	PLEK	***3.4***	Bt.15713.1.A1_at
	pleckstrin 2	PLEK2	***1.7***	Bt.28534.1.A1_at
	ankyrin repeat and SOCS box-containing 6	ASB6	***1.5***	Bt.27341.1.S1_at
	suppressor of cytokine signaling 2	SOCS2	***2.5***	Bt.13273.1.S1_at
	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	***1.9***	Bt.10831.1.S1_at
	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	1.5	Bt.4279.2.S1_at
	Rho guanine nucleotide exchange factor (GEF) 5	ARHGEF5	***2.6***	Bt.18132.1.A1_at
	epithelial cell transforming sequence 2 oncogene	ECT2	***1.6***	Bt.28366.2.S1_at
			1.6	Bt.28366.1.A1_at
ion transport	Mucolipin 2	MCOLN2	***2.0***	Bt.25669.1.S1_at
iron ion transport	solute carrier family 40 (iron-regulated transporter), member 1	SLC40A1	1.8	Bt.22759.1.S1_at
L-ascorbic acid biosynthetic process	aldo-keto reductase family 1, member A1 (aldehyde reductase)	AKR1A1	1.5	Bt.4449.1.S1_at
learning or memory and dendrite development	abl-interactor 2	ABI2	1.6	Bt.26877.1.A1_at
lipid catabolic process	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	PLA2G7	***1.9***	Bt.4063.1.S1_at
	platelet-activating factor acetylhydrolase 2, 40kDa	PAFAH2	1.8	Bt.4013.1.S1_at
lipid metabolic process	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	SRD5A1	2.0	Bt.27694.1.S1_at
	stearoyl-CoA desaturase 5	SCD5	***1.7***	Bt.13249.1.A1_at
	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)	DEGS1	***1.6***	Bt.6334.1.A1_at
	tenascin XB	TNXB	***2.4***	Bt.5398.1.S1_at
	phospholipase C-like 2	PLCL2	1.7	Bt.27012.1.A1_at
	phospholipase A1 member A	PLA1A	***2.7***	Bt.19884.1.A1_at
			2.3	Bt.19884.2.S1_at
	phospholipase C, beta 4	PLCB4	***1.6***	Bt.4552.1.S1_a_at
liver development	lipolysis stimulated lipoprotein receptor	LSR	***1.9***	Bt.12462.1.S1_at
L-serine biosynthetic process	phosphoserine aminotransferase 1	PSAT1	***2.2***	Bt.13588.1.A1_at
mannose metabolic process	renin binding protein	RENBP	1.8	Bt.2629.1.S1_at
MAPKKK cascade and activation of MAPK activity	mitogen-activated protein kinase kinase 6	MAP2K6	1.9	Bt.561.1.S1_at

metabolic process	solute carrier family 27 (fatty acid transporter), member 3	SLC27A3	***2.2***	Bt.1828.1.A1_at
	arylsulfatase B	ARSB	***1.9***	Bt.18689.1.S1_at
	Aldehyde dehydrogenase 1 family, member A2	ALDH1A2	***1.9***	Bt.19093.1.S1_at
	arylsulfatase B	ARSB	***1.7***	Bt.18689.2.A1_at
	Similar to Long-chain-fatty-acid--CoA ligase 3 (Long-chain acyl-CoA synthetase 3) (LACS 3)	LOC100138312	***1.7***	Bt.17121.1.S1_at
	AU RNA binding protein/enoyl-Coenzyme A hydratase	AUH	***1.5***	Bt.14059.1.A1_at
	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	AGPAT5	2.2	Bt.22170.1.S1_a_at
			2.1	Bt.16140.1.A1_at
			2.1	Bt.22170.2.A1_at
			2.0	Bt.22170.1.S1_at
	NEDD8 activating enzyme E1 subunit 1	NAE1	1.9	Bt.15600.2.S1_at
	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	AGPAT5	1.9	Bt.23626.1.S1_at
	Acyl-CoA synthetase short-chain family member 3	ACSS3	1.6	Bt.21084.1.S1_at
	N-acetyltransferase 13 (GCN5-related)	NAT13	1.6	Bt.23551.3.S1_at
	lysozyme (renal amyloidosis)	LYZ	***7.5***	Bt.209.3.S1_at
	branched chain aminotransferase 1, cytosolic	BCAT1	2.2	Bt.17278.3.S1_a_at
			1.8	Bt.17278.3.S1_at
			1.8	Bt.17278.1.A1_at
	enolase superfamily member 1	ENOSF1	1.9	Bt.3276.1.A1_at
			1.8	Bt.3276.2.S1_at
	Quinolinate phosphoribosyltransferase	QPRT	***1.8***	Bt.27138.1.S1_at
	dihydrodiol dehydrogenase (dimeric)	DHDH	1.9	Bt.22630.1.S1_at
			1.6	Bt.22630.2.A1_at
	dehydrogenase/reductase (SDR family) member 7	DHRS7	1.5	Bt.20151.1.S1_at
	dehydrogenase/reductase (SDR family) member 1	DHRS1	1.5	Bt.13376.1.S1_at
	DPH5 homolog (S. cerevisiae)	DPH5	1.7	Bt.21419.1.S1_at
	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)	AGPAT4	***1.7***	Bt.25871.1.A1_at
	phosphatidylglycerophosphate synthase 1	PGS1	***1.6***	Bt.13710.3.S1_a_at
	HIV-1 Tat interactive protein 2, 30kDa	HTATIP2	***1.5***	Bt.8789.1.S1_at
	Fatty acyl CoA reductase 1	FAR1	1.9	Bt.10230.2.A1_at
			1.7	Bt.10230.1.A1_at
			1.5	Bt.20738.1.S1_at
microtubule cytoskeleton organization	hook homolog 2 (Drosophila)	HOOK2	***1.8***	Bt.20294.1.S1_at
	tubulin, alpha 1a /// tubulin, alpha 1b /// tubulin, alpha 4a	TUBA1A /// TUBA1B /// TUBA4A	2.0	Bt.15534.1.S1_a_at
microtubule-based movement	Kinesin family member 21A	KIF21A	***1.6***	Bt.20970.1.A1_at
microtubule-based process	tubulin, delta 1	TUBD1	***1.5***	Bt.25963.1.A1_at

mitochondrial DNA replication	DnaJ (Hsp40) homolog, subfamily A, member 3	DNAJA3	1.5	Bt.1294.1.S1_at
modification-dependent protein catabolic process	speckle-type POZ protein	SPOP	1.9	Bt.8549.1.S2_at
	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)	UBE2G2	1.8	Bt.28242.1.S1_at
	ubiquitin-conjugating enzyme E2L 6	UBE2L6	2.0	Bt.16725.2.A1_at
			1.9	Bt.16725.1.S1_at
Mo-molybdopterin cofactor biosynthetic process	Gephyrin	GPHN	1.7	Bt.14464.1.A1_at
monocyte chemotaxis	chemokine (C-C motif) ligand 2	CCL2	1.6	Bt.2408.1.S1_s_at
morphogenesis of an epithelium	crystallin, gamma S	CRYGS	***1.9***	Bt.1647.1.S1_at
	carbonic anhydrase II	CA2	***5.8***	Bt.22854.1.S1_at
mRNA processing	RNA binding motif protein 23	RBM23	***1.6***	Bt.26842.1.S1_at
	small nuclear ribonucleoprotein polypeptide A	SNRPA	1.5	Bt.22762.1.S1_at
	mago-nashi homolog B (Drosophila)	MAGOHB	1.7	Bt.22385.2.S1_a_at
multicellular organismal development	shisa homolog 2 (Xenopus laevis)	SHISA2	***1.8***	Bt.22389.1.S1_at
	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	SEMA6D	1.6	Bt.19862.2.S1_at
	cysteine and glycine-rich protein 2	CSRP2	***1.8***	Bt.2262.1.S1_at
	chromosome 20 open reading frame 149 ortholog	C13H20orf149	2.3	Bt.23173.1.S1_at
	leukocyte cell derived chemotaxin 1	LECT1	***1.9***	Bt.1509.1.S1_at
	Fragile X mental retardation, autosomal homolog 1	FXR1	1.8	Bt.3127.1.S1_at
	dickkopf homolog 1 (Xenopus laevis)	DKK1	***2.3***	Bt.13880.2.S1_at
myelination	Myelin basic protein	MBP	***1.8***	Bt.16377.1.S1_at
			1.7	Bt.12606.2.S1_at
myeloid progenitor cell differentiation	myeloid leukemia factor 1	MLF1	1.9	Bt.21766.3.S1_at
			1.6	Bt.21766.2.S1_at
			1.5	Bt.21766.1.A1_at
negative regulation of cell proliferation	Kruppel-like factor 4 (gut)	KLF4	***1.6***	Bt.1424.1.S1_at
	scinderin	SCIN	***2.4***	Bt.3980.1.S1_at
negative regulation of cytokine-mediated signaling pathway	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	SIGIRR	***1.5***	Bt.1676.1.A1_at
negative regulation of endocytosis	protein kinase C and casein kinase substrate in neurons 2	PACSIN2	***1.9***	Bt.20341.1.S1_at
negative regulation of membrane protein ectodomain proteolysis	TIMP metallopeptidase inhibitor 3	TIMP3	***1.9***	Bt.5136.1.S1_at
negative regulation of microtubule depolymerization	MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish))	MID1IP1	***1.5***	Bt.11034.1.S1_at
negative regulation of protein amino acid phosphorylation	family with sequence similarity 129, member A	FAM129A	1.5	Bt.4645.1.S1_at
negative regulation of protein amino acid phosphorylation	FK506 binding protein 1A, 12kDa	FKBP1A	1.7	Bt.22329.1.S1_at

negative regulation of signal transduction	DNA-damage-inducible transcript 4-like	DDIT4L	***2.3***	Bt.22199.1.S1_at
	Regulator of G-protein signaling 5	RGS5	3.5	Bt.28182.1.A1_at
negative regulation of transcription from RNA polymerase II promoter	sorbin and SH3 domain containing 3	SORBS3	***1.5***	Bt.1405.1.S1_at
	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	***8.2***	Bt.1730.1.A1_at
	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	***3.6***	Bt.5230.1.S1_at
	nuclear receptor subfamily 2, group F, member 1	NR2F1	***1.6***	Bt.543.1.S1_at
	early growth response 1	EGR1	3.8	Bt.22265.1.S1_at
neuron migration	FYN oncogene related to SRC, FGR, YES	FYN	***1.8***	Bt.2359.1.A1_at
neuron remodeling	netrin 4	NTN4	***2.6***	Bt.26919.1.S1_at
neuropeptide signaling pathway	neuromedin B	NMB	***1.8***	Bt.10630.1.S1_at
	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 2	PLEKHA2	1.6	Bt.22283.1.S1_at
neurotransmitter secretion	forty-two-three domain containing 1 /// synaptosomal-associated protein, 25kDa	FYTTD1 /// SNAP25	1.6	Bt.20032.1.A1_at
Notch signaling pathway	Notch homolog 2 (Drosophila)	NOTCH2	1.5	Bt.9169.1.A1_at
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	UPF1 regulator of nonsense transcripts homolog (yeast)	UPF1	***1.6***	Bt.21786.1.S1_at
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	adenylate kinase 2	AK2	1.6	Bt.946.1.S1_at
	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	REV3L	1.7	Bt.25319.2.S1_at
nucleosome assembly	H1 histone family, member 0	H1F0	***1.9***	Bt.20929.2.A1_at
	histone H4	H4	***1.5***	Bt.113.1.S1_at
	histone H4-like	LOC617875	7.2	Bt.9531.1.S1_at
	histone cluster 1, H1c	HIST1H1C	***2.0***	Bt.2728.1.S1_at
one-carbon metabolic process	carbonic anhydrase IV	CA4	***5.5***	Bt.555.1.S1_at
	carbonic anhydrase IX	CA9	3.8	Bt.27842.1.S1_at
	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	MTHFD1L	1.6	Bt.7490.1.A1_at
organ morphogenesis	transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)	TGM1	***2.9***	Bt.736.1.A1_at
ossification and osteoblast differentiation	insulin-like growth factor 2 (somatomedin A)	IGF2	***1.6***	Bt.7676.3.S1_at
osteoblast differentiation	bone morphogenetic protein 2	BMP2	1.5	Bt.29956.1.A1_at
ovarian follicle development	kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	***2.8***	Bt.13860.1.S1_at
			2.6	Bt.13860.2.A1_at
ovarian follicle rupture	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	AGT	***2.6***	Bt.14715.1.S1_at
ovulation from ovarian follicle	ADAM metallopeptidase with thrombospondin type 1 motif, 1	ADAMTS1	***2.8***	Bt.12010.1.S1_at
			2.5	Bt.12010.2.S1_at
oxidation reduction	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	CYP3A4	***2.0***	Bt.82.1.A1_at

	aldehyde dehydrogenase 5 family, member A1	ALDH5A1	1.5	Bt.2173.1.S1_at
	Egl nine homolog 3 (C. elegans)	EGLN3	1.5	Bt.23232.1.S1_at
pattern specification process	aspartate beta-hydroxylase	ASPH	2.1	Bt.14648.1.A1_at
patterning of blood vessels	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	2.3	Bt.2524.1.S1_at
peptidyl-proline hydroxylation to 4-hydroxy-L-proline	prolyl 4-hydroxylase, alpha polypeptide II	P4HA2	1.7	Bt.4381.1.A1_at
phosphatidylcholine biosynthetic process	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	FABP3	2.0	Bt.4758.1.S1_at
	inositol(myo)-1(or 4)-monophosphatase 1	IMPA1	1.6	Bt.424.1.S1_at
phosphatidylinositol metabolic process	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	***2.9***	Bt.20732.1.A1_at
phospholipid biosynthetic process	lysophosphatidylcholine acyltransferase 3	LPCAT3	***2.4***	Bt.27204.1.S1_at
			1.6	Bt.26573.2.S1_at
placenta development	Norrie disease (pseudoglioma)	NDP	1.9	Bt.3307.1.A1_at
platelet activating factor biosynthetic process	choline phosphotransferase 1	CHPT1	1.6	Bt.22082.1.S1_at
polyamine biosynthetic process	ornithine decarboxylase 1	ODC1	2.9	Bt.7133.1.S1_at
positive regulation of endothelial cell proliferation	cadherin 13, H-cadherin (heart)	CDH13	***3.0***	Bt.6592.1.S1_at
positive regulation of I-kappaB kinase/NF-kappaB cascade	lectin, galactoside-binding, soluble, 1	LGALS1	4.5	Bt.5472.1.S1_at
positive regulation of leukocyte chemotaxis	Coagulation factor II (thrombin) receptor-like 1	F2RL1	***3.0***	Bt.28745.1.S1_at
positive regulation of natural killer cell mediated cytotoxicity directed against tumor cell target	poliovirus receptor-related 2 (herpesvirus entry mediator B)	PVRL2	1.9	Bt.4943.3.S1_a_at
potassium ion transport	Potassium channel tetramerisation domain containing 20	KCTD20	***1.5***	Bt.27607.1.A1_at
prenylcysteine catabolic process	prenylcysteine oxidase 1 like	PCYOX1L	1.8	Bt.26903.1.A1_at
prostaglandin biosynthetic process	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	***4.0***	Bt.2151.2.A1_at
	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	1.8	Bt.15528.1.S1_at
	prostaglandin D2 synthase 21kDa (brain)	PTGDS	4.7	Bt.1645.1.S1_at
	prostaglandin E synthase	PTGES	1.6	Bt.8953.1.S1_at
protein amino acid ADP-ribosylation	similar to poly (ADP-ribose) polymerase family, member 3	LOC507426	1.7	Bt.24799.1.S1_at
protein amino acid dephosphorylation	protein tyrosine phosphatase, receptor type, S	PTPRS	***2.2***	Bt.21399.3.S1_a_at
	protein tyrosine phosphatase, receptor type, M	PTPRM	***1.6***	Bt.26720.1.S1_at
	dual specificity phosphatase 10	DUSP10	2.3	Bt.2070.1.A1_at
			1.9	Bt.2070.2.A1_at

	Slingshot homolog 1 (Drosophila)	SSH1	***1.6***	Bt.25416.1.A1_at
	neutral cholesterol ester hydrolase 1	NCEH1	1.5	Bt.20045.1.A1_at
protein amino acid glycosylation	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	B3GALT2	5.5	Bt.7447.1.A1_at
			5.4	Bt.7251.1.S1_at
protein amino acid phosphorylation	renal tumor antigen	RAGE	***1.9***	Bt.18292.1.A1_at
	protein kinase-like protein SgK196	SGK196	***1.7***	Bt.20140.1.S1_at
	cyclin-dependent kinase 8	CDK8	***1.6***	Bt.20526.1.S1_at
	Serine/threonine kinase 33	STK33	***1.6***	Bt.25006.1.S1_at
	serine/threonine kinase 38 like	STK38L	***1.6***	Bt.21548.1.A1_at
	microtubule associated serine/threonine kinase 3	MAST3	***1.6***	Bt.9194.1.S1_at
	mitogen-activated protein kinase kinase kinase 8	MAP3K8	***1.5***	Bt.28561.1.S1_at
	NUAK family, SNF1-like kinase, 2	NUAK2	1.6	Bt.20360.1.A1_at
	WNK lysine deficient protein kinase 2	WNK2	1.6	Bt.21050.1.S1_at
	budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	1.6	Bt.21513.1.A1_at
	RIO kinase 3 (yeast)	RIOK3	1.6	Bt.22359.1.A1_at
	Mitogen-activated protein kinase 6	MAPK6	1.5	Bt.3448.3.S1_at
	serum/glucocorticoid regulated kinase 1	SGK1	***1.5***	Bt.16123.1.S1_at
			1.5	Bt.16123.2.S1_a_at
	TEK tyrosine kinase, endothelial	TEK	***4.1***	Bt.4390.1.S1_at
	protein kinase C, epsilon	PRKCE	***1.7***	Bt.13959.1.S1_at
	doublecortin-like kinase 1	DCLK1	3.6	Bt.14572.1.A1_at
	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	***1.9***	Bt.19517.1.S1_at
	p21 protein (Cdc42/Rac)-activated kinase 1	PAK1	***2.1***	Bt.5858.1.S1_at
	ribosomal protein S6 kinase, 90kDa, polypeptide 1	RPS6KA1	***2.4***	Bt.27200.1.S1_at
	ribosomal protein S6 kinase, 90kDa, polypeptide 5	RPS6KA5	***1.5***	Bt.13490.1.S1_at
	mitogen-activated protein kinase 13	MAPK13	2.3	Bt.800.1.S1_at
	G protein-coupled receptor kinase 4	GRK4	***2.2***	Bt.2996.2.S1_a_at
	megakaryocyte-associated tyrosine kinase	MATK	***1.9***	Bt.24440.1.S1_at
	G protein-coupled receptor kinase 5	GRK5	***1.9***	Bt.4263.1.S1_at
	casein kinase 1, alpha 1	CSNK1A1	2.0	Bt.4532.1.S1_at
	casein kinase 1, delta	CSNK1D	1.6	Bt.10184.1.S1_a_at
protein folding	FK506 binding protein 14, 22 kDa	FKBP14	***1.9***	Bt.28429.1.S1_at
	DnaJ (Hsp40) homolog, subfamily A, member 4	DNAJA4	***1.5***	Bt.26805.1.S1_at
	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	PTPLA	2.2	Bt.23355.1.S1_at
	peptidylprolyl isomerase D	PPID	1.7	Bt.7221.1.S1_at
	Thioredoxin domain containing 4 (endoplasmic reticulum)	TXNDC4	1.5	Bt.11930.1.S1_at
protein import into nucleus, docking	exportin 1 (CRM1 homolog, yeast)	XPO1	1.8	Bt.9586.1.A1_at
protein modification process	tubulin tyrosine ligase-like family, member 12	TTLL12	***2.3***	Bt.12406.1.S1_at

	neural precursor cell expressed, developmentally down-regulated 4	NEDD4	***1.8***	Bt.19024.1.A1_at
	WW domain containing E3 ubiquitin protein ligase 2	WWP2	***1.9***	Bt.23266.1.S1_at
	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	HERC1	***1.6***	Bt.8263.1.A1_at
	neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	1.5	Bt.3892.1.S1_at
	serine/threonine kinase 38	STK38	***2.1***	Bt.7505.1.S1_at
protein targeting	A kinase (PRKA) anchor protein 12	AKAP12	1.7	Bt.10458.2.S1_at
protein targeting to mitochondrion	cysteine-rich secretory protein LCCL domain containing 2 /// translocase of inner mitochondrial membrane 13 homolog (yeast)	CRISPLD2 /// TIMM13	***2.1***	Bt.1927.1.S1_at
protein thiol-disulfide exchange	ERO1-like (S. cerevisiae)	ERO1L	1.8	Bt.29332.1.A1_at
protein ubiquitination	ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	UBE2H	1.6	Bt.6350.1.A1_at
			1.6	Bt.4543.1.S1_at
proteolysis	cathepsin L1	CTSL1	***2.6***	Bt.10200.1.S1_at
	matrix metallopeptidase 11 (stromelysin 3)	MMP11	***2.3***	Bt.13703.1.S1_at
	complement factor I	CFI	***2.1***	Bt.13387.1.S1_at
	similar to transmembrane protein 27 /// transmembrane protein 27	LOC782259 /// TMEM27	***2.0***	Bt.21824.1.A1_at
	ADAM metallopeptidase domain 33	ADAM33	***1.8***	Bt.2566.1.S1_at
	protease, serine, 23	PRSS23	***1.7***	Bt.20330.1.S1_at
	haptoglobin	HP	***1.5***	Bt.12553.1.S1_at
	cathepsin A	CTSA	2.0	Bt.21410.1.S1_at
	legumain	LGMN	1.9	Bt.8947.1.S1_at
	cathepsin K	CTSK	1.9	Bt.23218.1.S1_at
	similar to Cathepsin O	CTSO	1.9	Bt.28045.1.S1_at
	cathepsin C	CTSC	1.9	Bt.8022.1.S1_at
	fibroblast activation protein, alpha	FAP	1.9	Bt.2717.1.A1_at
	peptidase (mitochondrial processing) beta	PMPCB	1.8	Bt.9797.1.S1_at
	serine carboxypeptidase 1	SCPEP1	1.7	Bt.6357.1.S1_at
	beta-site APP-cleaving enzyme 2	BACE2	1.6	Bt.7130.1.S1_at
	cathepsin L2	CTSL2	1.6	Bt.3987.1.S1_at
	Mitochondrial intermediate peptidase	MIPEP	1.5	Bt.3170.2.S1_at
	aminoacylase 1 /// similar to aminoacylase 1	ACY1 /// LOC790609	2.0	Bt.12885.1.S1_at
	Matrix metallopeptidase 16 (membrane-inserted)	MMP16	***1.6***	Bt.27248.1.A1_at
	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	MMP3	1.9	Bt.18504.2.S1_a_at
			1.5	Bt.18504.2.A1_at
	matrix metallopeptidase 13 (collagenase 3)	MMP13	3.1	Bt.39.1.S1_at
			2.5	Bt.26268.1.A1_at
	matrix metallopeptidase 1 (interstitial collagenase)	MMP1	***16.0***	Bt.72.1.S1_at

	endoplasmic reticulum aminopeptidase 1	ERAP1	1.6	Bt.28338.1.S1_at
	Tripeptidyl peptidase I	TPP1	1.6	Bt.25614.1.A1_at
	cathepsin B	CTSB	1.9	Bt.393.1.S1_at
	cathepsin B	CTSB	1.7	Bt.393.1.S2_at
pseudouridine synthesis	pseudouridylate synthase 7 homolog (S. cerevisiae)	PUS7	2.0	Bt.28327.1.A1_at
			1.5	Bt.2503.1.S1_at
purine nucleotide biosynthetic process	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	ATIC	1.8	Bt.1679.1.S1_at
			1.6	Bt.20537.1.A1_at
pyridine nucleotide biosynthetic process	nicotinate phosphoribosyltransferase domain containing 1	NAPRT1	***1.6***	Bt.23360.1.S1_at
pyrimidine dimer repair by nucleotide-excision repair	high-mobility group nucleosome binding domain 1	HMGN1	1.5	Bt.21491.1.S1_at
pyrimidine nucleotide metabolic process	uridine phosphorylase 1	UPP1	2.0	Bt.20327.1.S1_a_at
regulation of blood pressure	angiotensin II receptor-associated protein	AGTRAP	1.5	Bt.4462.1.S1_at
regulation of cell growth	nephroblastoma overexpressed	NOV	***2.4***	Bt.13482.1.S1_at
	Hypothetical LOC538515	MGC143103	***1.8***	Bt.20272.1.S1_at
	WNT1 inducible signaling pathway protein 2	WISP2	2.8	Bt.27689.1.A1_at
	insulin-like growth factor binding protein 4	IGFBP4	2.4	Bt.5237.1.S1_at
	insulin-like growth factor binding protein 6	IGFBP6	1.7	Bt.9958.1.S1_at
	discoidin domain receptor tyrosine kinase 1	DDR1	***2.6***	Bt.7540.3.S1_at
regulation of cell shape	CDC42 effector protein (Rho GTPase binding) 2	CDC42EP2	***2.2***	Bt.20259.1.S1_at
regulation of I-kappaB kinase/NF-kappaB cascade	sequestosome 1	SQSTM1	2.1	Bt.28031.1.S1_s_at
			1.5	Bt.4869.1.S1_at
regulation of Rab GTPase activity	TBC1 domain family, member 8 (with GRAM domain)	TBC1D8	***1.7***	Bt.6705.1.S1_at
regulation of Rho protein signal transduction	Rho guanine nucleotide exchange factor (GEF) 1	ARHGEF1	***1.7***	Bt.21775.1.S1_at
regulation of the force of heart contraction	ATPase, Na+/K+ transporting, alpha 1 polypeptide	ATP1A1	***2.0***	Bt.21223.1.S1_at
	myosin, light chain 3, alkali; ventricular, skeletal, slow	MYL3	2.6	Bt.23497.1.S1_at
regulation of transcription, DNA-dependent	nuclear receptor subfamily 3, group C, member 2	NR3C2	***1.9***	Bt.17332.1.S1_at
	teashirt zinc finger homeobox 1	TSHZ1	***1.6***	Bt.20064.2.S1_at
	zinc finger with KRAB and SCAN domains 5	ZKSCAN5	***1.5***	Bt.7940.1.S1_at
	teashirt zinc finger homeobox 1	TSHZ1	***1.5***	Bt.20064.1.S1_at
	paternally expressed 3	PEG3	***2.5***	Bt.5027.1.A1_at
	homeobox A4	HOXA4	1.5	Bt.28155.1.S1_at
	empty spiracles homeobox 2	EMX2	***1.7***	Bt.1664.1.A1_at
	TEA domain family member 2	TEAD2	***1.7***	Bt.3877.1.S1_at
	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	ETS2	***1.7***	Bt.5774.2.S1_at
	distal-less homeobox 4	DLX4	***2.6***	Bt.27619.1.A1_at
	Meis homeobox 1	MEIS1	1.8	Bt.25390.1.A1_at
	MYC associated factor X	MAX	1.5	Bt.24987.1.S1_at

	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	ETS1	***1.6***	Bt.12712.1.A1_at
	transcription factor 7 (T-cell specific, HMG-box)	TCF7	***2.5***	Bt.21037.1.S1_at
			2.3	Bt.21037.2.S1_at
regulation of translation	eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1	2.3	Bt.5389.1.S1_at
response to hypoxia	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	2.2	Bt.5313.1.S1_at
	nuclear receptor subfamily 4, group A, member 2	NR4A2	***1.8***	Bt.10060.1.S1_at
	plasminogen activator, urokinase	PLAU	2.5	Bt.2519.1.S1_at
	hypothetical protein MGC127538	MGC127538	***40.3***	Bt.2577.1.S1_at
response to molecule of bacterial origin	CD14 molecule	CD14	***1.6***	Bt.13789.1.A1_at
response to oxidative stress	Peroxidasin homolog (Drosophila)	PXDN	***2.0***	Bt.24695.1.S1_at
response to stress	heat shock transcription factor 2 binding protein	HSF2BP	***1.6***	Bt.25916.1.A1_at
response to yeast	pentraxin-related gene, rapidly induced by IL-1 beta	PTX3	2.4	Bt.10398.1.S1_at
Rho protein signal transduction	CDC42 effector protein (Rho GTPase binding) 1	CDC42EP1	1.7	Bt.5168.1.S1_at
RNA processing	tRNA nucleotidyl transferase, CCA-adding, 1	TRNT1	1.6	Bt.2317.1.S1_at
selenium metabolic process	selenoprotein P, plasma, 1	SEPP1	3.3	Bt.2220.2.A1_a_at
signal transduction	Ankyrin 3, node of Ranvier (ankyrin G)	ANK3	***3.6***	Bt.6086.1.S1_at
	phosphodiesterase 1A, calmodulin-dependent	PDE1A	***2.5***	Bt.512.1.S1_at
	fibrinogen-like 1	FGL1	***2.3***	Bt.13204.1.S1_at
	angiopoietin-like 2	ANGPTL2	***1.7***	Bt.21589.1.S1_at
	similar to mCG142710	LOC511229	***1.5***	Bt.22390.1.S1_at
	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	MLLT4	***1.5***	Bt.9936.1.S1_at
	fibrinogen-like 2	FGL2	4.8	Bt.9202.1.S1_at
	SPARC-like 1 (hevin)	SPARCL1	2.2	Bt.5153.1.S1_at
	tumor necrosis factor receptor superfamily, member 21	TNFRSF21	1.9	Bt.12694.1.S1_at
	phosphodiesterase 3B, cGMP-inhibited	PDE3B	1.9	Bt.25926.1.A1_at
	tenascin C	TNC	1.8	Bt.11061.1.S1_at
	zinc finger, AN1-type domain 6	ZFAND6	1.6	Bt.17584.1.S1_at
	Rho GTPase activating protein 21	ARHGAP21	1.5	Bt.7430.1.S1_at
	angiopoietin 4	ANGPT4	1.5	Bt.29197.1.S1_a_at
	frizzled homolog 1 (Drosophila)	FZD1	2.0	Bt.26635.1.S1_at
	frizzled homolog 1 (Drosophila)	FZD1	1.8	Bt.26635.2.S1_at
	similar to metastasis suppressor 1 /// metastasis suppressor 1	LOC788499 /// MTSS1	***1.6***	Bt.6483.1.A1_at
	endothelin receptor type A	EDNRA	***2.0***	Bt.4719.1.S1_at
	MAS-related GPR, member F	MRGPRF	***1.8***	Bt.24347.1.S1_at
	prostaglandin E receptor 2 (subtype EP2), 53kDa	PTGER2	1.8	Bt.12301.1.S1_at
	leukotriene B4 receptor	LTB4R	1.6	Bt.16889.1.A1_at

	guanine nucleotide binding protein (G protein), gamma 10	GNG10	1.5	Bt.2195.1.A1_at
	coagulation factor II (thrombin) receptor-like 2	F2RL2	***2.4***	Bt.1345.1.S1_at
			1.9	Bt.24447.2.S1_at
	guanine nucleotide binding protein (G protein), gamma 2	GNG2	2.0	Bt.4586.1.S1_at
	oxytocin receptor	OXTR	***1.7***	Bt.140.1.S1_at
	plexin D1	PLXND1	1.7	Bt.16053.1.S1_at
	Similar to IQ motif containing GTPase activating protein 2	LOC100140708	***2.0***	Bt.20947.1.S1_at
			1.8	Bt.20947.2.S1_at
	GTP binding protein overexpressed in skeletal muscle	GEM	1.8	Bt.28062.1.S1_at
skeletal system development	C-type lectin domain family 3, member B	CLEC3B	3.1	Bt.1366.1.S1_at
	collagen, type I, alpha 2	COL1A2	1.9	Bt.8124.1.S2_at
	transforming growth factor, beta 2	TGFB2	2.4	Bt.6438.1.A1_at
	parathyroid hormone-like hormone	PTHLH	1.8	Bt.12848.1.S1_at
	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	***3.1***	Bt.4714.1.S1_at
small GTPase mediated signal transduction	ADP-ribosylation factor-like 6	ARL6	***1.7***	Bt.27912.1.A1_at
	RasGEF domain family, member 1B	RASGEF1B	***1.8***	Bt.27826.1.S1_at
smoothened signaling pathway	dispatched homolog 1 (Drosophila)	DISP1	***1.6***	Bt.18249.1.A1_at
spermatogenesis	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	SERPINA5	***2.0***	Bt.23733.1.A1_at
spermine biosynthetic process	spermine synthase	SMS	2.1	Bt.14326.1.S1_at
spliceosomal snRNP biogenesis and mRNA processing	gem (nuclear organelle) associated protein 8	GEMIN8	1.9	Bt.2768.3.S1_at
			1.6	Bt.2768.1.A1_at
steroid biosynthetic process	farnesyl-diphosphate farnesyltransferase 1	FDFT1	2.1	Bt.21724.2.S1_a_at
	mevalonate kinase	MVK	1.6	Bt.26959.2.S1_at
sulfate assimilation	3'-phosphoadenosine 5'-phosphosulfate synthase 1	PAPSS1	***1.6***	Bt.11219.1.A1_at
T cell costimulation	CD80 molecule	CD80	***2.5***	Bt.27986.1.S1_at
temperature homeostasis	cell death-inducing DFFA-like effector a	CIDEA	1.7	Bt.20167.1.S1_at
thyroid hormone generation	thyroglobulin	TG	***2.7***	Bt.331.1.S1_at
tissue homeostasis	collagen, type XI, alpha 2	COL11A2	***3.9***	Bt.2303.1.S1_at
transcription	TWIST neighbor	TWISTNB	***1.8***	Bt.13392.1.S1_at
	polymerase (RNA) I polypeptide D, 16kDa	POLR1D	1.7	Bt.23590.1.S1_at
	akirin 2	AKIRIN2	1.6	Bt.11326.3.S1_at
	N-acetyltransferase 14 (GCN5-related, putative)	NAT14	***1.6***	Bt.8928.1.S1_at
	polymerase (RNA) III (DNA directed) polypeptide C (62kD)	POLR3C	1.7	Bt.14013.2.S1_at
	GTF2I repeat domain containing 2	GTF2IRD2	1.5	Bt.22655.1.A1_at
	limb bud and heart development homolog (mouse)	LBH	***2.3***	Bt.17942.1.S1_at
	DNA-damage-inducible transcript 3	DDIT3	1.5	Bt.9852.1.S1_at
	recombination signal binding protein for	RBPJ	1.5	Bt.1699.2.S1_a_at

	immunoglobulin kappa J region			
	RAR-related orphan receptor C	RORC	1.6	Bt.16638.2.S1_at
	nuclear transcription factor Y, beta	NFYB	***1.6***	Bt.26520.1.S1_at
	regulatory factor X, 2 (influences HLA class II expression)	RFX2	***1.6***	Bt.4053.1.S1_at
	interferon regulatory factor 1	IRF1	1.5	Bt.10077.1.S2_at
	nuclear receptor subfamily 1, group H, member 3	NR1H3	***2.2***	Bt.17265.1.A1_a_at
	polymerase (RNA) I polypeptide E, 53kDa	POLR1E	1.9	Bt.10981.2.S1_at
			1.5	Bt.10981.1.A1_a_at
translation	mitochondrial ribosomal protein S6	MRPS6	***2.7***	Bt.9428.1.S1_at
	Ribosomal protein L7-like 1	RPL7L1	***1.6***	Bt.14159.1.A1_at
	mitochondrial ribosomal protein L3	MRPL3	1.6	Bt.5225.1.S1_at
	RNA binding motif (RNP1, RRM) protein 3	RBM3	2.3	Bt.9571.2.S1_a_at
			1.7	Bt.9571.1.S1_at
	eukaryotic translation elongation factor 1 alpha 1	EEF1A1	1.8	AFFX-Bt-ef1a-M_at
	Eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	EIF2S2	1.6	Bt.3458.2.S1_at
	eukaryotic translation initiation factor 2, subunit 3 gamma, 52kDa	EIF2S3	1.5	Bt.21351.1.S1_at
			1.5	Bt.21351.1.S1_a_at
	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	EIF2B3	1.6	Bt.20153.1.S1_a_at
	eukaryotic translation initiation factor 3, subunit B	EIF3B	1.7	Bt.12614.1.A1_s_at
	mitochondrial translational release factor 1	MTRF1	1.8	Bt.26163.1.A1_at
	tryptophanyl-tRNA synthetase	WARS	1.5	Bt.2841.1.S1_at
			1.5	Bt.2841.2.S1_a_at
translational initiation	similar to Density-regulated protein	LOC785243	1.6	Bt.13216.1.A1_at
transmembrane receptor protein serine	A kinase (PRKA) anchor protein 4	AKAP4	***2.5***	Bt.218.1.S1_at
transmembrane transport	solute carrier family 46, member 3	SLC46A3	***1.8***	Bt.9454.1.S1_at
transport	lysosomal protein transmembrane 5	LAPTM5	***3.7***	Bt.16049.1.S1_at
	phosphatidylinositol transfer protein, membrane-associated 1	PITPNM1	***2.0***	Bt.18627.1.S1_at
	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	***1.8***	Bt.9593.1.S1_at
	solute carrier family 4, sodium bicarbonate cotransporter, member 7	SLC4A7	***1.9***	Bt.27851.1.S1_at
	solute carrier family 35 (UDP-galactose transporter), member A2	SLC35A2	***1.5***	Bt.25927.1.A1_at
	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	SLC2A5	***2.0***	Bt.19805.1.A1_at
	sorting nexin 10	SNX10	2.1	Bt.27822.2.S1_at
			2.1	Bt.27822.1.A1_at
	sorting nexin 8	SNX8	1.8	Bt.28467.1.S1_at

	cytochrome b-561	CYB561	***1.8***	Bt.4360.1.S1_at
	CYB5 protein	CYB5	1.9	Bt.5134.1.S1_at
			1.5	Bt.4434.1.A1_at
	solute carrier family 25, member 38	SLC25A38	***1.7***	Bt.6425.1.S1_at
	Adaptor-related protein complex 1, sigma 1 subunit	AP1S1	***1.6***	Bt.9086.1.S1_at
	FXYD domain containing ion transport regulator 6	FXYD6	***2.2***	Bt.5369.1.S1_at
	voltage-dependent anion channel 2	VDAC2	1.5	Bt.4099.1.S1_at
	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	ATP6V0D1	1.6	Bt.1575.1.S1_at
	Inositol 1,4,5-triphosphate receptor, type 2	ITPR2	***1.6***	Bt.28659.1.A1_at
	sideroflexin 3	SFXN3	***1.6***	Bt.11145.1.S1_at
	gamma-aminobutyric acid (GABA) A receptor, alpha 1	GABRA1	1.5	Bt.25393.1.A1_at
	lactotransferrin	LTF	***1.8***	Bt.4802.1.S1_at
	potassium channel, subfamily K, member 1	KCNK1	***2.5***	Bt.8363.1.A1_at
	potassium voltage-gated channel, Isk-related family, member 4	KCNE4	3.6	Bt.22411.1.A1_at
	potassium inwardly-rectifying channel, subfamily J, member 15	KCNJ15	2.8	Bt.27605.2.S1_at
			2.0	Bt.27605.1.A1_at
	potassium inwardly-rectifying channel, subfamily J, member 8	KCNJ8	1.6	Bt.13065.1.S1_at
	transmembrane protein 38B	TMEM38B	1.5	Bt.20214.2.S1_at
	solute carrier family 38, member 2	SLC38A2	***1.6***	Bt.9238.1.S1_at
	Solute carrier family 6 (proline IMINO transporter), member 20	SLC6A20	***2.0***	Bt.28747.1.S1_at
	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	SLC16A1	1.8	Bt.13537.1.A1_at
	hemoglobin, mu	HBM	1.6	Bt.5773.1.S1_a_at
	janus kinase and microtubule interacting protein 1	JAKMIP1	***3.9***	Bt.19810.1.A1_at
	SEC16 homolog B (S. cerevisiae)	SEC16B	***1.6***	Bt.1347.1.S1_at
	SEC22 vesicle trafficking protein homolog C (S. cerevisiae)	SEC22C	1.6	Bt.18918.2.A1_at
	retinol binding protein 1, cellular	RBP1	***2.5***	Bt.17810.1.S1_a_at
	RAB3B, member RAS oncogene family	RAB3B	***3.5***	Bt.388.1.S1_at
	RAB6B, member RAS oncogene family	RAB6B	***1.8***	Bt.29469.1.A1_at
	mitochondrial carrier homolog 2 (C. elegans)	MTCH2	1.6	Bt.4503.1.S1_at
	mitochondrial carrier triple repeat 1	MCART1	1.6	Bt.11943.1.S1_at
	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	SLC25A20	1.6	Bt.11770.1.S1_a_at
	solute carrier family 25, member 33	SLC25A33	1.5	Bt.22577.2.S1_at
	vacuolar protein sorting 26 homolog A (S. pombe)	VPS26A	1.6	Bt.2478.1.S1_at
	ERGIC and golgi 3	ERGIC3	1.6	Bt.18891.1.S1_at
tricarboxylic acid cycle	aconitase 1, soluble	ACO1	1.6	Bt.1344.1.S1_at

	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	SDHB	1.5	Bt.13128.1.S1_at
tRNA aminoacylation for protein translation	solute carrier family 22, member 17	SLC22A17	***1.6***	Bt.5055.1.S1_at
tRNA processing	dihydrouridine synthase 3-like (S. cerevisiae)	DUS3L	1.5	Bt.15641.2.A1_at
two-component signal transduction system (phosphorelay)	phosphodiesterase 8A	PDE8A	1.7	Bt.21054.1.A1_at
ubiquitin-dependent protein catabolic process	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	***2.4***	Bt.5408.1.A1_at
urea cycle and arginine metabolic process	arginase, type II	ARG2	***5.6***	Bt.8624.1.S1_at
ureteric bud development	SIX homeobox 1	SIX1	1.6	Bt.17204.1.S1_at
vasculogenesis	tryptophanyl tRNA synthetase 2, mitochondrial	WARS2	1.6	Bt.1708.1.S1_at
Wnt receptor signaling pathway	catenin, beta interacting protein 1	CTNNBIP1	***2.0***	Bt.14644.2.S1_at
			1.9	Bt.14644.1.A1_at
	wingless-type MMTV integration site family, member 10B	WNT10B	***1.7***	Bt.6354.1.S1_at
xenobiotic metabolic process	cathelicidin 6	CATHL6	1.6	Bt.15991.1.S1_at
Unknown function	Dickkopf homolog 1 (Xenopus laevis)	DKK1	***12.0***	Bt.13880.1.S1_at
	chloride channel accessory 2	CLCA2	***10.6***	Bt.15970.1.A1_at
	similar to RNA-binding protein 35A (RNA-binding motif protein 35A)	LOC788414	***8.2***	Bt.27030.1.S1_at
	Plexin C1	PLXNC1	***5.4***	Bt.26968.1.S1_at
	similar to Mal, T-cell differentiation protein /// mal, T-cell differentiation protein	LOC782642 /// MAL	***5.3***	Bt.4060.1.S1_at
	thyroid hormone responsive (SPOT14 homolog, rat)	THRSP	***5.1***	Bt.15842.1.S1_at
	monooxygenase, DBH-like 1	MOXD1	***5.0***	Bt.20519.1.A1_at
	annexin A3	ANXA3	***3.8***	Bt.6775.1.S1_at
	actin filament associated protein 1-like 2	AFAP1L2	***3.8***	Bt.22165.1.S1_at
	latent transforming growth factor beta binding protein 1	LTBP1	***3.7***	Bt.20820.1.A1_at
	heparanase	HPSE	***3.7***	Bt.8127.1.S2_at
	occludin	OCLN	***3.7***	Bt.13868.1.S1_at
	S100 calcium binding protein A4	S100A4	***3.6***	Bt.5493.1.S1_at
	CREB regulated transcription coactivator 1	CRTC1	***3.5***	Bt.12510.3.S1_at
	SH3 domain binding glutamic acid-rich protein like 2	SH3BGRL2	***3.5***	Bt.16036.1.S1_at
	hypothetical LOC513129	LOC513129	***3.2***	Bt.10124.1.S1_at
	chondroadherin	CHAD	***3.2***	Bt.347.1.S1_at
	similar to inositol polyphosphate-5-phosphatase F	LOC539889	***3.1***	Bt.15840.1.A1_at
	collagen, type IX, alpha 2	COL9A2	***3.1***	Bt.4235.1.S1_at
	SH3 domain and tetratricopeptide repeats 2	SH3TC2	***3.0***	Bt.711.1.A1_at
	midline 1 (Opitz/BBB syndrome)	MID1	***2.9***	Bt.16611.1.A1_at

	leucine rich repeat neuronal 1	LRRN1	***2.9***	Bt.17579.1.A1_at
	monooxygenase, DBH-like 1	MOXD1	***2.9***	Bt.20519.2.S1_at
	Similar to cell adhesion molecule JCAM	LOC511594	***2.8***	Bt.3194.1.S1_at
	interleukin 17 receptor B	IL17RB	***2.8***	Bt.24532.1.A1_at
	Raftlin family member 2	RFTN2	***2.7***	Bt.24894.1.S1_at
	furry homolog (Drosophila)	FRY	***2.7***	Bt.8327.1.S1_at
	Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	CELSR2	***2.6***	Bt.13493.1.A1_at
	desmoplakin	DSP	***2.6***	Bt.1818.1.S1_at
	hydrogen voltage-gated channel 1	HVCN1	***2.5***	Bt.797.1.S1_at
	acid phosphatase 5, tartrate resistant	ACP5	***2.5***	Bt.5193.2.S1_a_at
	RNA binding protein with multiple splicing	RBPMS	***2.5***	Bt.11525.2.A1_at
	retinoic acid receptor responder (tazarotene induced) 1	RARRES1	***2.5***	Bt.24933.1.S1_at
	Transmembrane protein 163	TMEM163	***2.5***	Bt.20951.1.A1_at
	fin bud initiation factor homolog (zebrafish)	FIBIN	***2.5***	Bt.3435.1.A1_at
	Plexin C1	PLXNC1	***2.4***	Bt.6608.1.S1_at
	Glycoprotein M6A	GPM6A	***2.4***	Bt.27428.1.A1_at
	heparanase	HPSE	***2.4***	Bt.8127.1.S1_at
	AMME chromosomal region gene 1-like	AMMECR1L	***2.4***	Bt.17508.1.S1_at
	tetraspanin 33	TSPAN33	***2.3***	Bt.7808.1.A1_at
	similar to C4b-binding protein alpha chain precursor (C4bp) (Proline-rich protein) (PRP)	LOC515150	***2.3***	Bt.12136.1.S1_at
	AMME chromosomal region gene 1-like	AMMECR1L	***2.3***	Bt.17508.2.A1_at
	Cerebellin 4 precursor	CBLN4	***2.3***	Bt.24465.1.A1_at
	Dedicator of cytokinesis 5	DOCK5	***2.2***	Bt.20310.1.S1_at
	similar to Protein FAM101B	LOC618459	***2.2***	Bt.3701.1.S1_at
	hemicentin 1	HMCN1	***2.2***	Bt.26051.1.A1_at
	zinc finger, DHHC-type containing 14	ZDHHC14	***2.2***	Bt.22230.1.S1_at
	ash1 (absent, small, or homeotic)-like (Drosophila)	ASH1L	***2.2***	Bt.5037.1.S2_at
	Solute carrier family 39 (zinc transporter), member 8	SLC39A8	***2.2***	Bt.22697.1.A1_at
	sushi-repeat-containing protein, X-linked 2	SRPX2	***2.2***	Bt.8109.2.A1_at
	Tetraspanin 12	TSPAN12	***2.2***	Bt.29152.2.A1_at
	SRY (sex determining region Y)-box 6	SOX6	***2.2***	Bt.24317.1.A1_at
	transmembrane protein 165	TMEM165	***2.2***	Bt.4994.1.S1_at
	proline-serine-threonine phosphatase interacting protein 2	PSTPIP2	***2.2***	Bt.28073.1.S1_at
	hypothetical protein LOC788205	LOC788205	***2.2***	Bt.2933.1.S1_at
	Trophoblast glycoprotein	TPBG	***2.2***	Bt.24902.1.S1_at
	src kinase associated phosphoprotein 1	SKAP1	***2.1***	Bt.20853.1.S1_a_at
	Integrin, alpha 6	ITGA6	***2.1***	Bt.4544.1.S1_at
	Solute carrier organic anion transporter family, member 4A1	SLCO4A1	***2.1***	Bt.27240.1.A1_at

	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	SERPINA1	***2.1***	Bt.982.1.S1_at
	integrin, alpha 10	ITGA10	***2.1***	Bt.6610.1.S1_at
	cytoplasmic FMR1 interacting protein 2	CYFIP2	***2.1***	Bt.7948.1.S1_at
	Golgi-localized protein	GOLSYN	***2.1***	Bt.28562.1.S1_at
	similar to LPS-responsive vesicle trafficking, beach and anchor containing	LOC524279	***2.1***	Bt.13411.1.S1_at
	Similar to guanylate binding protein 1	LOC511531	***2.1***	Bt.24012.1.A1_at
	serpin peptidase inhibitor, clade I (neuroserpin), member 1	SERPINI1	***2.1***	Bt.19231.1.S1_at
	S100 calcium-binding protein A10	S100A10	***2.1***	Bt.5037.1.S1_at
	lumican	LUM	***2.1***	Bt.2452.1.S1_at
	similar to nucleolar protein No55	LOC788823	***2.0***	Bt.1720.1.S1_at
	tandem C2 domains, nuclear	TC2N	***2.0***	Bt.25489.1.A1_at
	hyaluronan binding protein 4	HABP4	***2.0***	Bt.20020.1.S1_at
	tetraspanin 12	TSPAN12	***2.0***	Bt.29152.1.S1_at
	similar to C27H2.2a	LOC516658	***2.0***	Bt.3291.1.A1_at
	olfactomedin 1	OLFM1	***2.0***	Bt.18101.1.A1_at
	solute carrier family 38, member 5	SLC38A5	***2.0***	Bt.26713.1.S1_at
	IQ motif containing with AAA domain 1	IQCA1	***2.0***	Bt.26706.1.S1_at
	polo-like kinase 2 (Drosophila)	PLK2	***2.0***	Bt.10259.1.A1_at
	KN motif and ankyrin repeat domains 1	KANK1	***2.0***	Bt.18315.3.A1_at
	Uncharacterized protein C13orf33 homolog	MGC155285	***2.0***	Bt.22080.1.S1_at
	Influenza virus NS1A binding protein	IVNS1ABP	***2.0***	Bt.11631.2.S1_at
	similar to beta-1,4-N-acetyl-galactosaminyl transferase 4	LOC100137759	***1.9***	Bt.14237.1.A1_at
	solute carrier family 35, member D2	SLC35D2	***1.9***	Bt.28522.1.S1_at
	Minichromosome maintenance complex component 10	MCM10	***1.9***	Bt.16556.1.A1_at
	diacylglycerol kinase, iota	DGKI	***1.9***	Bt.22642.1.A1_at
	mal, T-cell differentiation protein-like	MALL	***1.9***	Bt.13290.1.S1_at
	nuclear receptor coactivator 7	NCOA7	***1.9***	Bt.28087.1.S1_at
	hypothetical LOC534560	MGC152232	***1.9***	Bt.14166.1.A1_at
	hypothetical LOC507695	LOC507695	***1.9***	Bt.28344.1.S1_at
	transmembrane protein 184B	TMEM184B	***1.9***	Bt.10312.1.A1_at
	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	SERPINA1	***1.9***	Bt.982.1.S1_a_at
	thymocyte selection-associated high mobility group box	TOX	***1.9***	Bt.8400.1.A1_at
	DKFZP564O0823 protein	DKFZP564O0823	***1.9***	Bt.24157.1.A1_at
	spondin 2, extracellular matrix protein	SPON2	***1.8***	Bt.18280.1.S1_at
	six transmembrane epithelial antigen of the prostate 1	STEAP1	***1.8***	Bt.16925.1.A1_at

	tetraspanin 5	TSPAN5	***1.8***	Bt.22126.1.S1_at
	BEN domain containing 6	BEND6	***1.8***	Bt.3099.1.S1_at
	transmembrane protein 176A	TMEM176A	***1.8***	Bt.15565.1.S1_at
	allograft inflammatory factor 1-like	AIF1L	***1.8***	Bt.10983.1.S1_at
	tight junction protein 2 (zona occludens 2)	TJP2	***1.8***	Bt.20601.1.A1_at
	Heat shock 70kD protein 12B	HSPA12B	***1.8***	Bt.27345.2.S1_at
	ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	***1.8***	Bt.13583.1.A1_at
	ankyrin repeat domain 10	ANKRD10	***1.8***	Bt.9069.1.S1_at
	similar to F47B8.14	LOC525174	***1.8***	Bt.8637.1.S1_at
	transmembrane protein 30B	TMEM30B	***1.8***	Bt.22042.1.S1_at
	Transducin (beta)-like 1X-linked	TBL1X	***1.8***	Bt.9988.1.S1_at
	indoleamine 2,3-dioxygenase 2	IDO2	***1.8***	Bt.18821.1.A1_at
	hypothetical LOC510399	MGC128008	***1.8***	Bt.22468.2.A1_at
	Phosphodiesterase 4D interacting protein	PDE4DIP	***1.8***	Bt.3736.1.A1_at
	Tight junction protein 2 (zona occludens 2)	TJP2	***1.8***	Bt.20601.2.S1_at
	kelch-like 8 (Drosophila)	KLHL8	***1.8***	Bt.16467.1.S1_at
	cyclin-dependent kinase 2 associated protein 1	CDK2AP1	***1.8***	Bt.2660.1.S1_at
	Musculoskeletal, embryonic nuclear protein 1	MUSTN1	***1.8***	Bt.9832.1.S1_at
	transmembrane protein 37	TMEM37	***1.8***	Bt.16583.1.S1_at
	S100 calcium binding protein A9	S100A9	***1.7***	Bt.16201.2.A1_at
	musculoskeletal, embryonic nuclear protein 1	MUSTN1	***1.7***	Bt.9832.1.S1_a_at
	Rab interacting lysosomal protein-like 2	RILPL2	***1.7***	Bt.4884.1.S1_at
	Chromosome 5 open reading frame 32 ortholog	C7H5orf32	***1.7***	Bt.17200.1.A1_at
	Hypothetical protein LOC508031	LOC508031	***1.7***	Bt.18649.2.A1_at
	dimethylarginine dimethylaminohydrolase 2	DDAH2	***1.7***	Bt.5132.1.S1_at
	Adhesion molecule with Ig-like domain 2	AMIGO2	***1.7***	Bt.12294.1.S1_at
	chondroitin sulfate proteoglycan 4	CSPG4	***1.7***	Bt.28247.1.A1_s_at
	similar to KIAA0405	FLRT2	***1.7***	Bt.2942.1.S1_at
	ornithine aminotransferase (gyrate atrophy)	OAT	***1.7***	Bt.5400.1.S1_at
	hypothetical LOC614490	LOC614490	***1.7***	Bt.1907.1.S1_at
	Hypothetical protein LOC783068	MGC151567	***1.7***	Bt.11151.1.S1_at
	similar to tripartite motif-containing 8	LOC782444	***1.7***	Bt.9660.1.A1_s_at
	transmembrane protein 51	TMEM51	***1.7***	Bt.15702.1.S1_at
	similar to TLH29 protein precursor	ISG12(B)	***1.7***	Bt.9779.1.S1_at
	sushi-repeat-containing protein, X-linked 2	SRPX2	***1.7***	Bt.8109.1.A1_a_at
	hypothetical protein LOC512679	KIAA1549	***1.7***	Bt.26111.1.A1_at
	small EDRK-rich factor 1A (telomeric)	SERF1A	***1.7***	Bt.26048.1.A1_at
	telomerase-associated protein 1	TEP1	***1.7***	Bt.21156.1.S1_at
	interleukin 13 receptor, alpha 1	IL13RA1	***1.7***	Bt.28750.1.S1_at
	S100 calcium binding protein A9	S100A9	***1.7***	Bt.16201.1.S1_at
	fibroblast growth factor 12	FGF12	***1.7***	Bt.25062.1.A1_at

	Hypothetical LOC613328	MGC139239	***1.7***	Bt.19380.1.A1_at
	nuclear receptor coactivator 7	NCOA7	***1.7***	Bt.1029.1.S1_at
	hypothetical LOC617744	LOC617744	***1.7***	Bt.3734.1.A1_at
	Platelet derived growth factor C	PDGFC	***1.7***	Bt.3014.1.A1_at
	similar to Spectrin beta chain, brain 2 (Spectrin, non-erythroid beta chain 2) (Beta-III spectrin)	LOC506239	***1.7***	Bt.4069.1.S1_at
	Coiled-coil domain containing 104	CCDC104	***1.7***	Bt.27498.1.S1_at
	cerebellar degeneration-related protein 2, 62kDa	CDR2	***1.7***	Bt.15847.1.S1_at
	hypothetical LOC534560	MGC152232	***1.6***	Bt.14166.2.S1_at
	Ubiquitin-like 4A	UBL4A	***1.6***	Bt.4658.2.A1_at
	Bromodomain containing 7	BRD7	***1.6***	Bt.3135.2.A1_at
	transmembrane 9 superfamily member 2	TM9SF2	***1.6***	Bt.15747.1.A1_at
	Dopey family member 1	DOPEY1	***1.6***	Bt.19612.1.S1_at
	Jumonji, AT rich interactive domain 2	JARID2	***1.6***	Bt.7008.1.S1_at
	cytochrome c oxidase subunit VIb testes-specific isoform	COX6B2	***1.6***	Bt.29868.1.S1_at
	Hypothetical protein LOC614841	LOC614841	***1.6***	Bt.27272.1.A1_at
	tripartite motif-containing 22	TRIM22	***1.6***	Bt.19790.1.A1_at
	CD151 molecule (Raph blood group)	CD151	***1.6***	Bt.5473.1.S1_at
	contactin associated protein-like 2	CNTNAP2	***1.6***	Bt.5618.1.A1_at
	RAB38, member RAS oncogene family	RAB38	***1.6***	Bt.27433.2.A1_at
	Hypothetical protein LOC614841	LOC614841	***1.6***	Bt.22149.1.S1_at
	MAP-kinase activating death domain	MADD	***1.6***	Bt.29596.1.S1_at
	similar to tripartite motif-containing 8	LOC782444	***1.6***	Bt.27075.1.A1_at
	similar to hCG1980844	LOC789183	***1.6***	Bt.21561.1.S1_at
	transmembrane protein 63A	TMEM63A	***1.6***	Bt.12310.1.S1_at
	IQ motif containing K	IQCK	***1.6***	Bt.12904.2.S1_at
	chondroitin sulfate proteoglycan 4	CSPG4	***1.6***	Bt.8114.1.A1_at
	membrane-associated ring finger (C3HC4) 9	MARCH9	***1.6***	Bt.18862.2.S1_at
	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	***1.6***	Bt.24434.1.A1_at
	transmembrane protein 185A	TMEM185A	***1.6***	Bt.24898.1.S1_at
	similar to family with sequence similarity 20, member C	LOC534672	***1.6***	Bt.11748.1.A1_at
	Olfactomedin 1	OLFM1	***1.6***	Bt.876.1.S1_at
	Dolichyl-phosphate mannosyltransferase polypeptide 3	DPM3	***1.6***	Bt.9914.1.S1_at
	trinucleotide repeat containing 6C	TNRC6C	***1.6***	Bt.19479.2.A1_a_at
	transforming, acidic coiled-coil containing protein 2	TACC2	***1.6***	Bt.29029.1.S1_at
	junctophilin 1	JPH1	***1.6***	Bt.16236.1.A1_at
	Epidermal growth factor receptor pathway substrate 8	EPS8	***1.6***	Bt.12691.1.A1_at
	myosin IXB	MYO9B	***1.6***	Bt.1774.1.S1_at

	EH-domain containing 3	EHD3	***1.6***	Bt.3273.1.A1_at
	MORC family CW-type zinc finger 4	MORC4	***1.6***	Bt.6579.1.S1_at
	formin binding protein 1	FNBP1	***1.6***	Bt.10381.1.S1_at
	zinc finger protein 385A	ZNF385A	***1.6***	Bt.8699.1.S1_at
	chromosome 3 open reading frame 63 ortholog	C22H3ORF63	***1.6***	Bt.25054.1.A1_at
	centrosome and spindle pole associated protein 1	CSPP1	***1.6***	Bt.13321.1.S1_at
	frizzled homolog 8 (Drosophila)	FZD8	***1.6***	Bt.22538.1.A1_at
			1.6	Bt.22538.2.S1_at
	Similar to solute carrier family 35, member F2	LOC533008	***1.6***	Bt.21079.1.A1_at
	similar to nuclear receptor binding SET domain protein 1 /// nuclear receptor binding SET domain protein 1	LOC100140853 /// NSD1	***1.6***	Bt.11047.1.S1_at
	transmembrane protein 119	TMEM119	***1.6***	Bt.20234.1.S1_at
	chromosome 14 open reading frame 38 ortholog	C10H14orf38	***1.6***	Bt.5630.1.S1_at
	protein kinase C, delta binding protein	PRKCDBP	***1.6***	Bt.2285.1.A1_at
	glycosyltransferase 8 domain containing 2	GLT8D2	***1.6***	Bt.24824.1.S1_at
	glomulin, FKBP associated protein	GLMN	***1.6***	Bt.25404.1.A1_at
	similar to ankyrin repeat domain 26 /// similar to ankyrin repeat domain 26	LOC526505 /// LOC785905	***1.5***	Bt.24642.1.A1_at
	praja ring finger 2	PJA2	***1.5***	Bt.17496.1.S1_at
	Microtubule-associated protein 9	MAP9	***1.5***	Bt.17898.2.A1_at
	RAN binding protein 9	RANBP9	***1.5***	Bt.21966.1.S1_at
	inositol polyphosphate-4-phosphatase, type II, 105kDa	INPP4B	***1.5***	Bt.26310.1.A1_at
	IQ motif containing K	IQCK	***1.5***	Bt.12904.1.S1_at
	collagen, type IV, alpha 5	COL4A5	***1.5***	Bt.16495.1.A1_at
	similar to Forkhead box protein D1 (Forkhead-related protein FKHL8) (Forkhead-related transcription factor 4) (FREAC-4)	LOC783819	***1.5***	Bt.19280.1.A1_at
	GDNF family receptor alpha 4	GFRA4	***1.5***	Bt.21175.1.S1_at
	similar to F11C1.5a	LOC524159	***1.5***	Bt.9500.1.S1_a_at
	similar to N-acetyltransferase 5	LOC100140405	***1.5***	Bt.16639.1.A1_at
	similar to multiple EGF-like-domains 9	MEGF9	***1.5***	Bt.9781.1.S1_at
	CUE domain containing 1	CUEDC1	***1.5***	Bt.12356.1.S1_at
	chromosome 6 open reading frame 115 ortholog	C9H6ORF115	***1.5***	Bt.5717.1.S1_at
	melanoma antigen family D, 1	MAGED1	***1.5***	Bt.5519.2.S1_at
	muskelin 1, intracellular mediator containing kelch motifs	MKLN1	***1.5***	Bt.27199.1.S1_at
	leucine rich repeat containing 17	LRRC17	7.8	Bt.4886.1.A1_at
	Hypothetical LOC505226	MGC134574	7.0	Bt.17517.1.S1_at
	transmembrane protein 220	TMEM220	4.0	Bt.28251.1.S1_at
	immunoglobulin superfamily, member 10	IGSF10	3.7	Bt.26774.1.S1_at

	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	NDUFA4L2	3.1	Bt.1746.1.S1_at
	prickle homolog 1 (Drosophila)	PRICKLE1	2.8	Bt.18126.2.S1_at
	Complement factor H-related 5	CFHR5	2.8	Bt.17612.2.S1_at
	growth and transformation-dependent protein	LOC617104	2.5	Bt.9210.1.S1_at
	complement factor H-related 4	CFHR4	2.4	Bt.17612.1.A1_at
	LIM domain and actin binding 1	LIMA1	2.4	Bt.10730.1.S1_at
	dystrobrevin, beta	DTNB	2.4	Bt.7860.2.S1_a_at
	Similar to uronyl-2-sulfotransferase	LOC535975	2.3	Bt.21924.2.S1_at
	prickle homolog 1 (Drosophila)	PRICKLE1	2.4	Bt.18126.1.A1_at
			2.3	Bt.12688.1.A1_at
			2.1	Bt.12688.2.A1_at
	high-mobility group nucleosomal binding domain 2	HMGN2	2.2	Bt.1758.1.S1_at
	WD repeat and FYVE domain containing 1	WDFY1	2.2	Bt.27929.1.S1_at
	CDV3 homolog (mouse)	CDV3	2.2	Bt.22025.2.A1_a_at
	histamine N-methyltransferase	HNMT	2.2	Bt.26700.1.S1_at
	similar to meningioma expressed antigen 5 (hyaluronidase)	LOC538561	2.2	Bt.28653.1.S1_at
	similar to C1orf51 protein	LOC539246	2.2	Bt.14114.1.A1_at
	heme binding protein 1	HEBP1	2.2	Bt.5047.1.S1_at
	similar to zinc finger protein 239	ZNF239	2.1	Bt.26640.1.S1_at
	CD68 molecule	CD68	2.1	Bt.2334.1.S1_at
	Calpain 6	CAPN6	2.1	Bt.10150.1.S1_at
	ankyrin repeat domain 29	ANKRD29	2.1	Bt.20382.1.S1_at
	transmembrane protein 100	TMEM100	2.1	Bt.1787.2.S1_at
	guanine nucleotide binding protein-like 2 (nucleolar)	GNL2	2.1	Bt.6819.2.S1_at
	Ubiquitin domain containing 2	UBTD2	2.0	Bt.27591.1.S1_at
	upstream binding transcription factor, RNA polymerase I	UBTF	2.0	Bt.28435.1.S1_at
	FERM domain containing 6	FRMD6	2.0	Bt.28695.1.S1_at
	cytochrome c oxidase subunit VIa polypeptide 1	COX6A1	2.0	Bt.23002.1.S1_at
	similar to hCG1777421	LOC787439	2.0	Bt.17329.1.A1_at
	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	2.0	Bt.5056.3.S1_at
	hypothetical LOC508425	MGC155170	2.0	Bt.8802.1.S1_at
	Membrane bound O-acyltransferase domain containing 2	MBOAT2	2.0	Bt.8669.2.S1_at
	apoptosis antagonizing transcription factor	AATF	2.0	Bt.21475.3.S1_a_at
	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	2.0	Bt.5056.2.A1_at
	KN motif and ankyrin repeat domains 2	KANK2	2.0	Bt.7727.1.S1_at
	WD repeat, sterile alpha motif and U-box domain	WDSUB1	2.0	Bt.8290.1.S1_at

	containing 1			
	immunoglobulin superfamily containing leucine-rich repeat	ISLR	2.0	Bt.1899.1.A1_at
	WD repeat domain 41	WDR41	1.9	Bt.16334.2.S1_a_at
	Zinc finger CCCH-type containing 13	ZC3H13	1.9	Bt.16450.1.A1_at
	Insulin-like growth factor binding protein, acid labile subunit	IGFALS	1.9	Bt.24635.2.A1_at
	NAD(P)H dehydrogenase, quinone 2	NQO2	1.9	Bt.20318.1.S1_a_at
	Carbonic anhydrase IX	CA9	1.9	Bt.28317.1.S1_at
	Ras-related GTP binding D	RRAGD	1.9	Bt.21765.1.S1_at
	Membrane associated guanylate kinase, WW and PDZ domain containing 2	MAGI2	1.9	Bt.17191.1.A1_at
	cell adhesion molecule 1	CADM1	1.9	Bt.3806.1.A1_at
	myocilin, trabecular meshwork inducible glucocorticoid response	MYOC	1.9	Bt.4703.1.S1_at
	phosphatidylethanolamine-binding protein 4	PEBP4	1.9	Bt.14398.1.S1_at
	coronin, actin binding protein, 1C	CORO1C	1.9	Bt.656.2.S1_at
	growth arrest-specific 6	GAS6	1.8	Bt.712.1.S1_at
	dystrobrevin, beta	DTNB	1.8	Bt.7860.1.S1_s_at
	N-myc (and STAT) interactor	NMI	1.8	Bt.12285.2.S1_a_at
	growth hormone inducible transmembrane protein	GHITM	1.8	Bt.7007.1.S1_at
	procollagen C-endopeptidase enhancer	PCOLCE	1.8	Bt.7955.1.S1_at
	sarcalumenin	SRL	1.8	Bt.11687.1.S1_a_at
	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	1.8	Bt.3863.1.S1_at
	arrestin domain containing 4	ARRDC4	1.8	Bt.9792.1.S1_at
	S100 calcium binding protein A1	S100A1	1.8	Bt.1178.1.S1_at
	uronyl-2-sulfotransferase	UST	1.8	Bt.21924.1.A1_at
	Similar to nucleoporin 62	LOC516074	1.8	Bt.19355.1.A1_at
	sarcalumenin	SRL	1.8	Bt.11687.1.S1_at
	C1q and tumor necrosis factor related protein 7	C1QTNF7	1.8	Bt.19274.1.A1_at
	similar to meningioma expressed antigen 5 (hyaluronidase)	LOC538561	1.8	Bt.11492.1.A1_at
	synaptosomal-associated protein, 23kDa	SNAP23	1.8	Bt.23682.1.S1_at
	hypothetical protein LOC100140348	LOC100140348	1.8	Bt.21142.1.S1_at
	immediate early response 2	IER2	1.8	Bt.22194.2.S1_at
	myosin IB	MYO1B	1.8	Bt.979.1.A1_at
	chromosome 1 open reading frame 85 ortholog	C3H1orf85	1.8	Bt.6386.3.S1_a_at
	FERM domain containing 6	FRMD6	1.7	Bt.28695.2.A1_at
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	DDX17	1.7	Bt.18993.1.A1_at
	BMP binding endothelial regulator	BMPER	1.7	Bt.27778.1.A1_at
	phosphatidic acid phosphatase type 2A	PPAP2A	1.7	Bt.6626.1.S1_at
	sushi-repeat-containing protein, X-linked	SRPX	1.7	Bt.2046.1.S1_at

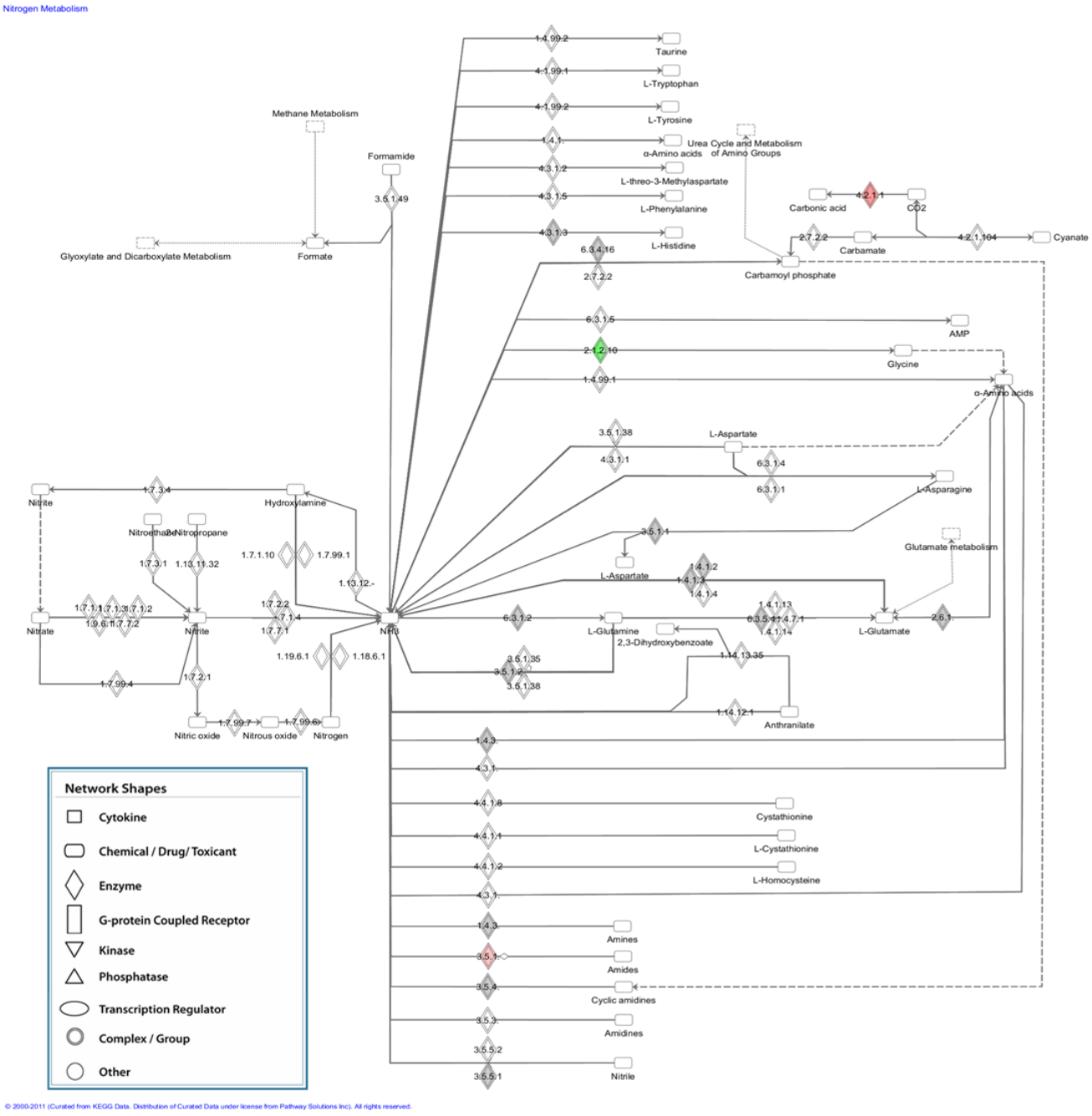
	glypican 4	GPC4	1.7	Bt.19508.1.A1_at
	Basic leucine zipper and W2 domains 1	BZW1	1.7	Bt.20692.1.A1_at
	junctional adhesion molecule 3	JAM3	1.7	Bt.8492.1.A1_at
	Similar to HZFW1	LOC788986	1.7	Bt.17673.1.A1_at
	similar to GC-rich sequence DNA-binding factor (GCF) (Transcription factor 9) (TCF-9)	LOC521363	1.7	Bt.28626.1.S1_at
	calcium/calmodulin-dependent protein kinase ID	CAMK1D	1.7	Bt.17491.1.A1_at
	apoptosis antagonizing transcription factor	AATF	1.7	Bt.21475.1.S1_at
	nipsnap homolog 3A (C. elegans)	NIPSNAP3A	1.7	Bt.26872.1.S1_at
	cold inducible RNA binding protein	CIRBP	1.7	Bt.23273.1.S1_at
	Trimethylguanosine synthase homolog (S. cerevisiae)	TGS1	1.7	Bt.9299.1.A1_at
	amyloid beta (A4) precursor-like protein 2	APLP2	1.7	Bt.23248.1.S1_at
	Signal sequence receptor, alpha	SSR1	1.7	Bt.28965.1.A1_at
	ribosomal RNA processing 9, small subunit (SSU) processome component, homolog (yeast)	RRP9	1.7	Bt.9939.2.S1_at
	DDB1 and CUL4 associated factor 4	DCAF4	1.7	Bt.29346.1.S1_at
	chromosome 1 open reading frame 113 ortholog	C3H1orf113	1.7	Bt.3415.1.A1_at
	Hypothetical LOC781004	LOC781004	1.7	Bt.13556.2.A1_x_at
	similar to ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative function)	LOC512304	1.7	Bt.22797.1.A1_at
			1.7	Bt.14001.1.A1_at
	UBX domain protein 8	UBXN8	1.7	Bt.28321.1.A1_at
	SH3-domain binding protein 5 (BTK-associated)	SH3BP5	1.7	Bt.20680.1.S1_at
	dopey family member 2	DOPEY2	1.7	Bt.28524.1.S1_at
	Zinc finger CCCH-type containing 13	ZC3H13	1.7	Bt.16450.2.A1_at
	heat-responsive protein 12	HRSP12	1.7	Bt.1556.1.S1_at
	torsin A interacting protein 1	TOR1AIP1	1.6	Bt.19402.1.A1_at
	cartilage acidic protein 1	CRTAC1	1.6	Bt.27196.1.A1_at
	multiple EGF-like-domains 10	MEGF10	1.6	Bt.3996.1.S1_at
	Nephronectin	NPNT	1.6	Bt.7393.1.S1_at
	N-myc (and STAT) interactor	NMI	1.6	Bt.12285.3.S1_a_at
	insulin-like growth factor binding protein, acid labile subunit	IGFALS	1.6	Bt.24635.1.S1_at
	transmembrane protein 126A	TMEM126A	1.6	Bt.24931.1.A1_at
	pleckstrin homology domain containing, family O member 2	PLEKHO2	1.6	Bt.4588.1.S1_at
	RNA binding motif protein 18	RBM18	1.6	Bt.20213.2.S1_at
	S100 calcium binding protein A1	S100A1	1.6	Bt.1178.1.S1_a_at
	chromosome 8 open reading frame 70 ortholog	C14H8ORF70	1.6	Bt.8903.1.S1_at
	NMDA receptor regulated 1	NARG1	1.6	Bt.16099.2.S1_at
	Fibroblast growth factor 11	FGF11	1.6	Bt.26644.2.S1_at
	mitochondrial ribosomal protein S35	MRPS35	1.6	Bt.13205.2.S1_at

	similar to SLIT3	LOC615883	1.6	Bt.22400.1.A1_at
	similar to Krueppel-like factor 2 (Lung krueppel-like factor)	LOC520939	1.6	Bt.2590.1.A1_at
	leucine-rich PPR-motif containing	LRPPRC	1.6	Bt.11322.1.A1_at
	abhydrolase domain containing 14A	ABHD14A	1.6	Bt.20453.2.S1_a_at
	COBW domain containing 2	CBWD2	1.6	Bt.21610.1.S1_at
	chromosome 2 open reading frame 7 ortholog	C11H2ORF7	1.6	Bt.22510.1.S1_at
	collagen, type VI, alpha 3	COL6A3	1.6	Bt.12498.1.A1_at
	Asparaginase like 1	ASRGL1	1.6	Bt.685.1.A1_at
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	DDX10	1.6	Bt.29345.1.A1_at
	protein phosphatase 1, regulatory (inhibitor) subunit 7	PPP1R7	1.6	Bt.1867.2.S1_at
	synaptosomal-associated protein, 23kDa	SNAP23	1.6	Bt.10994.1.S1_at
	SH3-domain binding protein 5 (BTK-associated)	SH3BP5	1.6	Bt.8619.1.S1_at
	zinc finger protein 12	ZNF12	1.6	Bt.23663.1.S1_at
	zinc finger, AN1-type domain 3	ZFAND3	1.6	Bt.23708.1.A1_at
	Ras-related GTP binding B	RRAGB	1.6	Bt.19793.1.A1_at
	hypothetical protein LOC613274	LOC613274	1.6	Bt.13584.2.S1_at
	transmembrane 4 L six family member 5	TM4SF5	1.6	Bt.20586.1.S1_a_at
	basic leucine zipper and W2 domains 1	BZW1	1.6	Bt.23604.1.S1_at
	inositol 1,4,5-triphosphate receptor interacting protein	ITPRIP	1.6	Bt.26784.1.S1_at
	GPN-loop GTPase 1	GPN1	1.6	Bt.13501.2.S1_at
	NAD(P)H dehydrogenase, quinone 2	NQO2	1.6	Bt.20318.2.S1_a_at
	similar to high-mobility group box 3	LOC532409	1.6	Bt.11711.1.A1_at
	transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	1.6	Bt.1789.1.S1_at
	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	ELOVL4	1.6	Bt.17635.1.A1_at
	transferrin receptor 2	TFR2	1.6	Bt.6186.1.S1_at
	Zinc finger protein 106 homolog (mouse)	ZFP106	1.6	Bt.27226.1.S1_at
	Kruppel-like factor 11	KLF11	1.6	Bt.16916.2.A1_at
	LIM and senescent cell antigen-like domains 2	LIMS2	1.6	Bt.21349.1.S1_at
	hypothetical protein LOC100270756	LOC100270756	1.6	Bt.14198.1.S1_at
	Hypothetical LOC784866	LOC784866	1.6	Bt.18219.1.A1_at
	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	PSMD9	1.6	Bt.941.1.S1_at
	hypothetical protein LOC535260	FAM151B	1.6	Bt.27442.1.S1_at
	Similar to Cytoplasmic dynein 1 light intermediate chain 2 (Dynein light intermediate chain 2, cytosolic) (LIC53/55) (LIC-2)	LOC519789	1.6	Bt.22658.1.A1_at
	endosulfine alpha	ENSA	1.5	Bt.1710.1.S1_s_at

	latent transforming growth factor beta binding protein 3	LTBP3	1.5	Bt.5011.1.S2_at
	brain protein I3	BRI3	1.5	Bt.12436.1.S1_at
	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	CCRN4L	1.5	Bt.27843.1.S1_at
	AHNAK nucleoprotein 2	AHNAK2	1.5	Bt.25793.1.S1_at
	Rab9 effector protein with kelch motifs	RABEPK	1.5	Bt.20961.2.S1_at
	FGGY carbohydrate kinase domain containing	FGGY	1.5	Bt.24969.1.S1_at
	Golgi membrane protein 1	GOLM1	1.5	Bt.5523.1.S1_at
	carboxymethylenebutenolidase homolog (Pseudomonas)	CMBL	1.5	Bt.3203.1.S1_at
	renalase, FAD-dependent amine oxidase	RNLS	1.5	Bt.27736.1.S1_at
	DTW domain containing 1	DTWD1	1.5	Bt.27055.1.S1_at
	similar to dapper 1	LOC538778	1.5	Bt.2726.2.S1_at
	trimethylguanosine synthase homolog (S. cerevisiae)	TGS1	1.5	Bt.9299.2.S1_at
	cystatin B (stefin B)	CSTB	1.5	Bt.24354.1.S1_at
	mitochondrial ribosomal protein S33	MRPS33	1.5	Bt.22772.2.S1_at
	family with sequence similarity 82, member B	FAM82B	1.5	Bt.13819.2.A1_a_at
	osteoglycin	OGN	1.5	Bt.5341.1.S1_at
	coiled-coil domain containing 90A	CCDC90A	1.5	Bt.6391.1.A1_at
	cirrhosis, autosomal recessive 1A (cirhin)	CIRH1A	1.5	Bt.24879.1.A1_at
	synemin, intermediate filament protein	SYNM	1.5	Bt.9424.1.S1_at
	EF-hand domain family, member D2	EFHD2	1.5	Bt.15561.1.S1_at
	Similar to MAM domain containing 2	LOC539199	1.5	Bt.25769.1.A1_at
	GPN-loop GTPase 1	GPN1	1.5	Bt.13501.3.S1_at
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	DDX41	1.5	Bt.5258.1.S1_at
	transmembrane 4 L six family member 1	TM4SF1	1.5	Bt.6087.2.S1_a_at

Fold change in bold: up-regulation; not in bold: down-regulation

Appendix 7 Nitrogen Metabolism Pathway



Red: Molecule that is upregulated (ie. has a positive (+) expression value) and whose expression value meets the user defined cutoff. Green: Molecule that is downregulated (ie. has a negative (-) expression value) and whose expression value meets the user defined cutoff. Gray: Molecule neither up nor down-regulated or does not meet the user-defined cut off. White: Molecule that is not user specified, but incorporated into the network through relationships with other molecules. Molecule color intensity: The intensity of green and red molecule colors indicates the degree of down or upregulation, respectively. A greater intensity of green represents a higher degree of downregulation, and a greater intensity of red represents a higher degree of upregulation.