Studies on the Function of the NGFIB Subfamily of Nuclear Receptors in Adipose Tissue

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Abstract

The NGFIB family of nuclear receptors consists of three members: Nurr1, nur77 and NOR-1. Genes from this family are classified as immediate early genes in that they are rapidly and transiently induced by a variety of stimuli, including neurotransmitters, growth factors, cAMP and fatty acids. Although identified as having no putative ligand binding domain, regulation of these nuclear receptors may be at the level of phosphorylation or RXR heterodimerisation. Nurr1 and nur77 are expressed in adipose tissue at levels greater than in liver, spleen, lung and kidney, in both human and mouse tissue. During adipogenesis in both 3T3-L1 cells and primary sub-cutaneous murine preadipocytes, there is induction of Nurr1 and nur77 expression between 60 and 120 minutes after which expression subsides. Using different protocols to induce differentiation it was demonstrated that isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor which elevates intracellular cAMP, was a critical requirement for inducing the transient Nurr1 expression as well as adipogenesis. In order to investigate the effect of Nurr1 expression in adipogenesis, an adenovirus mediated over-expression system was employed. Over-expression of Nurr1 resulted in a decrease in adipogenesis, as well as decreases in the expression of PPARγ and C/EBPα. Examination of adipocyte morphology illustrated that cells over-expressing Nurr1 had less lipid accumulation, as assessed by Oil red O and by confocal microscopy. Furthermore mRNA analysis of adipocyte markers showed reduced levels of Hormone Sensitive Lipase (HSL), CD36 and Perilipin expression. In addition, the effect on adipocyte function, namely lipolysis and glucose uptake, was also investigated. Results suggest that Nurr1 does not significantly alter glucose uptake but does elevate lipolysis. Subsequent to the findings above, microarray analysis has been performed on control and Nurr1 over-expressing 3T3-L1 cells at two time points (72hrs and 192hrs), post differentiation. Analysis of the microarray data confirmed the earlier findings on adipogenesis and has identified a number of additional pathways, in which Nurr1 would appear to play a role, namely – NO pathway, steroidogenesis and cytokine/prostaglandin regulation. Preliminary work has been performed with siRNA to knock-down the gene

expression of Nurr1 in order to investigate potential consequences on adipogenesis. In conclusion, a supposed role for Nurr1 has been demonstrated in adipogenesis and lipid accumulation and also adipocyte secretory and metabolic function, with implications for insulin resistance at the adipocyte level.

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ACTH	Adrenocorticotrophic hormone
ADD1/SREBP1c	Adipocyte determination and differentiation-dependent factor/sterol regulatory element binding protein 1c
ADH2	Alcohol dehydrogenase 2
AF-1/2	Activation function-1/2
AhR	Arylhydrocarbon receptor
AMPKγ3	Adenosine monophosphate kinase γ3
BAT	Brown adipose tissue
C/EBP	CAAT/Enhancer binding protein
COUP TF	COUP transcription factor
CPM	Counts per minute
CPT1	Carnitine palmitoyltransferase
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	corticotrophin releasing hormone
Cyp11B2	Cytochrome P450 11B2
Cyp21	Cytochrome P450 21
DA	Dopamine
DBD	DNA binding domain
Dex	Dexamethasone
DHR38	Drosophila homolog receptor 38
DR5	Direct repeat 5
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinase 1/2
FBS	Foetal bovine serum
FXR	Faresnoid X receptor
GFP	Green fluorescent protein
GH	Growth hormone
GLUT1/4	Glucose transporter 1/4

GSK	GlaxoSmithKline
HPA	Hypothalamic pituitary adrenal
HRE	Hormone response element
HSD3B2	3β-hydroxysteroid dehydrogenase type 2
HSL	Hormone Sensitive Lipase
IEG	Immediate early gene
IL	Interleukin
IMBX	Isobutylmethylxanthine
IRS-1	Insulin receptor substrate-1
KLF	Krüppel-Like factor
LBD	Ligand binding domain
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
MSK1	Mitogen- and stress-activated protein kinase
NBRE	NGFIB response element
NGFIB	Nerve growth factor induced -B
nGRE	Negative glucocorticoid receptor response element
NR	Nuclear receptor
NurRE	nur77 response element
Ocn	Osteocalcin
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PGC1	PPAR gamma co-activator 1
PGE2	Prostaglandin E2
PGFα2	Prostaglandin Fα2
PI3K	Phosphatidylinositol-3 kinase

PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
Pref-1	Preadipocyte factor 1
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PVN	Paraventricular nucleus
RA	Retinoic acid
RANTES	Regulated upon activation normal T cell expressed and secreted
RAR	Retinoic acid receptor
RELMα	Resistin-like molecule α
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
TH	Thyroid hormone
TMRE	Tetramethylrhodamineethylester
TNFα	Tumor necrosis factor α
NEFA	Non esterified fatty acids
UCP	Uncoupling protein
VDR	Vitamin D receptor
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue

Chapter 1 - Introduction and Literature Review

What is a Nuclear Receptor?

The study of the members of the nuclear receptor family, their cellular distribution and role(s) is an exciting and novel area of research. Many excellent reviews of their regulatory role in both central and peripheral (metabolic and inflammatory) functions are available. A selection of these reviews have been summarised to best portray the broad scope of their regulatory effects (Aranda 2001, Evans 1988, Mangelsdorf 1995, Truss 1993, Tenbaum 1997, Dean 1996, Beato 2000, Thompson 2003, Robinson-Rechavi 2003).

The nuclear receptors (NR) comprise a large family of proteins that regulate gene transcription and mediate expression responsible for the biological effects in response to NR activation. This superfamily, consisting of over 150 members across multiple species, includes classic endocrine hormone receptors, such as those for estrogens, androgens, glucocorticoids, T3/T4 thyroid hormones, retinoids and Vitamin D3 (Aprilletti 1998, Beato 2000, Hager 2001, Mangelsdorf 1995, Zhang 2000). There are also those that respond to intermediates of lipid metabolism, e.g. peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR) and farnesoid X receptor (FXR) (Hihi 2002, Waxman 1999, Kliewer 1999, Repa 1999). And finally there are those, for which to date no ligand has been identified eg. COUP-TF's and NGFIB's (Pereira 2000, Maruyama 1998). For these receptors there may be an undiscovered ligand or there is also the possibility that at some stage in evolution they have lost their ability to bind ligand and now are regulated by some other means – phosphorylation, for example (Privlasky 2004). The last two decades has seen a total of 48 human nuclear receptors cloned and sequence comparison has illustrated these receptors exhibit an extensive homology in both structure and function.

Ligands have been identified for many of the orphan receptors and it is now known that compounds such as fatty acids, leukotrienes, prostaglandins and cholesterol derivatives can regulate gene expression through certain nuclear receptors and many of these ligands are produced intracellularly as metabolic products.

Nuclear receptor domain structure

A typical nuclear receptor consists of:

1. A variable NH_2 –terminal region, A/B, variable in sequence and length.
2. A conserved DNA-binding domain (DBD), region C
3. A linker hinge region D
4. A conserved E region that contains the ligand-binding domain (LBD)
5. Some receptors contain a COOH-terminal extension (region F) of unknown function.

These receptors also include regions required for transcriptional activation which are referred to as Activation Function-1 (AF-1) and Activation Function-2 (AF-2). AF-1, located in the N-terminal A/B region, contributes to constitutive ligand-independent activation by certain receptors. AF-2 which is located at in the E region/LBD is ligand dependent and conserved among most members of the nuclear receptor superfamily, although it is not ligand dependent for orphans. (See Figure 1.1)

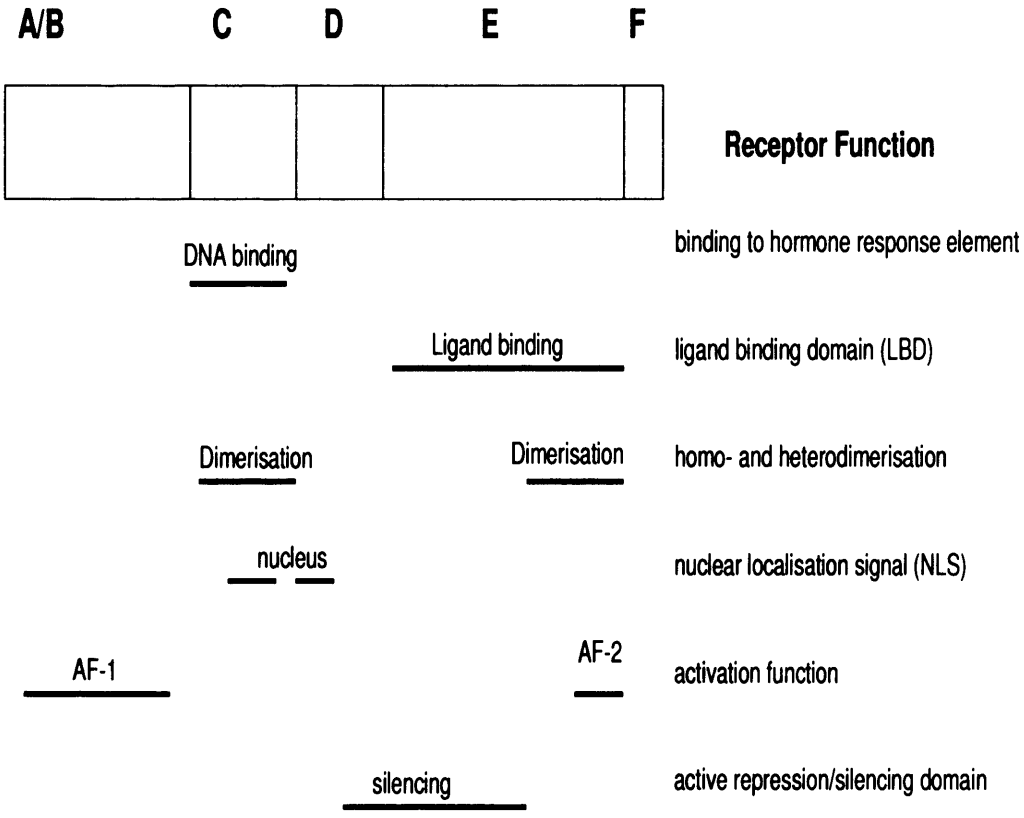


Fig. 1.1. - Schematic representation of nuclear receptor structure

Reproduced from S.Tenbaum & A Baniahmad; (1997) Int J Biochem Cell Biol 29:1325-41.

1. The A/B region

The N-terminal A/B region harbours one or more autonomous transcriptional activation function (AF-1), which when linked to a heterologous DNA binding domain, can activate transcription in a constitutive manner. In some cases, multiple receptor isoforms are generated by alternative splicing of the A/B region e.g. TRβ1 and TRβ2 (Williams 2000, Murata 1998). This domain can also contribute to the cell specific activity, possibly by interacting through cell type-specific factors (Dowhan 1996). This region is very weakly conserved in evolutionary terms.

2. The DNA binding domain (DBD)

The DBD is the most conserved domain of nuclear receptors and confers the ability to recognise specific target DNA sequences, known as response elements, and activate genes. This domain consists of 60-70 amino acids containing 2 zinc fingers and a COOH-terminal extension. The region contains nine cysteines, as well as other residues which are conserved across the nuclear receptor superfamily, which are required for high affinity DNA binding. In each zinc finger, four of the cysteines form one tetrahedrical zinc ion and both fingers fold together to form a compact interdependent structure (Berg 1989). Within the zinc finger motif are several sequence elements, termed P-, D-, T- and A-Boxes, which either define or contribute to: 1) response element specificity, 2) the dimerisation interface within the DBD and 3) contacts with the DNA backbone and residues flanking the core DNA recognition sequences respectively. The actual core of the DBD contains 2 α-helices - the first one at the beginning of the third conserved cysteine, which binds the major groove of DNA, making contacts with specific bases and the second spans the carboxy terminus of the second zinc finger.

3. The hinge region

This region is very poorly conserved between the nuclear receptor sub-families and serves as a hinge between the DBD and LBD. It is thought to allow rotation of the DBD in relation to other regions and may help dimerisation. This domain has also been shown to contain nuclear localisation signals for certain receptors.

4. The ligand binding domain (LBD)

As the name suggests this domain is responsible for the binding of ligands. However, this domain has many functions in addition to ligand binding. It can mediate homo- and heterodimerisation, interactions with heat shock proteins, ligand–dependent recruitment of co-activators or co-repressors which results in transcriptional activation or transcriptional repression respectively. The LBD contains 2 conserved regions - "a signature motif" and the carboxy terminal AF-2 motif, responsible for the ligand dependent transcriptional activation (Danielian 1992). Many of the nuclear receptor family crystal structures have been solved and the findings show that the receptors share a very similar structure. The region consists of 12 α-helical regions, termed H1-12, folded into a three-layered antiparallel helical sandwich. A central core layer of three helices is packed between two additional layers to form a cavity, the ligand binding pocket, which is mainly hydrophobic and the size of the pocket varies widely among the receptors. Helix 12 of the LBD contains the AF-2 domain and the position of which changes upon ligand binding, making the structure more compact.

5. COOH-terminal

This region displays little evolutionary conservation. Recent evidence suggests that the F region may play a role in co-activator recruitment to the E domain and in determining the specificity of the ligand binding domain coactivator interface (Pters 1999, Sladek 1999).

Hormone response elements

Nuclear receptors regulate transcription by binding to derivatives of the same hexameric DNA core motif, 5'-PuGGTCA (Pu = A or G), known as hormone response elements (HREs). These HREs are usually located in the 5'-flanking region of the target gene. In most cases they are found relatively close to the core promoter but can in some cases be located in enhancer regions several kilobases upstream of the transcription start site. Nuclear receptors can bind their response element as monomers, but most tend to bind as either homo- or heterodimers with RXR to a DNA sequence comprising typically of two core hexameric motifs.

These motifs can be configured as palindromes, inverted palindromes or as direct repeats. The factors which determine the specificity of the HRE are:

- The space between the half-sites.
- Some receptors use the same half site sequence but different spacing.
- The sequence of the half site.

The concept of the response element was initially suggested by studies on mouse mammary tumour virus (MMTV) and glucocorticoid receptor with research on thyroid hormone and retinoic acid response elements, providing additional evidence (Koenig 1987, Ringold 1979, Leid 1992). This was later developed into a model in which the HRE's for the vitamin D receptor (VDR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR) are composed of direct repeats spaced by 3, 4 or 5 nucleotides (i.e. DR3, DR4 and DR5 respectively) (Umesono 1991). An example of the variety of response elements is shown in Figure 1.2.
Although most of the discussion described here focuses on transcriptional activation, NRs do have the ability to repress gene expression in a ligand-dependent manner. Transrepression can be brought about by negative response elements, such as the nGRE in the POMC promoter (Davis 1994), transcriptional antagonism e.g. COUP-TF's (Tsai 1997) or by cross-talk with different signalling pathways such as AP-1 and NfκB (Pfahl 1993, Wissink 1997).

Fig. 1.2 - Variety of response elements arrangements

Arrangement of Half-Sites

Palindrome:

5'-AGGTCA /TGACCT-3'

Direct Repeat

5'-AGGTCA(n)$_x$AGGTCA-3'

Inverted Repeat

5'-TGACCT(n)$_x$AGGTCA-3'

Half-Site Structure

AGGTCAnAGGTCA	-DR1	PPRE, RXRE	
AGGTCAnnAGGTCA	-DR2	RARE	
AGGTCAnnnAGGTCA	-DR3	VDRE	*Different spacing*
AGGTCAnnnnAGGTCA	-DR4	TRE	
AGGTCAnnnnnAGGTCA	-DR5	RARE	
AGAACAnnnTGTTCT		GRE, PRE, ARE, MRE	
AGGTCAnnnTGACCT		ERE	*Same spacing different half-sites*
*AA*AGGTCA		NBRE	
*T/CCA*AGGTCA		SF-1 RE	*Single Half sites*

Co-activators and Co-repressors

Regulation of gene transcription by nuclear receptors requires the recruitment of proteins characterised as coregulators, with ligand dependent exchange of co-repressors for co-activators serving as the basic mechanism for switching gene repression to activation, involving modulation of the chromatin structure. These proteins do not bind DNA directly but enable the recruitment of the basic transcriptional machinery to nuclear receptor regulated genes (Torchia 1998). Co-activators and co-repressors have opposing effects on histone acetylation, with activators associated with histone acetylation, whilst repressors mediate histone deacetylation.

Ligand binding is the crucial molecular event that switches the function of a number of NR's from active repression to transcriptional activation. However, ligand binding is not necessarily sufficient to induce dissociation of co-repressors; in fact it would appear the AF-2 domain serves to trigger the release. In some cases helix 12 containing the AF-2 domain is fully inhibitory for co-repressor binding for NR's. NR's lacking this AF-2 act as constitutive repressors e.g. RevErbA Alpha (Sap 1989).

The NGFIB subfamily of nuclear receptor

The NGFIB subfamily in higher eukaryotes comprises of 3 closely related members – Fig. 1.3. nur77 was the first to be identified, as a gene induced by nerve-growth factor (NGF) in the rat PC12 cell line (Milbrandt 1988, Hazel 1988). Nurr1 was subsequently isolated from a neonatal mouse brain cDNA library, using low hybridisation conditions with a probe consisting of the DNA-binding domain (DBD) of COUP-TF1, another orphan nuclear receptor (Law 1992). And finally, NOR-1 was identified from cultured rat fetal forebrain cells (Ohkura 1994). Genes from this family are classified as immediate early genes in that they are all rapidly and transiently induced by a variety of stimuli, including neurotransmitters (Arender 1989), growth factors (Milbrandt 1988, Bartel 1989), cAMP (Kovalovsky 2002) and fatty acids (Arender 1989, Bartel 1989). This family have also been shown to function as constitutive activators *in vitro* (Davis 1994, Wilson 1991). These 3 receptors comprise the NGFIB subfamily and are known by many other names – see figure 1.4.

As described earlier, these receptors consist of a central DBD, a potential LBD and a variable N-terminus. The DBD contains 2 highly conserved zinc fingers that bring about the specific interaction between the receptor and DNA response elements. The LBD is essential for hormone/ligand recognition; however as to date no ligand has been discovered for a member of this family. In fact crystallography studies of Nurr1 suggest that the ligand-binding pocket is too small to accommodate the docking of a ligand. Studies have shown a pocket of approximately 100Å^3, which when compared to other nuclear receptors is extremely small (Wang 2003). For example, the pockets of the PPAR nuclear receptors range from 1100Å-1600Å^3 (Nolte 1998). The LBD region of the NGFIB subfamily has also been shown to contain bulky hydrophobic regions, involved in dimerisation, which fill the pocket. These hydrophobic residues are conserved throughout the family so the absence of a ligand-binding pocket was also predicted for nur77 and NOR-1. Recent data certainly supports this, as the LBD crystal structure for rat nur77 is indeed filled with bulky hydrophobic side

chains (Flaig 2005). DHR38, the drosophila orthologue for NOR-1, also revealed no ligand binding pocket (Baker 2003).

Within the NGFIB subfamily itself, the DBD exhibits more than 90% homology between the 3 members. In relation to other members of the nuclear receptor superfamily at least 50% identity is maintained. The C-terminus of all the NGFIB subfamily exhibits 50-60% homology.

These nuclear receptors can bind as heterodimers with RXR (Perlmann 1995) (except NOR-1) to direct repeat elements (AGGTCA) spaced by 5 nucleotides (DR5), or as monomers to the extended half-site DNA sequence, termed the NGFIB response element (NBRE 5'-AAAGGTCA-3')(Wilson 1991, Perlmann 1995). Recognition of the additional adenine residues depends on the A-Box in the DBD. It has also been reported that homodimers of nur77 can bind to a novel response element termed the NurRE, which was identified within the POMC promoter. The NurRE has a palindromic structure containing 2 half sites separated by 6bp, which are quite similar to the NBRE (75%). This response element is nearly 50x more sensitive to Nur77 action than the NBRE (Philips 1997).

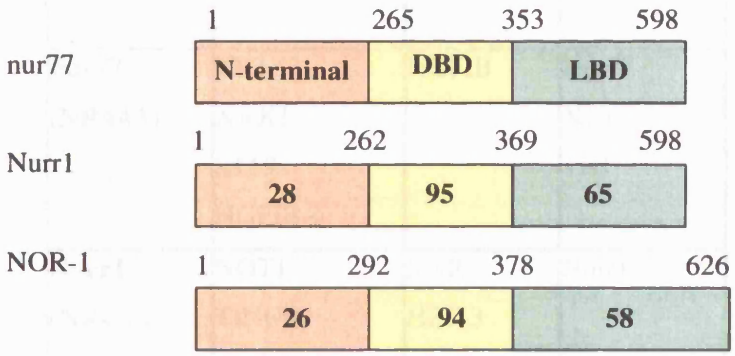


Fig. 1.3 NGFIB family homology

Box diagram showing homology of human family members. Numbers in each box represent %age homology. Numbers above diagrams represent the amino acid residue of each domain.

	Human	Rat	Mouse
nur77 **(NR4A1)**	TR3 NAK1 ST59 NGFIB-α	NGFIB	Nur77 N10 Tis1
Nurr1 **(NR4A2)**	NOT1 TINUR NGFIB-β	RNR HZF-3	Nurr1
NOR-1 **(NR4A3)**	Minor TEC NGFIB-γ	NOR-1 NOR-2	

Fig. 1.4 NGFIB family nomenclature

Species nomenclature for all three members of the NGFIB family.

Regulation of the NGFIB family

NGFIB and phosphorylation

As stated previously, based on the crystal structure there is no apparent ligand binding pocket for any of the NGFIB subfamily (Wang 2003). It has been suggested that NGFIB activity could be regulated through phosphorylation as there are multiple phosphorylation sites in the N-terminus of the receptor. There are no apparent sites contained within the C-terminus. Nurr1 has an unusually large N-terminal domain which has been shown to mediate transcriptional activation as well as cell type specific activity through the AF2 region in the C-terminal domain (Castro 1999).

Phosphorylation of NRs in general has been shown to happen in all three major domains, the N-terminal activation function (AF-1), the ligand binding and the DNA binding domains. A recent review provides an overview of the role of phosphorylation in nuclear receptor activity (Rochette-Egly 2003). DNA binding, protein stability and regulation of activation/repression have all been shown to be factors that are influenced by the phosphorylation state of the nuclear receptor (Weigel 1996). Phosphorylation occurs mainly through serine/threonine (Sheridan 1989) and tyrosine residues (Migliaccio 1986) in nuclear receptors. Potential phosphorylation sites (Fig 1.5) in the N-terminal domain of Nurr1 reveals a consensus site for serine/threonine mitogen-activated protein kinases (MAPK) in a region conserved between Nurr1, nur77 and NOR-1.

Fig. 1.5 Potential MAPK consensus region (amino acids refer to Nurr1)

	124	133
Nurr1	**PSSPPTPSTP**	
nur77	**PCSAPSPPTP**	
Nor1	**QSPPSTPTTP**	
Consensus	**PXS/TP**	

It has been suggested that Nurr1 AF-1 activity is in part dependent on a MAPK phosphorylation site (Nordzell 2004). Also of interest, in nur77 MAPK

dependent phosphorylation within the DBD has been shown to negatively regulate DNA binding and activity (Davis 1997, Fahrner 1990, Hazel 1991, Hirata 1993, Katagiri 1997, Li 1997). This region is highly conserved between Nurr1 and nur77 and it is likely that a similar inhibitory effect may also be significant in Nurr1. Thus, MAPK signalling pathways may impart both positive (AF-1) and negative (inhibition of DNA binding) influences on the transcriptional activity of Nurr1 (Nordzell 2004).

More recently it has been demonstrated that downstream of ERK1/2, MSK1 and MSK2 are involved in the transcriptional activation of this family (Darragh 2005). MSKs have previously been shown to activate transcription of genes with CRE's, suggesting a possible control mechanism for this family. Nurr1 and NOR1 have one or more classical CRE sites in their promoters and are reported to be CREB dependent genes (Ohkubo 2000, McEvoy 2002). In contrast nur77 does not appear to have a CRE in its promoter, although it does have two AP-1 like elements, which have sequences similar to CRE and have been reported to bind members of the CREB family of transcription factors (Fass 2003).

NGFIB and heterodimerisation with RXR

As described earlier, Nurr1 and nur77 have been shown to bind to a DR5 element as a heterodimer with RXR, which has been classified as a promiscuous dimerisation partner (Schulman 1997) and also described as a "master regulator" (Mangelsdorf 1995). Three types of RXR heterodimers exist (Kastner 1995, Mangelsdorf 1995). In some heterodimers, such as RXR/VDR, RXR is a completely silent partner. In others such as RXR/RAR, RXR is a conditionally silent partner (formation of the heterodimer prevents binding of RXR ligand). However, in contrast RXR acts as a fully active and competent partner of heterodimers with orphan receptors such as nur77 and Nurr1. The nur77/Nurr1-RXR heterodimer is permissive to ligand binding such that they can be activated in response to RXR ligand. Perhaps surprisingly, it has been shown that NOR-1 does not have the ability to form stable heterodimers with RXR (Zetterstrom 1996). NOR-1 can however interfere with Nurr1-RXR heterodimer signalling as

described in Zetterstrom at al. It is unclear as to why NOR-1 fails to heterodimerise with RXR. One theory is that it lacks three critical amino acids in helix 9, which have been identified as being involved in the dimer interface in the crystal structure of RXR ligand binding domain homodimer (Bourguet 1995).

It is known that nur77 and Nurr1 heterodimerise with RXR through the C-terminal domain as this region has been shown to bind to RXR when linked to the Gal4 DBD binding domain in a mammalian two-hybrid experiment (Perlmann 1995). The recent discovery that Nurr1 has a collapsed ligand binding pocket is very interesting in the RXR heterodimerisation story (Wang 2003). It is possible that Nurr1/nur77 are regulated through the RXR partner and its ligands. There is evidence to suggest that in primary neuronal cultures, RXR ligands increase the number of surviving dopamine cells and that this requires ligand binding to RXR in the context of Nurr1-RXR heterodimers (Wallen-Mackenzie 2003). Another recent paper demonstrates that the effects of RXR agonists and antagonists are dependent on the presence of nur77, since these effects are not observed in nur77 deficient mice (Ethier 2004).

Pharmacological modulation of NGFIB

Despite the evidence accumulating that this family are likely to be ligand-independent nuclear receptors, recent literature supports the discovery of activators or modulators for this family. These include 6-mercaptopurine, 1,1-Bis(3'-indoyl)-1-(p-substituted phenyl)methanes, methotrexate and PGA2. Although the authors show activation predicted to be through the LBD, it is possible that the NGFIB's are modulated by these compounds by some other means in addition to direct interaction with the ligand binding pocket. For example this family has been shown to be modulated through adenosine receptors or through the AF-1 domain, via an indirect mechanism which in turn mediates transactivation, co-activator recruitment and activation by metabolites such as 6-mercaptopurine (Wansa 2003, Chintharlapalli 2005, Ralph 2005, Kagaya 2005).

Functions of the NGFIB family

The expression of the NGFIB family, (nur77, Nurr1 and NOR-1) are far more ubiquitous than originally thought and their function(s) may, to some extent, be determined by the cell in which they are expressed and the prevailing physiological and pathphysiological milieu.

NGFIB and dopamine signalling

Initial reports show that Nurr1 was expressed predominantly in the dopaminergic neurons in the brain. The onset of this expression is just before E10.5, prior to the appearance of the dopaminergic marker enzyme tyrosine hydroxylase (TH) at E11.5. TH is the rate-limiting enzyme for the biosynthesis of dopamine. Nurr1 expression continues through normal adulthood suggesting a requirement for normal function of mature dopaminergic neurons (Ethier 2004).

Using Nurr1 null mice, it has been shown that Nurr1 is essential for the differentiation of ventral mesencephalic dopaminergic neurons, which can be observed via the agenesis of cell markers such as TH, the retinoic acid converting enzyme ADH2 and the receptor tyrosine kinase, c-ret (Le 1999, Castillo 1998, Saucedo-Cardenas 1998). The Nurr1 homozygote knock-out is embryonic lethal (Zetterstrom 1997).

The dopaminergic neurons in Nurr1 null mice adopt a normal ventral localisation and neuronal phenotype characterised by the expression of the homeobox domain protein Ptx-3 at E11.5. However, these dopaminergic precursor cells fail to differentiate and commit to the dopaminergic phenotype (Le 1999, Castillo 1998, Saucedo-Cardenas 1998).

It has been established that both Nurr1 and retinoids can induce cell cycle arrest and morphological differentiation of MN9D cells. MN9D cells show properties of immature neuronal cells that can mature and extend neurites under certain culturing conditions. They also have characteristic dopaminergic properties, but do not express Nurr1. By over-expressing Nurr1 in these cells the authors show how it can influence basic dopaminergic functions such as dopamine content and production. This mechanism is dependent on DNA binding but does not require dimerisation with RXR (Castro 2001, Hermanson 2003).

Some reports indicate that Nurr1 contributes to the maintenance of mature DA neurons. Consequently DA neurons of Nurr1 heterozygous mice are more vulnerable to dopaminergic toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Le 1999). Recent studies have demonstrated that nitric oxide mediates this increased susceptibility (Imam 2005). Abnormal motor behaviours in aged Nurr1 heterozygous mice (>15 months) were associated with decreased DA levels, a decrease in DAergic neurons and reduced expression of Nurr1 and DA, suggesting a motor impairment that is analogous to a Parkinsonian deficit (Jiang 2005). Mutations in Nurr1 have also been identified in familial cases of Parkinson's disease. These mutations are situated in the 5' untranslated region of Nurr1 and have been shown to decrease the expression of Nurr1, providing further compelling evidence for a clinically relevant role of Nurr1 in human adult brain (Le 2003).

NGFIB and their role in bone

Nurr1 and the other family members are expressed in primary osteoblasts and cell lines. Their expression has been shown to be significantly induced in response to parathyroid hormone (PTH), with NOR-1 induction more sensitive to PTH treatment (Tetradis 2001a). As expected for immediate early genes this induction is rapid and transient, with nur77 mRNA and protein levels peaking earlier and declining faster than Nurr1 (Tetradis 2001b). This induction is evident in both MC3T3-E1 cells and also in mouse calvarie organ cultures (Tetradis 2001a). The PTH effect is mediated mainly through cAMP PKA signalling (Tetradis 2001a, Pirih 2003). Tetradis et al have shown that Nurr1 can be induced by a number of agents in osteoblasts as well as the cAMP PKA signalling previously reported. The strongest inducers were activators of the cAMP-PKA-coupled pathway (forskolin, PTH, PTHrP & PGE2) followed by PKC- and calcium-coupled signalling activators (PMA, ionomycin, PGF2α & fluprosentol) (Tetradis 2001a, Pirih 2003, Pirih 2004). New data also support these findings as they show induction of the NGFIB family by PTH *in vivo*. mRNA levels of all three genes

peaked earlier and were more transient *in vivo* compared to those reported in *in vitro* experiments (Pirih 2005).

Genes with NGFIB response elements in their promoter/enhancer regions include alkaline phosphatase, bone sialoprotein, osteocalcin, type I (a)1 and type I (a)2 collagen, insulin growth factor, collagenase, mouse osteopontin(OPN) and osteocalcin(Ocn) (Tetradis 2001a, Pirih 2003, Lammi 2004). Studies have shown binding of Nurr1 to the mouse osteopontin promoter as both a monomer and heterodimer (with RXR), and this transactivation is independent of RXR heterodimerisation, although RXR ligands have been shown to enhance transactivation induced through Nurr1 (Lammi 2004). In addition Lammi et al have reported that Nurr1 is involved in mediating PTH-induced OPN expression. OPN is not required for bone formation but it has been suggested to have an important role in bone remodelling in response to mechanical stress, such as postmenopausal osteoporosis (Rittling 1998, Yoshitake 1999, Terai 1999). Recently Pirih et al have also shown that Nurr1 can mediate transactivation through the rat Ocn promoter. The Nurr1 response element on the Ocn promoter appears to behave as a true monomeric site, suggesting the possibility that dimerisation (with RXR) is not required for osteoblastic gene transactivation (Pirih 2004).

The above observations are interesting from a functional point view as neither of these genes is required for normal skeletal development but more for skeletal homeostasis. Osteopontin ablation impairs bone remodelling following ovariectomy (Yoshitake 1999) and PTH treatment (Ihara 2001), while osteocalcin knockout mice develop osteopetrosis due to inapt skeletal remodelling (Ducy 1996). These genes are also expressed at different points of development indicating that Nurr1 is likely to regulate multiple genes along the osteoblastic lineage (Liu 2003). Therefore it could be hypothesised that Nurr1 may have a role in bone remodelling through the regulation of the OPN and Ocn promoter.

NGFIB and HPA axis

Another area where the NGFIB subfamily is proposed to play a role is in the regulation of the hypothalamic pituitary adrenal (HPA) axis. There are several lines of evidence to support this theory. The axis is regulated at the level of the hypothalamus by corticotrophin releasing hormone (CRH), which is synthesized in the hypothalamic paraventricular nucleus (PVN). Upon stimulation of the PVN, CRH is released and transported to the anterior pituitary where it causes an increase in the synthesis of pro-opiomelanocortin (POMC), which is the precursor to many molecules including adrenocorticotrophic hormone (ACTH). Other ACTH derived peptides include β-endorphin, β-lipotrophin, γ-lipotrophin and the melanin-stimulating hormones. ACTH is a primary inducer of adrenal steroidogenesis and the subsequent release of glucocorticoids, androgens and the mineralocorticoid aldosterone. In order to maintain homeostasis of the HPA axis, glucocorticoids inhibit CRH and POMC synthesis and secretion at the hypothalamic and anterior pituitary level.

The NGFIB family have been implicated at multiple levels in the regulation of the HPA axis – Fig. 1.7. This is partly evident from their expression in HPA tissues. Studies show expression of Nurr1 in the anterior pituitary (Saucedo-Cardenas 1996) and adrenal cortex (Davis 1994, Bassett 2004). Nurr1 is also constitutively expressed in the PVN (Saucedo-Cardenas 1996) and nur77 is rapidly induced by stress and Interleukin-1β in the PVN (Honakaniemi 1994, Chan 1993). Furthermore, administration of CRH to conscious rats significantly increases the level of nur77 expression in the PVN (Parkes 1993). In addition, nur77 has been shown to be expressed in both the adrenal cortex and medulla (Davis 1994). nur77 induction has also been implicated in the transcriptional induction of steroid-21α-hydroxylase, a rate limiting step in glucocorticoid synthesis (Wilson 1993, Kelly 2004).

An NBRE has been located in the POMC promoter which happens to overlap with a negative glucocorticoid receptor response element (nGRE) which is responsible for mediating the glucocorticoid negative feedback loop (Figure 1.6). It is thought that repression of POMC transcription is mediated in part by GR

dependent inhibition of NGFIB subfamily activation of the POMC promoter (Davis 1994). A second nur77 specific response element (NurRE) has also been located in the POMC promoter which contributes to activation of this promoter by nur77 (Lin 1996).

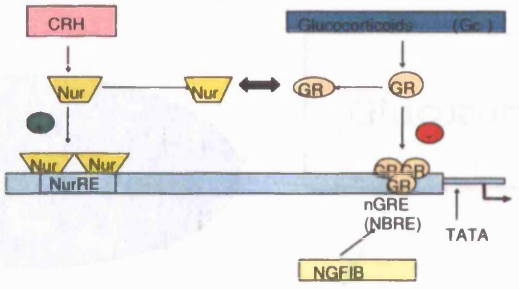


Fig. 1.6 Adapted from Drouin et al (1998) J Steroid Biochem Molec Biol 65:59-63.

Diagram showing NurRE and putative NBRE present in the POMC promoter.

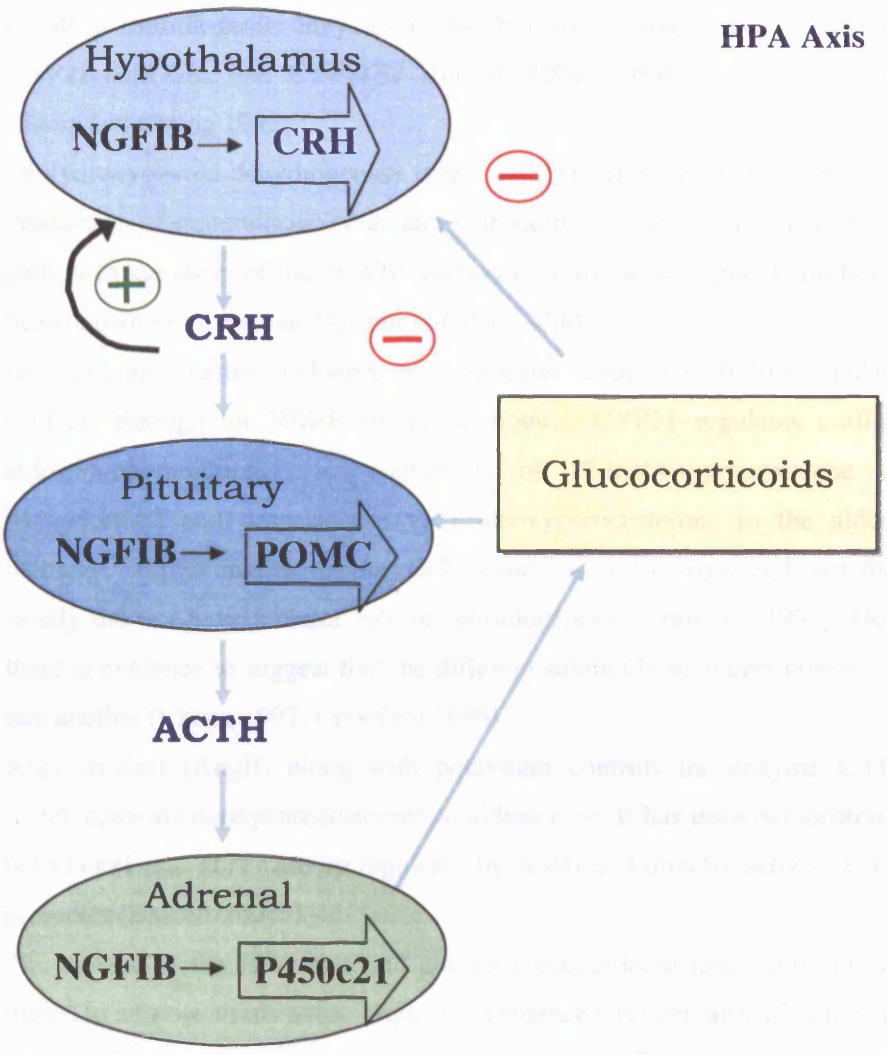


Fig. 1.7 NFGIB and HPA axis
Overview of the HPA axis and NGFIB family involvement

The NGFIB subfamily has also been implicated in the transcriptional regulation of other steroidogenic enzymes in the human adrenal gland. These include CYP21, HSD3B2 and CYP11B2 (Bassett 2004a, 2004b, 2004c, Wilson 1993, Chang and Chung 1995).

3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), an enzyme essential for the production of mineralocorticoids and glucocorticoids, shows a similar expression pattern to members of the NGFIB subfamily in the adrenal gland and has indeed been shown to be regulated by nur77 (Martin 2004).

As mentioned earlier, evidence also indicates a role for NGFIB regulation of CYP21, through an NBRE in its promoter. CYP21 regulates cortisol and aldosterone production by conversion of 17-hydroxyprogesterone to 11-deoxycortisol and progesterone to 11-deoxycorticosterone in the aldosterone pathway. Initial studies on the nur77 knock-out mice suggested that this sub-family did not have a major role in steroidogenesis (Crawford 1995). However, there is evidence to suggest that the different subfamily members compensate for one another (Cheng 1997, Crawford 1995).

AngiotensinII (AngII) along with potassium controls the enzyme CYP11B2, which converts deoxycorticosterone to aldosterone. It has been demonstrated that both Nurr1 and nur77 are up regulated by AngII and directly activate CYP11B2 promoter (Bassett 2004b).

The control of the HPA axis and plasma glucocorticoid levels are both closely linked to adipose tissue mass. Recently, evidence has been mounting to support a role for CRH in the regulation of energy balance (Richard 2002), including a direct influence on human adipocytes, regulation of 11β-hydroxysteroid dehydrogenase type 1 activity (Friedberg 2003) and cortisol formation (Masuzaki 2001). Expression of CRH receptors and CRH-like peptides has been demonstrated in adipose tissue (Seres 2004). This data, along with the recent publication describing the influence of CRH on adipocyte cortisol production (Friedberg 2003), suggests systemic and autocrine/paracrine effects of the CRH system in adipose tissue homeostasis.

NGFIB and inflammation

Previously it has been described how the NGFIB subfamily members are involved in mediating the CRH activation of POMC gene transcription. It has also been demonstrated that Nurr1 is involved in the regulation of CRH expression in joint inflammatory disease. Elevated levels of CRH are produced locally in inflamed synovial tissue (Crofford 1993). In addition enhanced expression of Nurr1 is evident in synovial fluid of subjects with rheumatoid arthritis compared with normal subjects (McEvoy 2001). This suggests that Nurr1 plays a role in transcriptional regulation of synovial fluid homeostasis. McEvoy et al have hypothesised that Nurr1 may represent a point of convergence of at least two signalling pathways involved in mediating inflammatory signals through cytokine induction of this nuclear receptor (McEvoy 2002).

The NGFIB family are also induced by inflammatory stimuli in activated macrophages. Evidence points to the NF-κB pathway as one of the principle regulators of this inducible family. In response to these inflammatory stimuli, NGFIB receptors may function as intracellular switches between transcriptional programs of activation, differentiation, or self-destruction of macrophages via interactions with the aforementioned NF-κB and glucocorticoid signalling. It is postulated that these receptors may be important mediators of inflammatory signalling in the artery wall (Pei 2005). TNFα, LPS and IL-1 induce nur77 which in turns regulates plasminogen activator inhibitor 1 (PAI-1) expression. PAI-1 regulates intravascular fibrinolysis and high plasma levels are associated with an increased risk for vascular disease (Gruber 2003).

NGFIB and other putative functional roles

Literature is accruing on the many putative functional physiological roles for this family of receptors. It has become apparent that they are involved in cell development, homeostasis and cell death as well as implications in a number of diseases – Parkinson's disease, cancer, atopic dermatitis, rheumatoid arthritis and other inflammatory diseases (Wu 2002, Ohkubo 2000, Kolluri 2003, Kagaya 2005, Murphy 2001, Pei 2005). A role has also been proposed for nur77 in

lipolysis in skeletal muscle cells – this will be further discussed in the next section. Collectively the functions described above would suggest a highly adaptive and multi-functional role for these receptors in human biology.

NGFIB and their putative role in adipogenesis

Obesity is a prevalent health hazard in industrialised countries and is closely associated with a number of pathological disorders including non-insulin-dependent diabetes, hypertension, cancer, gall bladder disease and atherosclerosis (Albu 1998, Bray 2003, Bray 2002, Dittrick 2005). A greater insight into the molecular basis of obesity, including that of the differentiation of the preadipocyte cell to an adipocyte (adipogenesis) would significantly increase our understanding of the aetiology of the disease. The extracellular and intracellular signalling pathways involved with the early events of adiposity differentiation and growth are unclear. However, much has been reported on the several transcription factors playing a major role in the regulation of adipogenesis, notably those of two families: CAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) (Cao 1991, Chawla 1994a, 1994b - these events will be discussed in greater detail in Chapter 3). Conversely, we know very little about transcription factors that inhibit adipogenesis. There are reports on factors such as Pref-1 and Wnt signalling (see Chapter 3) which are considered to inhibit adipogenesis. However, to date there is no reported evidence of transcriptional control of inhibition of adipogenesis.

Preliminary data produced in-house, prior to embarking on this project, suggested that the NGFIB family of orphan nuclear receptors may play a role in the regulation of adipogenesis. Each member of the family is highly expressed in human adipose tissue (Figure 1.8). There is also some data to suggest that it Nurr1 is induced transiently during differentiation of adipocytes (Figure 1.9). As cited earlier, the NGFIB family has been proposed to have a role regulating the HPA axis and CRH. CRH has also been proposed to play a role in adipose tissue homeostasis (Seres 2004). The role of the NGFIB family in the HPA axis and CRH regulation must also be considered in the context of adipogenesis. As

mentioned above - ACTH is a primary inducer of adrenal steroidogenesis and the subsequent release of glucocorticoids, androgens and the mineralocorticoid, aldosterone. Glucocorticoids play an important role in regulating adipose tissue function and recent work also predicts a role for MR/aldosterone in adipogenesis (Rondinone 1993, Caprio 2005). The NGFIB family are implicated at many levels with enzymatic control of the generation of these hormones.

Recently, nur77 has been implicated in the regulation of energy homeostasis by regulating lipolysis in skeletal muscle. It has been shown that nur77 can be induced by β-adrenergic agonists such as isoprenaline (Lim 1995, Maxwell 2005). Given that β-adrenergic agonists and receptors are involved in regulation of cold- and diet- induced thermogenesis and induce increases in energy expenditure, this would suggest cross-talk between the NGFIB family and the adrenergic pathways. Using siRNA for nur77 it was demonstrated that there was a down regulation of genes and proteins associated with energy expenditure and lipid homeostasis, AMPKγ3, UCP3, CD36, adiponectin receptor 2, GLUT4 and caveolin 3 (Maxwell 2005). These are examples of mRNAs encoding proteins involved in the regulation of energy expenditure, lipid and carbohydrate metabolism.

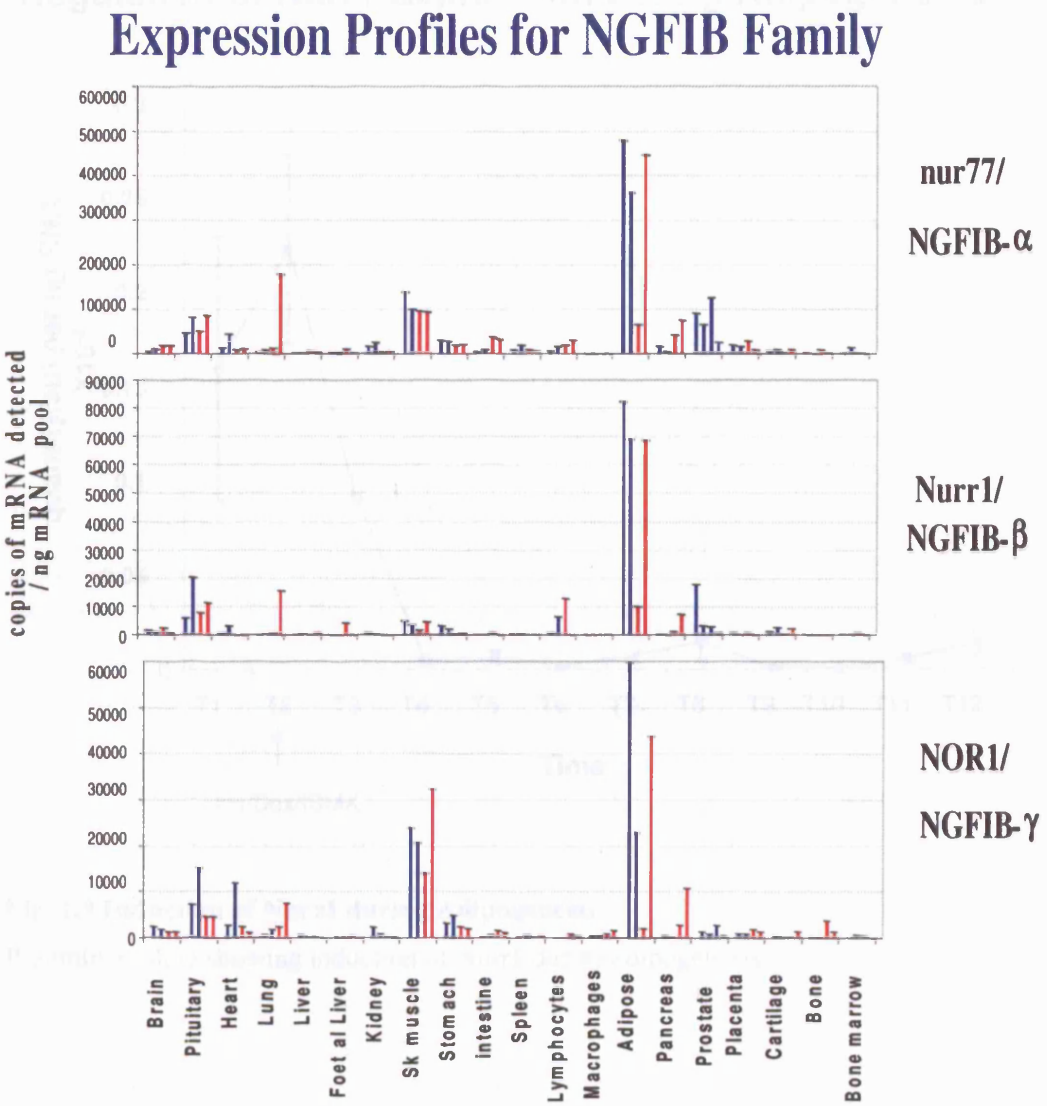


Fig. 1.8 Expression analysis of NGFIB family

TaqMan analysis of NGFIB expression in human tissues. 4 samples per tissue 2 male (blue) and 2 female (red).

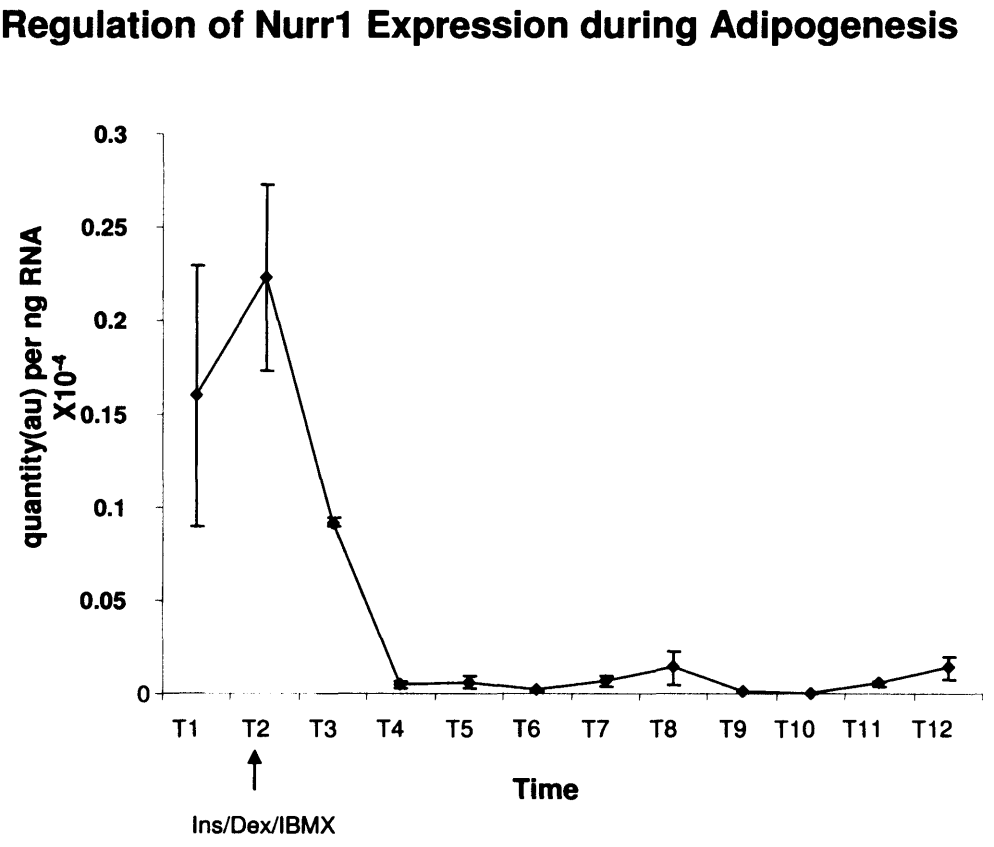


Fig. 1.9 Induction of Nurr1 during Adipogenesis

Preliminary data showing induction of Nurr1 during adipogenesis.

Aims of study

Given the known functions of NGFIB family and potential role in metabolic homeostasis this project aimed to investigate the effect of the NGFIB family on adipogenesis and adipocyte function.

The aims were as follows:

1. To confirm expression of Nurr1/nur77 in adipose tissue in mouse. The original expression data was obtained from human tissue and although no difference is expected, confirmation would be required.

2. To investigate the induction of Nurr1/nur77 during adipogenesis and try to elucidate the mechanism of induction. Differentiation cocktail to be added to 3T3-L1 cells and RNA to be collected at appropriate timed intervals. This study will also be repeated in a primary cell model.

3. To analyse the effects of adenoviral over-expression of Nurr1 in 3T3-L1 cells on the regulation of a number of adipogenic and adipocyte marker genes. mRNA will be isolated at various time points during the differentiation period and cDNA synthesised for SybrGreen analysis.

4. To analyse the effect of over-expression of Nurr1 on metabolic functions, lipolysis and glucose uptake in mature differentiated adipocytes.

5. To use microarray analysis of differentiating 3T3-L1 cells over–expressing Nurr1 to investigate changes in the expression of multiple genes. Cells will be differentiated as above (3) and RNA extracted at 72hrs (T72) and 192 hrs (T192) post-induction of differentiation. Microarray to be performed at The Institute of Child Health, London.

6. To analyse the outcome of down-regulating the expression of Nurr1 and nur77 during the differentiation period in 3T3-L1 cells using siRNA technology. siRNA's for Nurr1 and nur77 are commercially available.

This thesis describes the expression of Nurr1 (and to a lesser extent nur77) and its effect(s) on the differentiation of 3T3-L1 adipocytes in vitro. It also describes the

induction of Nurr1/nur77 in 3T3-L1 and primary adipocytes and possible mechanisms of induction. The effects of over-expression are then analysed by SybrGreen analysis, microarray studies, investigation of lipolysis and glucose uptake. Finally, a small section describes some initial gene silencing studies of the NGFIB family using siRNA tools.

These results should throw some light on the putative physiological role(s) for this novel family of transcription factors in adipocyte and adipose tissue function, as well as the consequences of their overexpression or down-regulation in these cells.

Chapter 2: Expression of NGFIB subfamily in murine adipose tissue and during adipogenesis

Introduction

Adipose Tissue

Traditionally, adipose tissue was thought of as nothing more than a passive reservoir for energy storage, however recent and ongoing research has shown adipose tissue to be a complex highly active metabolic, immune and endocrine organ. Siiteri first identified adipose tissue as a major site for the metabolism of sex steroids (Siiteri 1987). The subsequent identification and characterisation of leptin in 1994 (Zhang 1994) established adipose tissue as an endocrine system expressing and secreting signals (adipokines) that regulate a myriad of physiological functions. These adipokines are capable of endocrine, paracrine and/or autocrine effects. In addition adipose tissue expresses numerous receptors allowing it to respond to the afferent signals from traditional hormone systems as well as the central nervous system (Mohamed-Ali 1998, Ailhaud 2000, Fruhbeck 2001, Ahima 2000, Frayn 2003, Kershaw 2004).

Adipocytes are now thought to account for about 50% of the cellular make up of the tissue; it also contains connective tissue matrix, nerve tissue, stromal vascular cells and immune cells which all function together as an integrative unit (Frayn 2003). In addition to its endocrine function, adipose tissue provides thermal insulation, which is evident in many large marine animals, and protection for many body organs.

There are two types of adipose tissue, distinguished histologically and functionally: brown adipose tissue (BAT) and white adipose tissue (WAT) (Cinti 2000, Cinti 2001, Frayn 2004). They both contain the triacylglycerol storage unit of adipocytes. BAT is distinguished from WAT macroscopically by its brown appearance, which reflects both increased vascularisation and its greater mitochondrial mass. In contrast, WAT typically has adipocytes with a single large lipid vacuole, unilocular, as opposed to the multilocular adipocytes of BAT, with far less mitochondria and a small volume of cytoplasm.

BAT's main function is in adaptive thermogenesis, which is the production of heat through the actions of uncoupling protein 1 (UCP1) (Nicholls 1978, Desautels 1978, Enerback 1997). In humans, BAT thermogenesis is important at birth and early infancy, however, with age BAT diminishes, leaving only very small amounts of active tissue (Lean 1986). The site for adaptive thermogenesis in humans has been shown to be localised to skeletal muscle and possibly involves another uncoupling protein – UCP3, although the effect of UCP3 on energy expenditure may be secondary to its primary functional role (Lin 1998, Clapham 2000). In contrast, in rodents and other small animals the contribution of BAT thermogenesis to energy expenditure can help these animals survive periods of cold and energy can be conserved during periods of inactivation such as hibernation.

On the other hand WAT does not express UCP1 and 3, only small amounts of UCP2, whose role has yet to be fully elucidated, so therefore is not involved in heat thermogenesis. Despite WAT accounting on average for 9-28% (sex dependent), in regular healthy individuals, it is characterised by low O_2 consumption compared to BAT. Its main physiological role is the storage of excess energy in the form of triacylglycerides. The amount of lipid stored in the WAT is determined by the balance between body consumption and expenditure. If intake constantly exceeds output then adipocytes will expand enormously, with the ability to differentiate into new adipocytes from precursor cells when required for additional storage (Gregoire 1998, Rosen 2000) and also apoptosis of adipocytes when fat stores decrease (Sorisky 2000). Normal health relies on having WAT in relatively normal amounts – 9-18% in male and 14-28% in female (DiGirolamo 2000). Conditions in which there is too little WAT, lipodystrophy, currently on the increase with the use of highly active antiretroviral therapy for HIV (Loew 2003) have just as harmful metabolic consequences as having an excess of WAT such as obesity, which is associated with insulin resistance, hyperglycemia, dyslipidemia and hypertension (Grundy 2004).

As with most biological systems, the physiology, anatomy and function of adipose tissue can be researched in number of ways: humans, animals (rat and

mouse) or cell lines. In an ideal situation studies on humans would be the gold standard, however there are ethical and heterogeneity implications which also must be considered. Animals pose less of an ethical problem and are more genetically homogenous as well as having the ability to maintain them in a controlled environment for the duration of their lives. Numerous strains of mouse and rat animal models have contributed greatly to our understanding and knowledge of obesity and its associated diseases. The final method available is the use of cell lines. As well as adipose tissue obtained from human volunteers and animals, from which the adipocyte can be isolated and grown are a number of immortalised pre-adipocyte cell lines. Most frequently used are the cell lines – 3T3-L1 and 3T3-F442A, for which the mechanisms of induction of adipogenesis are described below and also further in Chapter 3.

Undeniably all three forms of study have contributed greatly to our understanding of the adipocyte, adipose tissue and its function.

In vitro induction of adipogenesis

Adipogenesis is the program of events which brings about the conversion of a cell from a fibroblastic pre-adipocyte to a fully formed mature lipid laden adipocyte. The mechanisms of this event and the regulation of the process are discussed in more detail in Chapter 3.

Differentiation of primary cultures and cell lines is commonly enhanced by treating postconfluent cells with a differentiation cocktail, containing a combination of insulin (Green 1975), isobutylmethylxanthine (Russell 1976), dexamethasone (Rubin 1978) and serum (Green 1974), a procedure which involves three separate signal transduction pathways. Insulin enhances the lipid forming capability of the cells and also has a role in promoting differentiation in primary preadipocytes. Insulin binds to its receptor and activates a tyrosine kinase signalling pathway which in turn phosphorylates insulin receptor substrate-1, and to a lesser degree –2 and -3 (IRS-1, IRS-2, IRS-3). Phosphorylated IRS initiates several signalling cascades including mitogen-activated protein kinase (MAPK) and phosphoinositide 3 kinase (PI3K) (Saltiel 2001).

Isobutylmethylxanthine inhibits phosphodiesterases, blocks A1 adenosine receptors, increases cAMP levels and potentiates the glucocorticoid effect in 3T3-L1 cells (Russell 1976, Rubin 1978, Schiwek 1987, Schmidt 1990).

Dexamethasone, a potent inducer of adipogenesis, is a synthetic glucocorticoid steroid, responsible for the expression of C/EBPδ (Cao 1991) and PPARγ (Wu 1996). However, glucocorticoids are conditional inducers as the glucocorticoid induced leucine zipper protein (GILZ) antagonises adipocyte differentiation by binding to a CEBP binding site and inhibiting the transcription of PPARγ2 and LPL genes (Shi 2003). Dexamethasone is also involved in the down regulation of the Pref-1 gene, an inhibitor of adipogenesis (see Chapter 3)(Smas 1999).

Serum contains a number of adipogenic factors such as growth hormone(GH), with FBS exhibiting the best adipogenic activity (Kuri-Harcuch 1978).

Thus the aims and objectives of this chapter are to:

(i) Confirm and contrast the expression of Nurr1/nur77 in various mouse tissues and adipose tissue.

(ii) Assess if Nurr1/nur77 are induced in 3T3-L1 and primary pre-adipocyte cells, following addition of adipogenic cocktail.

(iii) Analyse the cocktail components for their ability to induce Nurr1/nur77.

Experimental Procedures

Cell culture

3T3-L1 cells were obtained from ATCC (VA, USA). 3T3-L1 preadipocytes are an adherent cell line and were cultured routinely in either T75 or T175 flasks (Nunc, VWR International LTD UK) in either 15ml or 35 ml of Dulbecco's Modified Eagle's Medium (Invitrogen, Paisley UK) supplemented with 10% Foetal Bovine Serum (Invitrogen) and maintained at 37°C and 5% CO_2. Before reaching confluency cells were trypsinised (Trypsin/EDTA Invitrogen) and re-seeded at a split of 1:5. Cells were fed every 48 hours.

Primary Pre-adipocytes from murine C57BL/6J kindly obtained from Jan Kopeky's Laboratory (Academy of Sciences, Czech Republic).

Differentiation

Differentiation experiments were performed in 24 well plates (Nunc VWR International LTD UK). Preadipocytes were seeded at 3 x 10^4 cells per well. Cells were allowed to attach and grown to confluency. Post confluent cells were then treated with the adipocyte differentiation induction medium – dexamethasone 2.5nM (Sigma Aldrich, Dorset UK), isobutylmethylxanthine 0.5mM (IBMX Sigma Aldrich) and insulin 5mg/ml (Sigma Aldrich). For the induction of Nurr1 studies, RNA was isolated at T0, T30mins, T60mins, T90mins, T120mins, T240 mins, T480 mins and thereafter every 24 hours. For the cocktail analysis studies the cells were treated with each component of the cocktail individually and in varying combinations.

Primary pre-adipocytes were seeded at 5 x 10^4 cells per well. Cells were allowed to attach and grown to confluency. Post-confluent cells were treated with:

1. Dexamethasone (2.5nM), IBMX (0.5mM), insulin (5mg/ml) and BRL49653 (100nM).
2. Dexamethasone (2.5nM), IBMX (0.5mM), insulin (5mg/ml)
3. Dexamethasone (2.5nM)
4. IBMX (0.5mM)
5. Insulin (5mg/ml).

RNA was isolated at T0, T30, T60, T90, T120, T240, T480 mins following induction.

Isolation of RNA from adipocytes

Cells were lysed in 500µl Trizol (Invitrogen) and stored at -80°C, whilst sample collection was completed. Samples were then thawed on ice ready for RNA extraction. To each sample 100µl of chloroform was added and sample tubes mixed thoroughly. Tubes were then placed on ice for 15-20mins to allow separation of the phases. Samples were then centrifuged at room temperature for 10mins at 12000 x g. Following centrifugation, the upper aqueous phase is carefully removed to a new RNAse/DNAse free tube. 0.7 volume of isopropanol was added to each. To facilitate a better precipitation of RNA, tubes were incubated for 1-2 hours at -20°C. Samples were then centrifuged at 12000 x g for 10 mins to pellet the RNA. Supernatants were carefully removed so as not to dislodge the delicate pellet and washed with 100µl of 70% ethanol. Following another centrifugation step (5mins 12000 x g), supernatant was removed and the pellet air-dried. Finally, the pellet was resuspended in 25µl DEPC water.

Isolation of RNA from murine C57BL/6J tissue

Tissues were collected from WT C57BL/6J mice and quickly snap frozen in liquid nitrogen. Each tissue was then ground in a pestle and mortar into a fine powder and added to 1ml of Trizol (Invitrogen). RNA extracted as before.

Isolation of RNA from murine C57BL/6J epididymal and sub-cutaneous adipose tissue.

Adipose tissue isolated from the epididymus (Epi AT) and sub-cutaneous (SC AT) regions was chopped very finely in a Petri-dish. This was then transferred to a universal containing 9ml of DMEM (Invitrogen) and 1ml of 30mg/ml collagenase (Worthington Type 2X3N6770, VWR International Ltd, UK) and incubated with shaking at 37°C for 45mins. The digested material was then filtered into a 15ml centrifuge tube and centrifuged at 3000rpm for 5 mins at 4°C. The adipocytes were removed from the upper fraction and flash frozen. Supernatant was removed and the pellet (containing stromal vascular fraction)

resupended in 1ml of DMEM and flash frozen. 1ml of Trizol was added to each sample for RNA extraction. RNA extracted as before.

cDNA synthesis

Prior to cDNA synthesis, all RNA samples were subjected to DNAse treatment to remove any contaminating DNA in the sample. To each RNA sample, 1 unit of DNAse (Promega, Southampton UK) and 3µl of 10x Buffer was made up to a final volume of 35µl with DEPC water and incubated at room temperature for 30mins. Reaction was stopped by the addition of 1µl of Stop reagent and incubated at 65°C for 5 mins. Tubes were then returned to ice.

The concentration of RNA was measured by optical density at 260nm; an OD of 1 is equivalent to 40ug/ml RNA in an undiluted sample. All readings were performed on a NanoDrop spectrophotometer (Labtech, East Sussex UK). 1µl of undiluted sample is placed on the machine's aperture and an OD 260/280 performed, calculating the concentration per µl and also the quality of RNA. Pure RNA would have and A260/A280 ratio of approximately 2. Deviations from this indicate protein or RNA contaminants.

1µg of RNA was mixed with 1µl (0.5µg) of oligo dT (Invitrogen) and made up to 12µl with DEPC water and annealed at 72°C for 15 mins. The samples were then cooled slowly on ice for 15mins. To this 5 x First Strand Buffer, 2µl DTT (100mM), 1 µl dNTP's (10mM) and 1µl of SuperScript II Reverse Transcriptase (Invitrogen) were added, giving a total volume of 20µl. Samples were then incubated at 42°C for 45 mins, followed by a heat inactivation step at 70°C for 15 mins and then cooled at 4°C. To each reaction 80µl of DEPC water was added, giving a final concentration of 10ng/µl.

SybrGreen RT-PCR

Semi-quantitative polymerase chain reaction was carried out using SybrGreen and specific primers for β-Actin, Nurr1, and nur77 (Sigma Genosys, Suffolk UK). Master Mix was prepared containing 12.5µl of 2x SybrGreen Master Mix

(Applied Biosystems, Warrington UK), 1µl of forward primer (10mM), 1µl of reverse primer (10mM), 5.5µl of dH_2O and 5µl of cDNA. Semi- quantitative PCR was performed on the ABI 7700 (Applied Biosystems, Warrington UK). Genomic standards (Promega) were run in parallel to allow relative quantification.

Primer sequences:

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
β-Actin	gagctatgagctgcctgacg	agtttcatggatgccacagga
Nurr1	tcgacatttctgccttctcc	ccactctcttgggttccttg
nur77	ggtatccgaaagtgggcaga	cggaggatgaagagttccag

Fig. 2.1. Primer sequences for SybrGreen PCR analysis

Statistical analysis

Results are expressed as mean±SD or SEM for normally distributed values or median (interquartile range) for skewed data. Comparisons between groups were made by two-tailed Student's *t*-test, Mann-Whitney U test or Wilcoxon rank test, as appropriate. $P < 0.05$ was considered to be statistically significant. In the induction of adipogenesis experiments expression levels are expressed as percent compared to levels of the mean T0 value.

Results

Expression of Nurr1 and nur77 in murine C57BL/6J tissue

Extensive expression analysis has been reported for members of this family of NR's and most papers cite basal expression of these family members in brain (Law 1992, Xiao 1996, Watson 1989). Expression has also been reported in muscle tissues and testes for nur77 (Watson 1989). As previously indicated, members of this family can be induced rapidly and transiently in most other tissues. Additional data which has been made available (GSK in-house) indicates that as well as being constitutively expressed in brain; these genes are expressed abundantly in human adipose tissue (whole) as well as some evidence of expression in mouse adipose tissue. To confirm these findings, tissue was isolated from murine C57BL/6J and analysed for the expression of Nurr1 and nur77.

Expression was detected for Nurr1 in brain and at lower levels in adipose tissue with heart, kidney and lung expression at very low but detectable levels (Fig. 2.2). nur77 was expressed at similar levels in the brain and to a lesser degree in heart and lung but much higher levels were detected in adipose tissue in particular adipose isolated from the epididymal area (Fig. 2.3). The fact the levels were higher for nur77, than Nurr1 in adipose, mirror previous in-house data from human tissues. Adipose tissue was also separated into its constituent fractions – stromal vascular and adipocytes. Nurr1 expression was 10 fold higher in the stromal vascular fraction compared to the adipocytes (Fig. 2.4). nur77 showed similar expression levels in both the stromal vascular and the adipocytes (Fig. 2.5), although slightly higher in sub-cutaneous stromavascular fraction. Levels do appear higher than adipose tissue but this could be down to a concentration of the samples. Data normalised to β-actin expression and expressed as Nurr1/ β-actin +/- SEM.

Induction of Nurr1 and nur77 in 3T3-L1 cells following addition of differentiation cocktail

Nurr1 is an immediate early gene whose expression is normally induced approximately 2 hrs after the induction stimuli has been applied. To assess if the same was true for Nurr1 and nur77 expression in 3T3-L1 cells, the differentiation cocktail was added to post confluent cells and mRNA extracted at T0, T30, T60, T90, T120, T240, and T480 minutes and analysed for the expression of both Nurr1 and nur77.

Following addition of the differentiation cocktail to the 3T3-L1 cells, a rapid and transient induction of both mRNAs was detected. The expression pattern for both these genes was as expected with a peak of expression between T60 and T120 mins after which expression levels subsided (Fig. 2.6 and 2.7). No detectable levels of NOR-1 could be detected in these cells. Data is presented as % induction because the numerical values obtained in the different experiments had a degree of variation. This variation could be due to the degree of induction of the NGFIB member, efficiency of reverse transcription step or the efficiency of the actual SybrGreen PCR. Although it may appear that 100% induction is not achieved, the results presented are the average of a number of experiments.

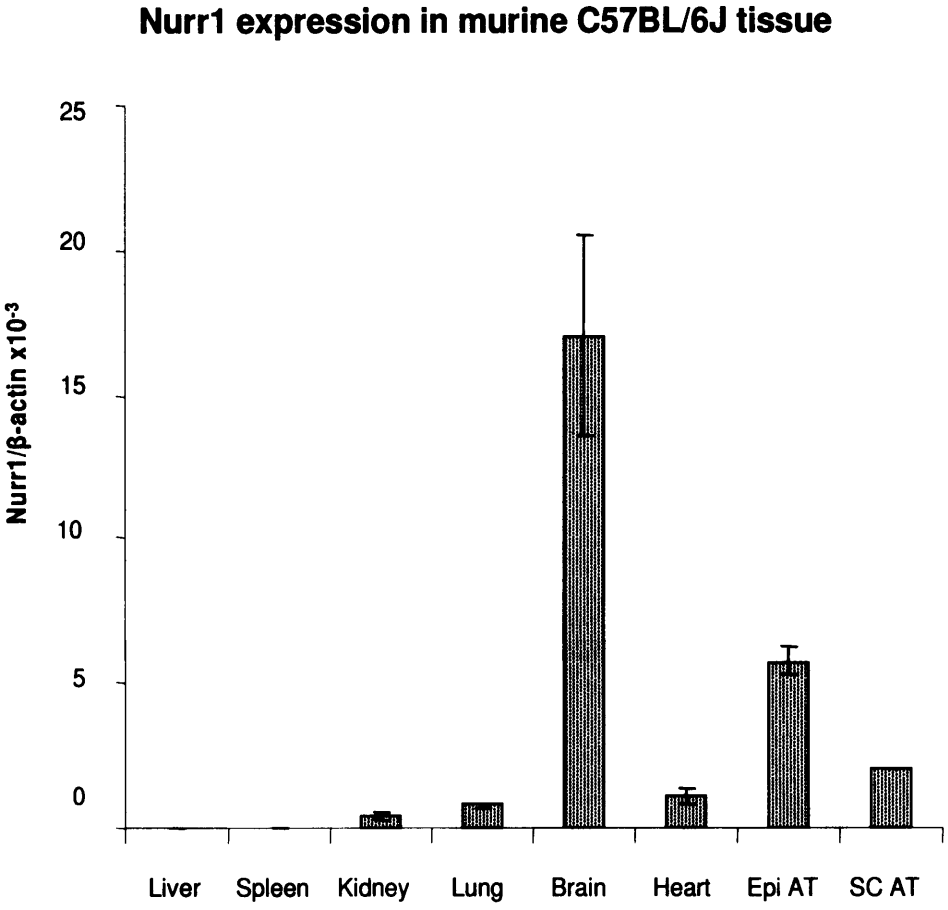


Fig. 2.2 Expression of Nurr1 in murine C57BL/6J tissue

Expression of Nurr1 in murine C57BL/6J tissue as assessed by SybrGreen analysis. Data normalised to β-actin expression and expressed as ratio of Nurr1/β-actin +/- SEM (n=10). Compared to expression in liver, the kidney, lung, brain, heart, and adipose tissues had significantly elevated expression levels (all $p<0.001$).

(Epi AT = Epididymal adipose tissue, SC AT = Sub-cutaneous adipose tissue).

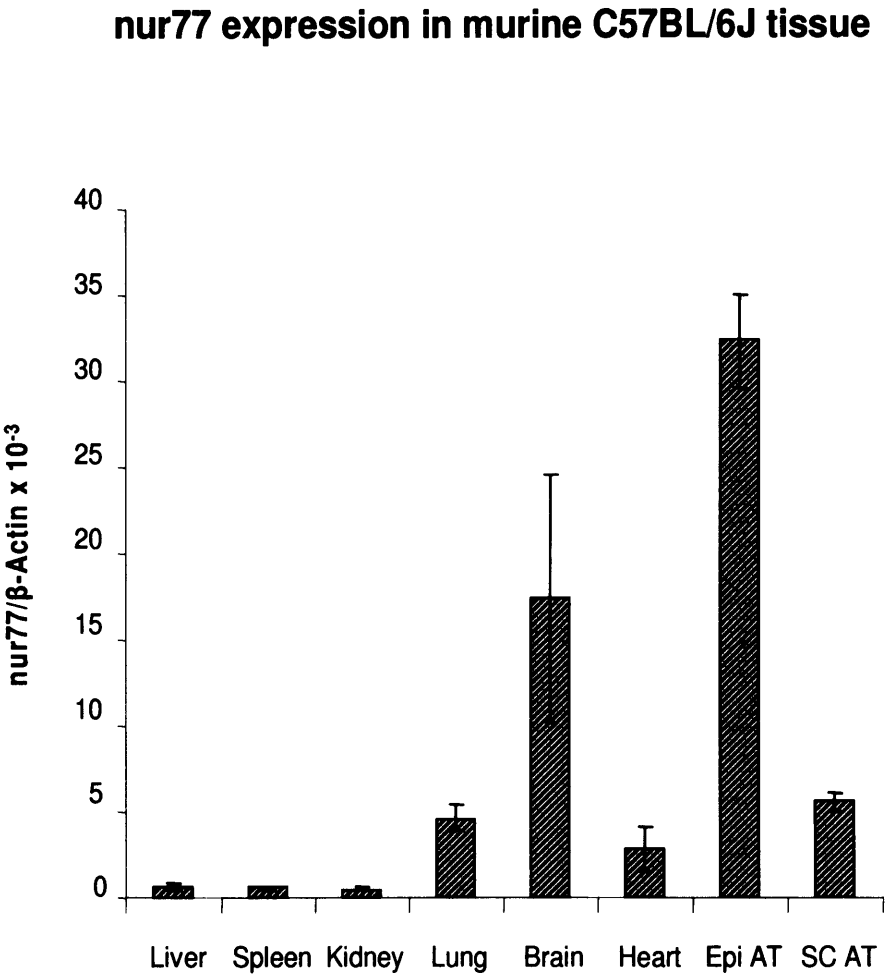


Fig. 2.3 Expression of nur77 in murine C57BL/6J tissue

Expression of nur77 in murine C57BL/6J tissue as assessed by SybrGreen analysis. Data normalised to β-actin expression and expressed as nur77/ β-actin +/- SEM (n=10). Compared to the liver, the lung, brain, heart and adipose tissues showed elevated expression (all $p<0.001$).

(Epi AT = Epididymal adipose tissue, SC AT = Sub-cutaneous adipose tissue).

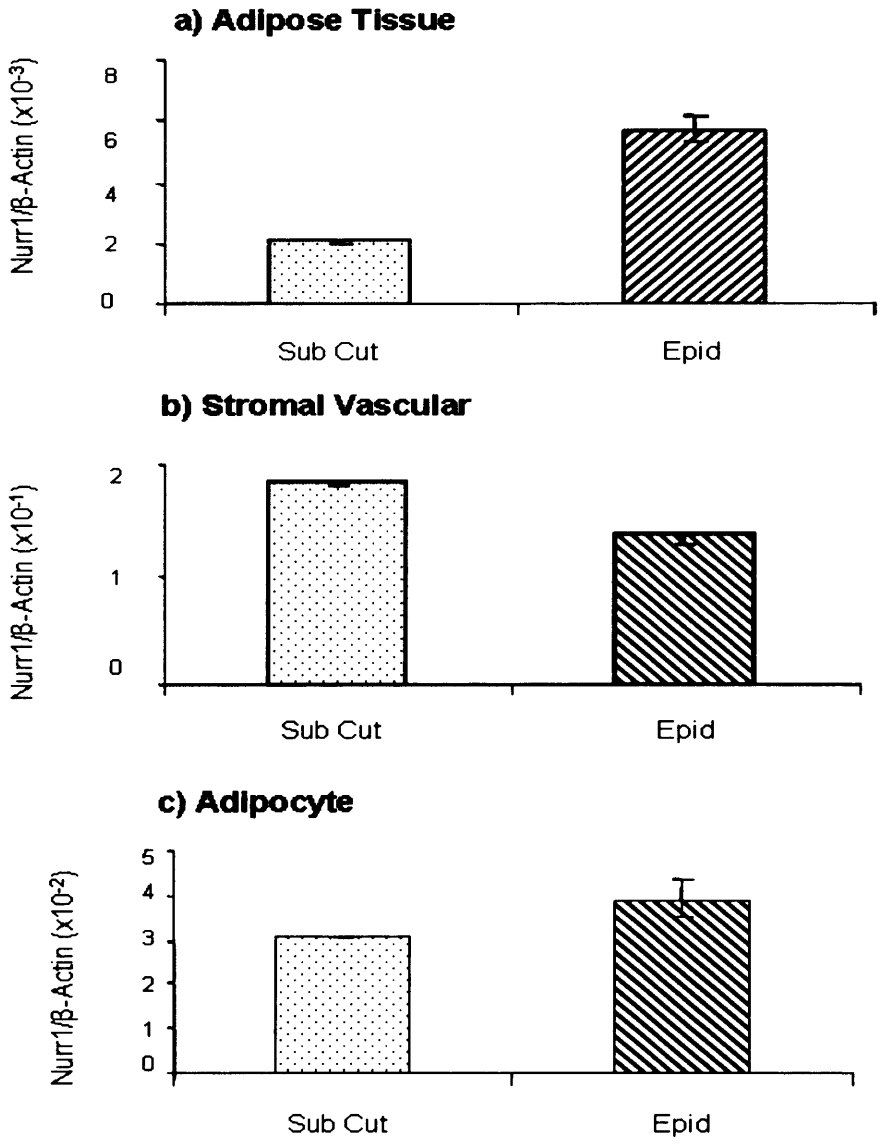


Fig. 2.4 Expression of Nurr1 in murine C57BL/6J adipose tissue fractions

Expression of Nurr1 in adipose tissue (a), stromal vascular (b) and adipocyte (c) (n=10) as assessed by SybrGreen analysis. Data normalised to β-actin expression and expressed as Nurr1/ β-actin +/- SEM. Expression of Nurr1 in stromovascular fraction of both depots higher than seen in isolated adipocytes (all $p<0.01$).

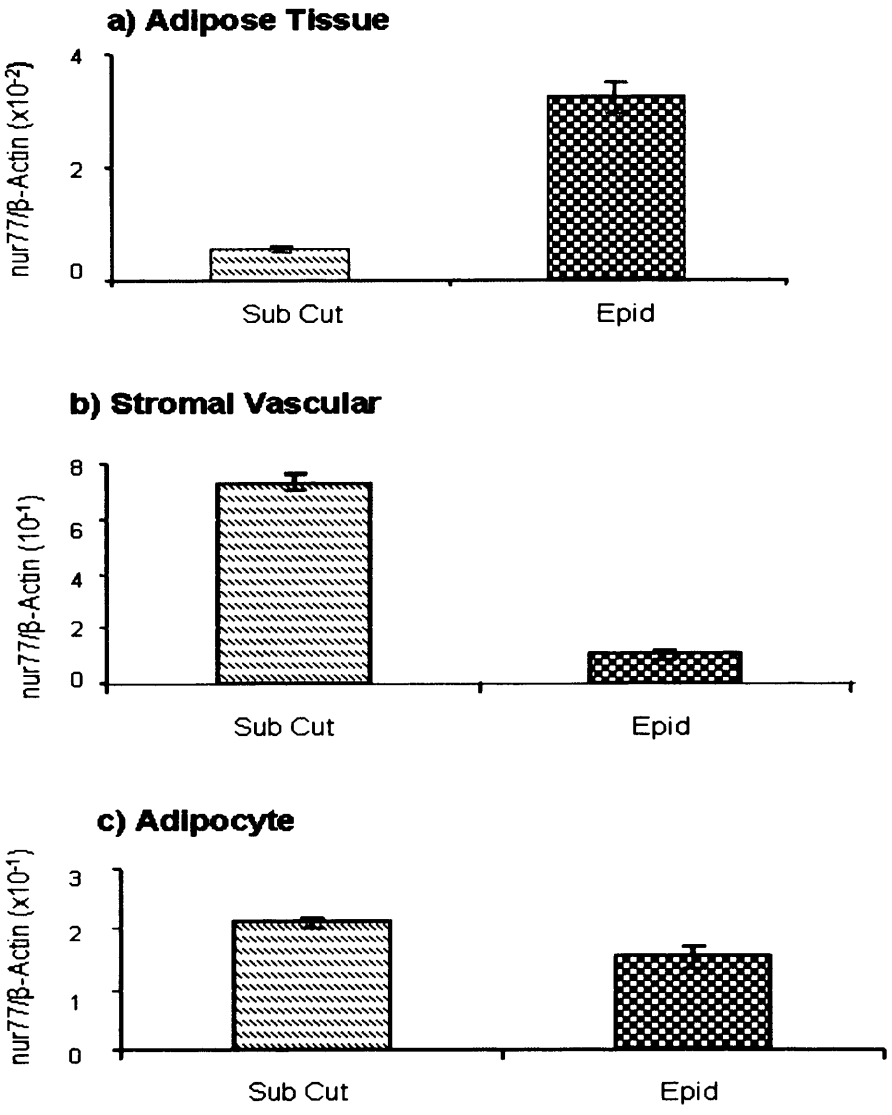


Fig. 2.5 Expression of nur77 in murine C57BL/6J adipose tissue fractions

Expression of nur77 in adipose tissue (a), stromal vascular (b) and adipocyte (c) (n=10) as assessed by SybrGreen analysis. Data normalised to β-actin expression and expressed at nur77/ β-actin +/- SEM. nur77 expression only elevated in stromovascular fraction of sub-cutaneous tissue ($p<0.01$).

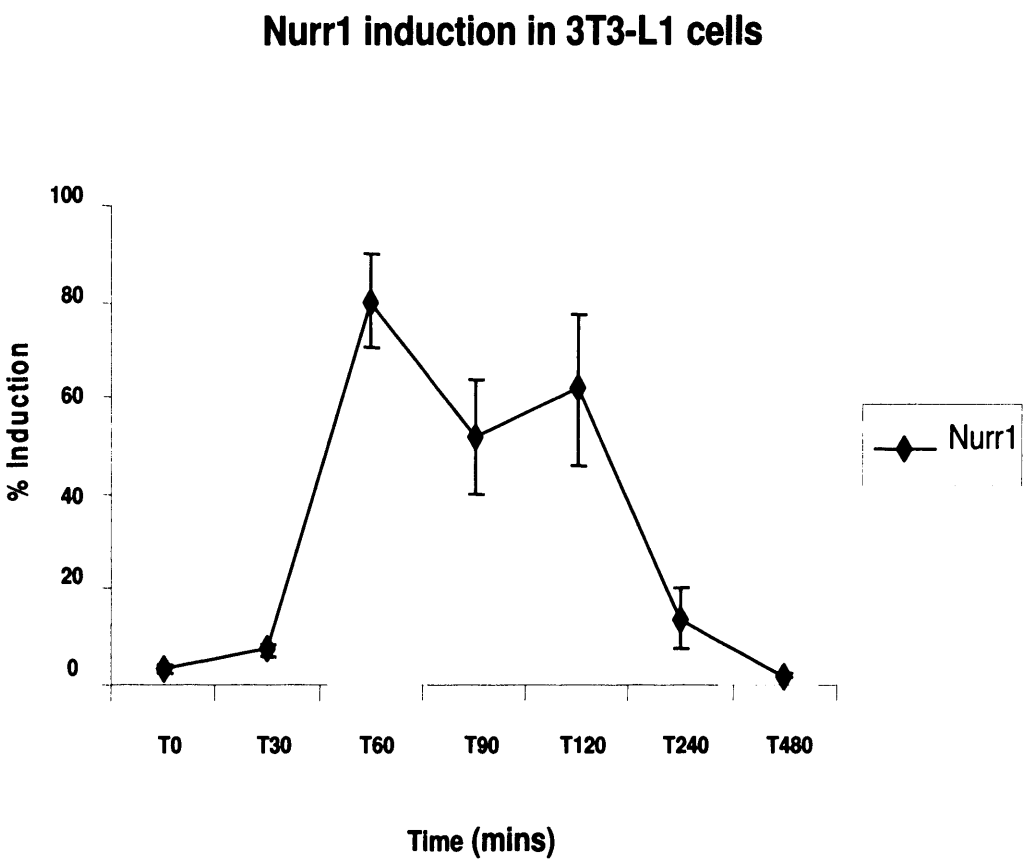


Fig. 2.6 Induction of Nurr1 during adipogenesis

Graph shows induction of Nurr1 following addition of differentiation cocktail to 3T3-L1 cells, as assessed by SybrGreen analysis. Data expressed as mean % induction compared to mean value of T0, following normalisation to β-actin, +/- SEM, n=4. T0 v T60-T120 $p<0.001$; T0 v T240-480 $p>0.05$=NS.

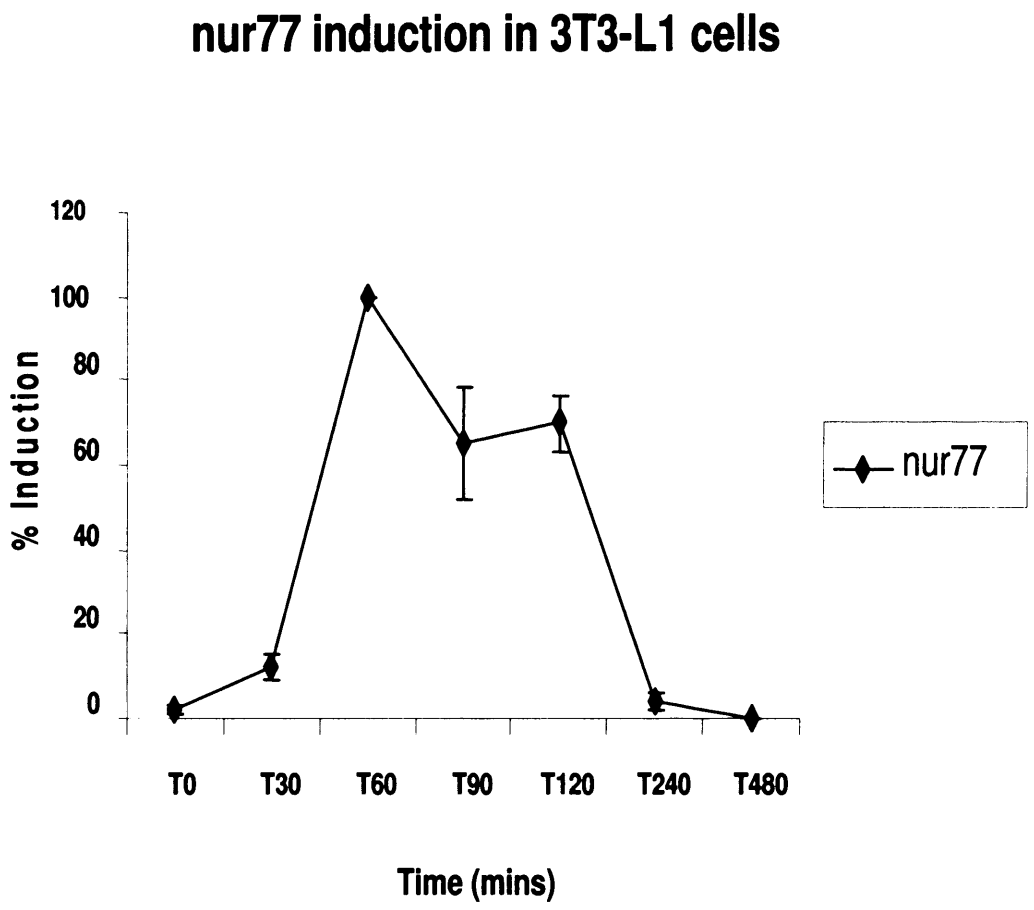


Fig. 2.7 Induction of nur77 during adipogenesis

Graph shows induction of nur77 following addition of differentiation cocktail components to 3T3-L1 cells, as assessed by SyBrgreen analysis. Data expressed as mean % induction of mean T0 value, following normalisation to β-actin, +/- SEM, n=4. T0 v T60-T120 $p<0.001$; T0 v T240-480 $p>0.05$=NS.

Analysis of cocktail components on Nurr1 and nur77 induction

As described previously the components of the adipogenic include dexamethasone, isobutylmethylxanthine and insulin. It is widely known that there are number of inducers of the NGFIB family of receptors ranging from neurotransmitters to membrane depolarization (Milbrandt 1988, Arender 1989, Kovalovsky 2002, Bartel 1989, Davis 1994). The aim of the experiment is to discover whether or not it is a combination of all three elements which is responsible for the induction of Nurr1 and nur77, or 2 components or just one of these inducers. The induction experiment was set-up exactly as before but the inducers were added individually or as a combination of two. RNA isolated at the same time points T0, T30, T60, T90, T120, T240mins and assessed for induction levels of Nurr1 and nur77.

The results for each gene (Fig. 2.8-2.13) are distinct with Nurr1 exhibiting the more specific induction. The principal inducer of Nurr1 is IBMX, which correlates well with previous data which shows that elevation of cAMP levels induce the expression of this family. This will be discussed further in the next section. Addition of insulin to the IBMX has little effect on the induction, whilst dexamethsone in combination with IBMX negatively impacts the expression of Nurr1.

nur77 demonstrates a different pattern of induction compared to Nurr1. Each member of the cocktail seems to in someway induce nur77 whether added individually or as a combination.

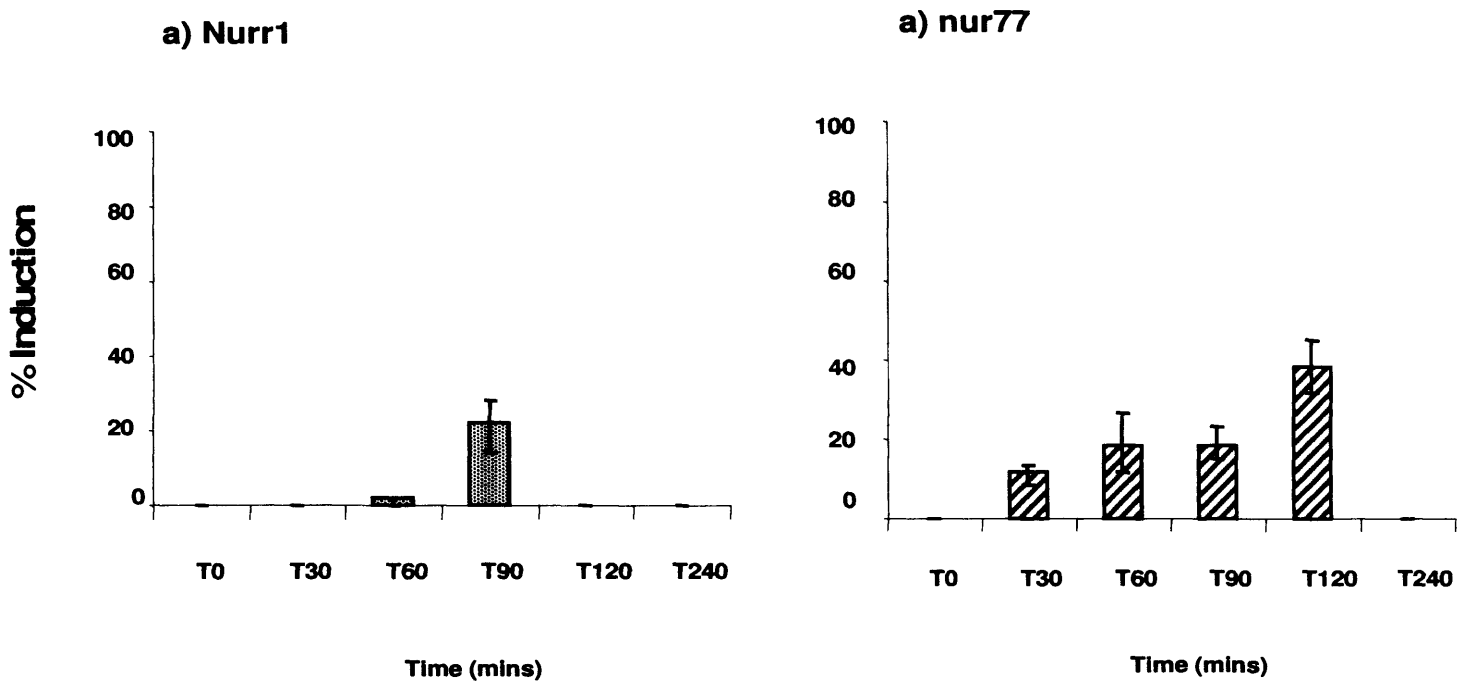


Fig. 2.8 Induction of NGFIB family in response to insulin

Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using insulin only. Data expressed as mean % induction, following normalisation to β-actin, +/- SEM, n=4

a) Nurr1, significantly elevated at T60 and T90 compared to T0 prior to returning to T0 levels. b) nur77, stays significantly elevated from T30-T120, compared to T0. All $p<0.001$.

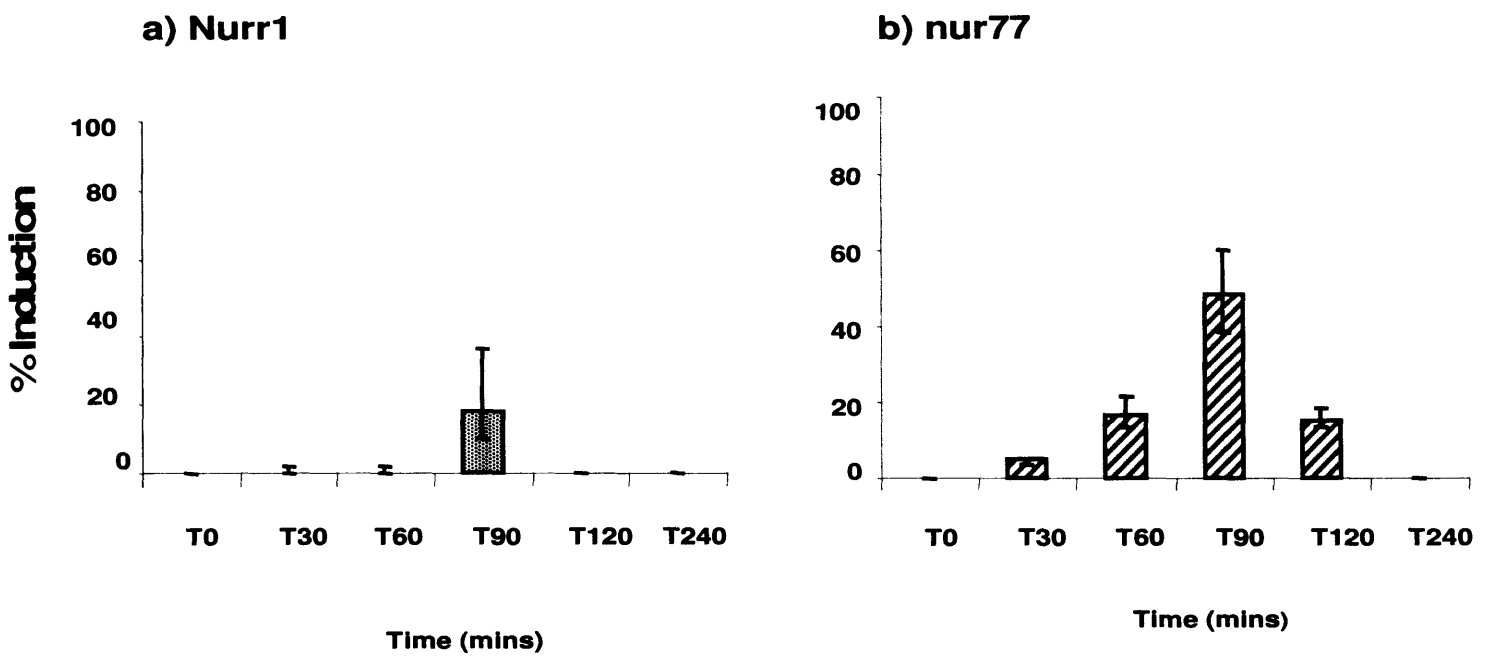


Fig. 2.9 Induction of NGFIB family in response to dexamethasone
Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using dexamethasone only. Data expressed as mean % induction compared to mean T0 values, following normalisation to β-actin, +/- SEM, n=4.
a) Nurr1, only elevated at T90 b) nur77, elevated at T30-T120. All $p<0.001$.

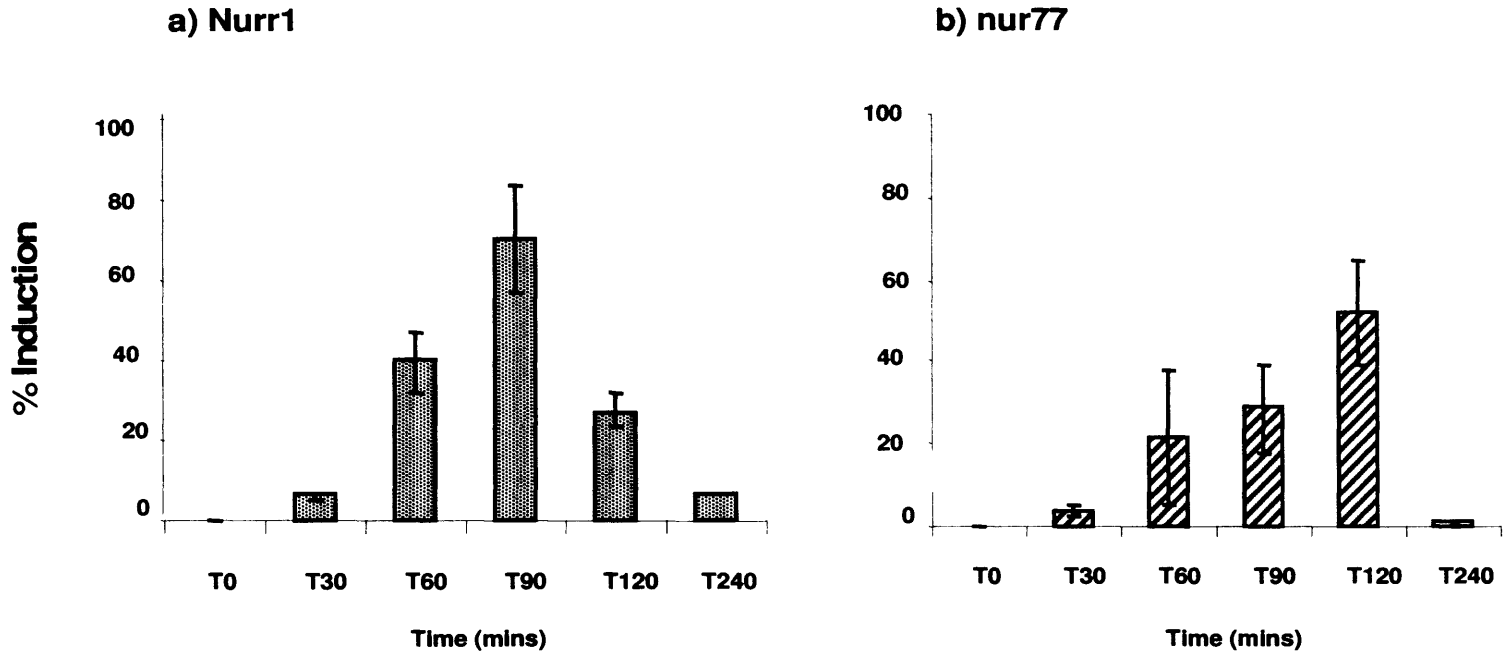


Fig. 2.10 Induction of NGFIB family in response to IBMX
Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using IBMX only. Data expressed as mean % induction compared to mean T0, following normalisation to β-actin, +/- SEM, n=4
a) Nurr1, significantly elevated at T30-T240; b) nur77, significantly elevated at T30-T120, all $p<0.001$.

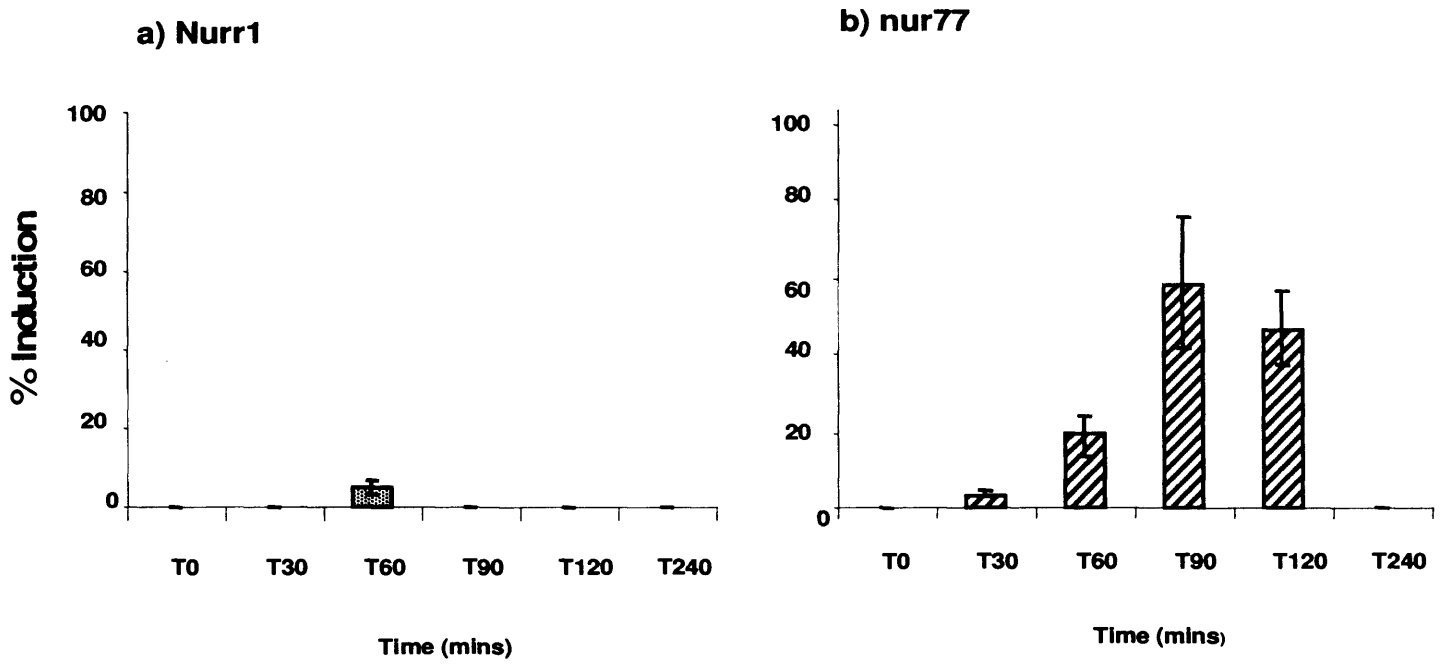


Fig. 2.11 Induction of NGFIB family in response to insulin and dexamethasone
Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using insulin and dexamethasone only. Data expressed as mean % induction compared to mean T0 values, following normalisation to β-actin, +/- SEM, n=4
a) Nurr1, only significantly elevated at T60, $p=0.04$; b) nur77, significantly elevated at T30-T120, $p=0.03$ to $p<0.01$.

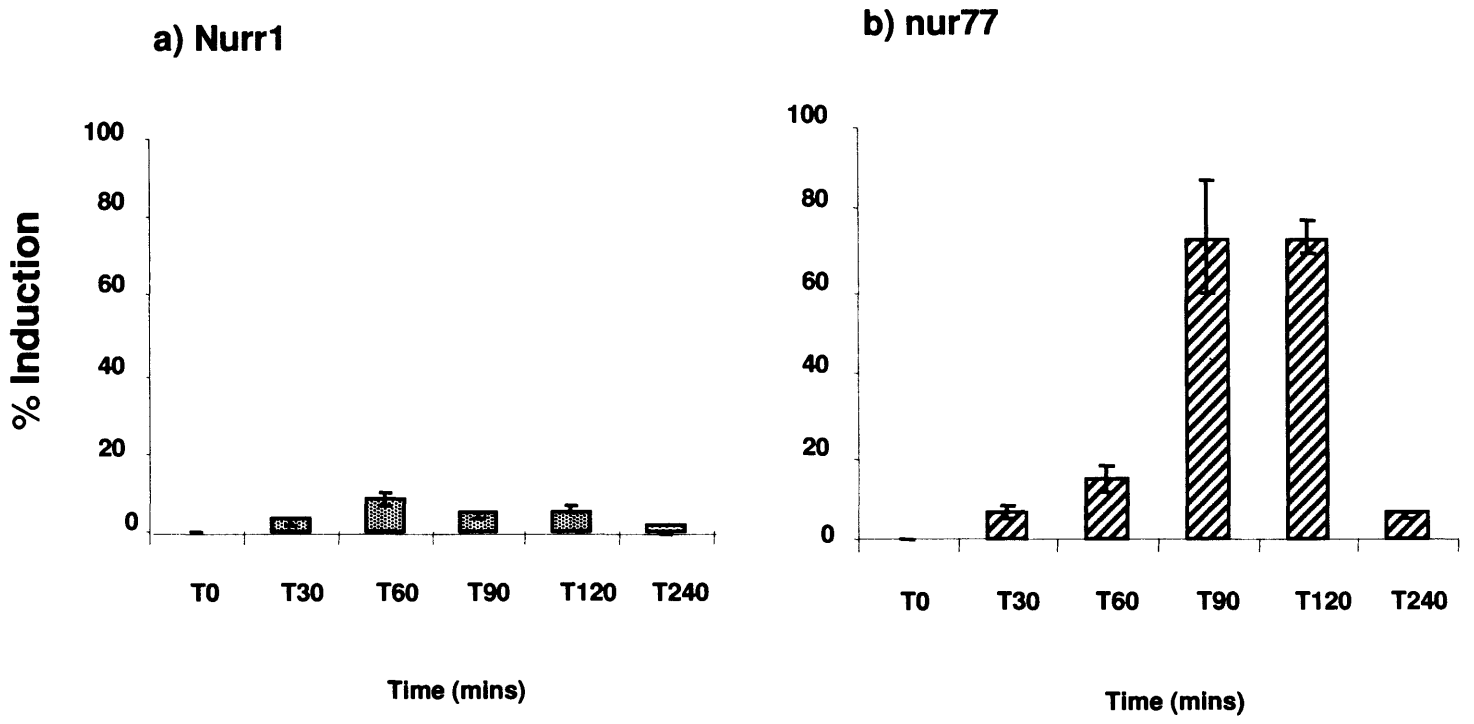


Fig. 2.12 Induction of NGFIB family in response to dexamethasone and IBMX
Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using dexamethasone and IBMX only. Data expressed as mean % induction compared to mean T0 value, following normalisation to β-actin, +/- SEM, n=4
a) Nurr1, b) nur77. All $p<0.01$

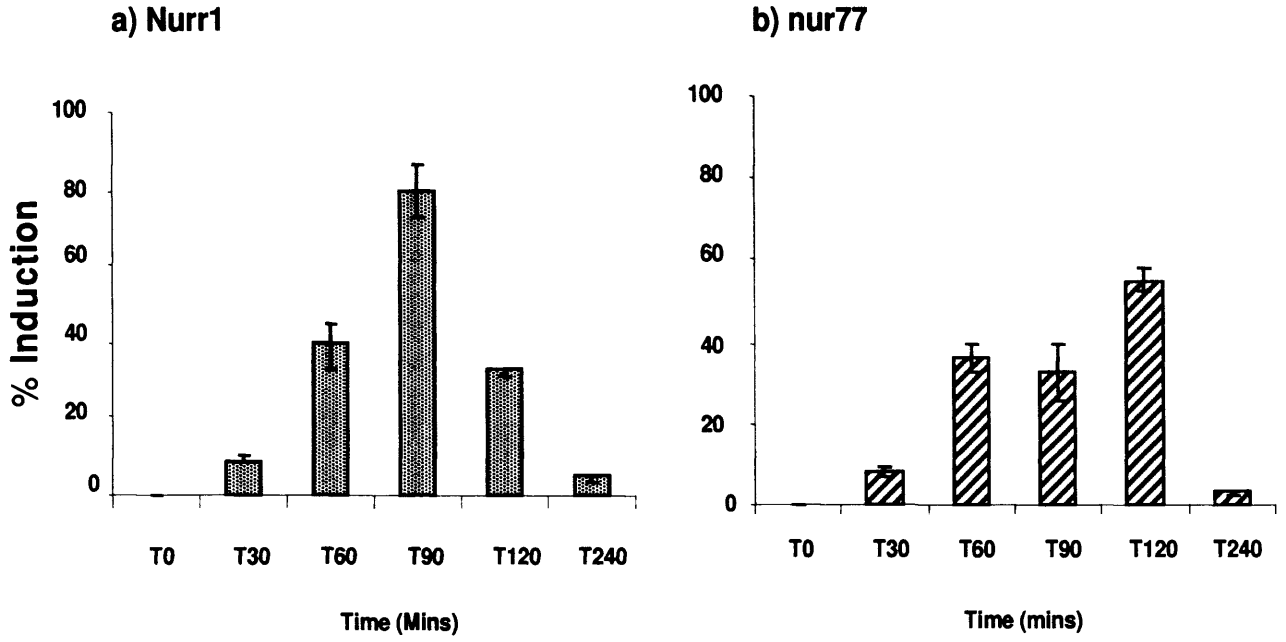


Fig. 2.13 Induction of NGFIB family in response to insulin and IBMX
Nurr1 and nur77 induction levels as assessed by SybrGreen analysis following induction using insulin and IBMX only. Data expressed as mean % induction compared to mean T0, following normalisation to β-actin, +/- SEM, n=4
a) Nurr1, b) nur77 All $p<0.01$

Induction of Nurr1 and nur77 in primary mouse pre-adipocytes and analysis of cocktail components.

Primary pre-adipocytes were analysed for their ability to induce expression of Nurr1 and nur77 and an investigation into the effects of the individual cocktail components was also performed. Primary mouse pre-adipocytes were set up exactly as the 3T3-L1 system and post confluency, differentiation was initiated. The pre-adipocytes require the PPARγ agonist BRL49653 for differentiation, therefore differentiation was induced with and without the compound. The cocktail was also analysed as individual components.

To date all of the expression analysis has been performed in the immortalised 3T3-L1 cell line and whilst this has been used as an ideal system to elucidate the adipogenesis program, it must be remembered that it may not always parallel events which takes place *in vivo* accurately.

Nurr1 and nur77 are indeed induced in a similar manner in primary mouse pre-adipocytes as 3T3-L1 cells (Fig 2.14 and 2.15). Nurr1 exhibits a peak of expression at approximately 60mins followed by a return to basal in the cells without BRL49653. However, the cells induced in combination with BRL49653 appear to show sustained expression of Nurr1. nur77 expression parallels that found in 3T3-L1 cells with a peak of expression between 60-90mins and a return to basal levels in both differentiation cocktail alone and in the presence of BRL49653.

Analysis of the cocktail shows that IBMX would appear to be the primary inducer for both Nurr1 and nur77 (Fig. 2.16-2.17). Addition of either insulin or dexamethsone has a negligible effect on the induction on either Nurr1 or nur77.

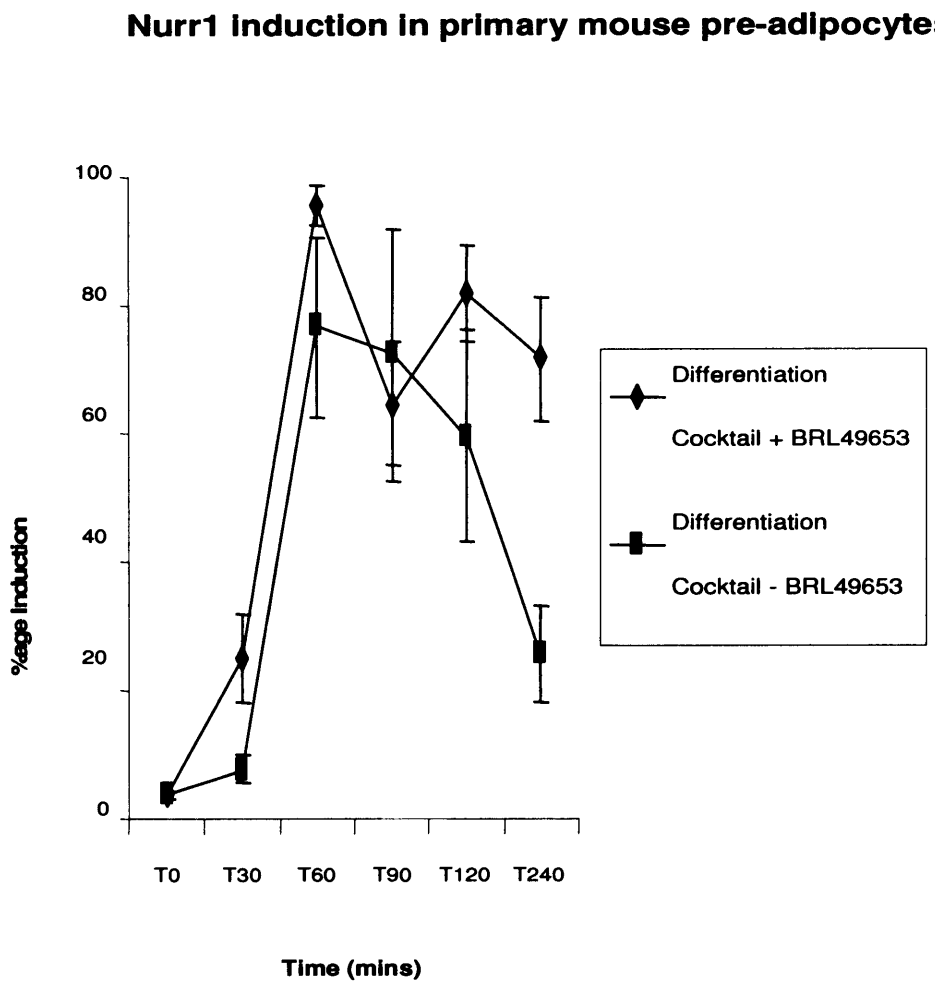


Fig. 2.14 Induction of NGFIB family members during adipogenesis in primary mouse pre-adipocytes

Graph shows induction of Nurr1 following addition of differentiation cocktail to primary mouse pre-adipocytes, as assessed by SybrGreen analysis. Data expressed as mean % induction compared to mean T0 value, following normalisation to β-actin, +/- SEM, n=3. All $p<0.001$ after T30 and remains elevated up to T240.

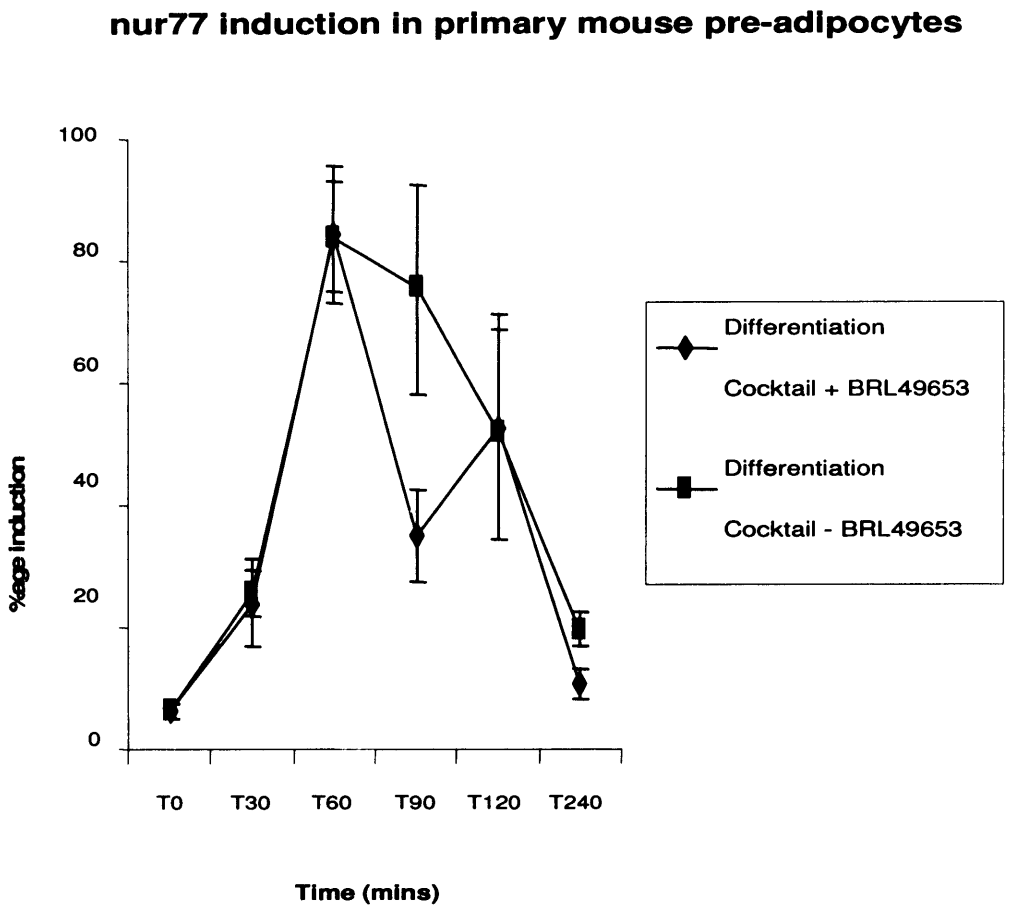


Fig. 2.15 Induction of NGFIB family members during adipogenesis in primary mouse pre-adipocytes.

Graph show induction of nur77 following addition of differentiation cocktail to primary mouse pre-adipocytes, as assessed by SybrGreen analysis. Data expressed as mean % induction of mean T0 level, following normalisation to β-actin, +/- SEM, n=3. All $p<0.01$ from T30 to T240.

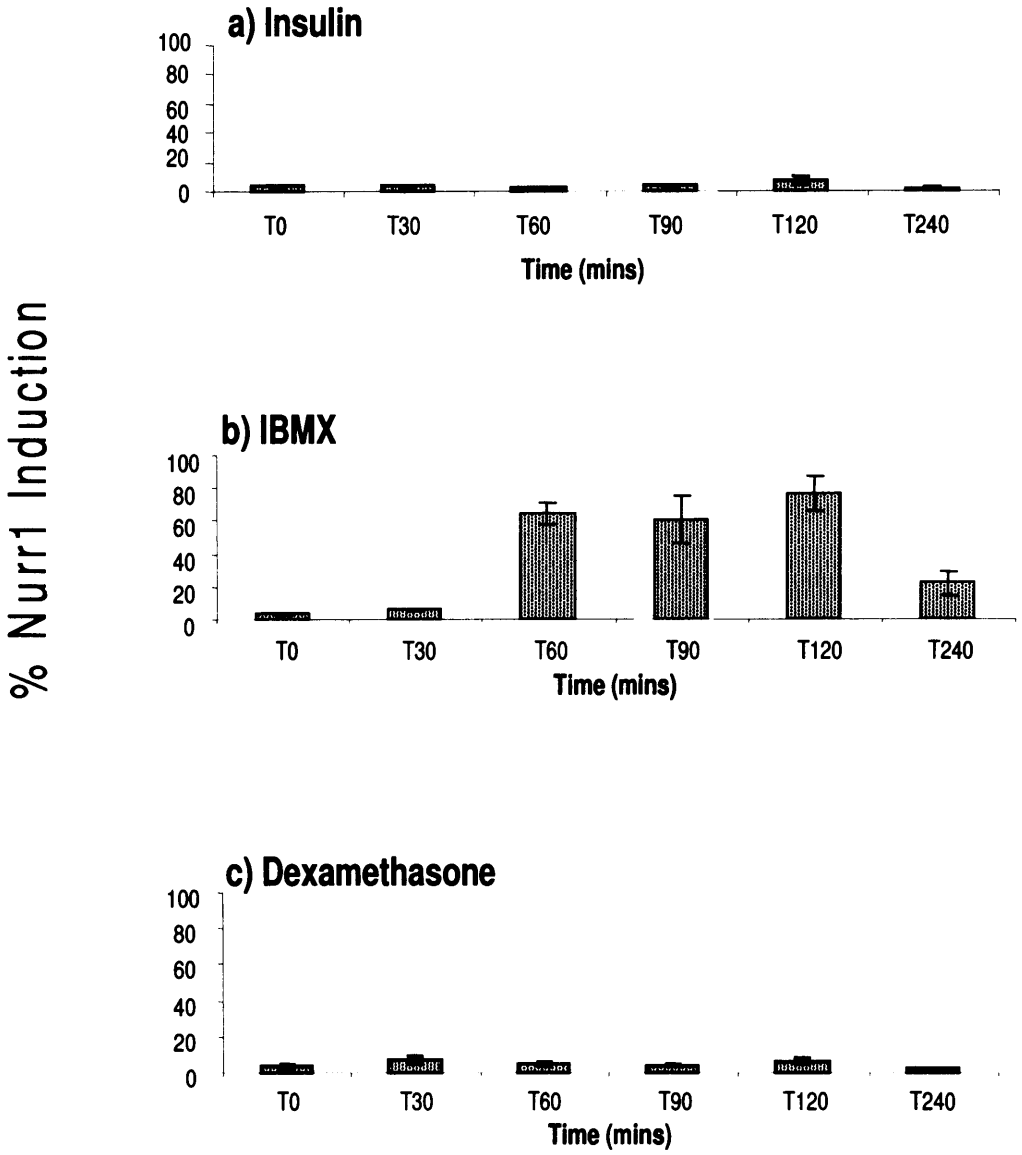


Fig. 2.16 Analysis of differentiation cocktail on Nurr1 expression in primary pre-adipocytes

Nurr1 induction as assessed by SybrGreen analysis following the addition of the individual cocktail components. a) Insulin all $p>0.05$. b) IBMX, all $p<0.01$ at times T60-T240, c) Dexamethasone, all $p>0.01$. Data expressed as mean % induction of mean T0 value, following normalisation to β-actin, +/- SEM, n=3.

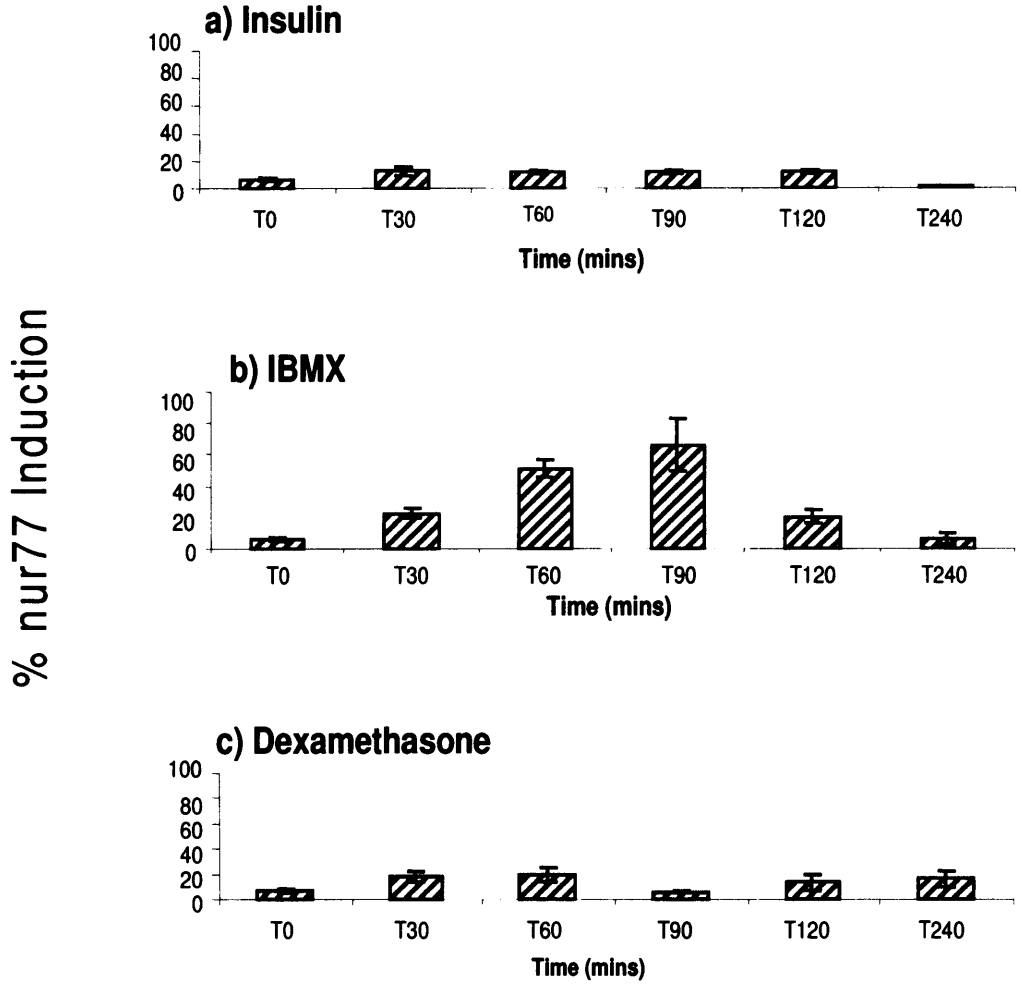


Fig. 2.17 Analysis of differentiation cocktail on nur77 expression in primary pre-adipocytes

nur77 induction as assessed by SybrGreen analysis following the addition of the individual cocktail components. a) Insulin $p<0.05$ at T30-120. b) IBMX, $p<0.01$ at times T30-T120, c) Dexamethasone, $p<0.01$ at T30, T60, T120 and T240. Data expressed as mean % induction compared to T0, following normalisation to β-actin, +/- SEM, n=3.

Discussion

Expression of the NGFIB family in mouse tissue

Previous in-house data as presented earlier in this report (Fig. 1.8) illustrates the expression of each member of the NGFIB family in human tissues. Highest levels of expression are evident, surprisingly in adipose tissue, which has not been reported before, as well as expression in brain, skeletal muscle with lower levels detected in a variety of other tissues. In mouse tissue, Nurr1 exhibits highest expression in the brain, which correlates well with previous data by others (Law 1992, Xiao 1996, Watson 1989). nur77 is expressed at similar levels in the brain as Nurr1 but its highest expression appears to be in adipose tissue (Fig 2.2 and 2.3). These studies have confirmed that both Nurr1 and nur77 are indeed expressed at appreciable levels in mouse adipose tissue in both the epididymal and the sub-cutaneous depots. Nurr1 expression is higher in stromal vascular fraction than adipocytes, whilst nur77 appears to be expressed at similar levels in both fractions (Fig 2.4 and 2.5). This may suggest a role for this family in the regulation of adipose tissue function.

Induction of Nurr1 in 3T3-L1 cells following addition of differentiation cocktail

The NGFIB family are transcription factors which are classified as immediate early genes illustrated by a rapid and transient induction of expression by a variety of stimuli and intracellular signalling cascades (Milbrandt 1988, Arender 1989, Kovalovsky 2002, Bartel 1989, Davis 1994). However, constitutive expression is observed in the adult mouse brain (Xiao 1996, Watson 1989) but as described earlier, expression can also be induced. The induction of immediate early genes (IEG) occurs in the absence of de novo proteins synthesis and cannot be blocked by protein synthesis inhibitors (Platenik 2000). Extensive research in the area of the c-fos and c-jun IEG's has demonstrated that the downstream effects of these genes are critical for the proper functioning of many cellular

systems, although the exact regulatory mechanisms still remain elusive (Sng 2004).

Following the addition of differentiation cocktail to 3T3-L1 cells, a rapid induction of Nurr1 and nur77 mRNA levels was detected, with levels peaking between 60 minutes and 120 minutes, which parallels that seen in other tissues and systems (Davis 1994, Philips 1997, Platenik 2000). Expression then returns to basal levels by approximately 4 hours and further expression of these genes is not apparent during the differentiation process (Fig 2.6 and 2.7). Tetradis et al reported the induction of Nurr1 and nur77 maximally at 60mins following addition of 10nM PTH and demonstrated that pretreating the cells with cycloheximide, to prevent protein synthesis, did not inhibit the induction of Nurr1 (Tetradis 2001, Philips 1997b). The transient induction of Nurr1 and nur77 in 3T3-L1 cells precedes the induction of other genes involved in the adipogenesis program. This suggests that the family could be involved in the very early events of adipogenesis. This role could be one of two:

- Transient induction of the NGFIB subfamily could be responsible for maintaining the cells in an undifferentiated state and their induction could bring about the switching off of genes such as Wnt's, Pref-1 or other unidentified genes expressed in undifferentiated pre-adipocytes.
- Transient induction of the NGFIB subfamily could be responsible for the induction of genes involved early in the adipogenesis program.

Analysis of cocktail components on Nurr1 and nur77 induction in 3T3-L1 cells

As previously cited, the cocktail used to initiate differentiation in 3T3-L1 cells contains a combination of insulin, isobutylmethylxanthine and glucocorticoids as well as the serum provided in the growth medium. Whilst a combination of all three induce the expression of both Nurr1 and nur77 (Fig. 2.6 & 2.7), the purpose of this experiment is to analyse which component(s) of the cocktail are the primary inducers of expression. Surprisingly the results for each gene are entirely

distinct (Fig. 2.8-2.13). In this experiment, it was observed that the principal inducer of Nurr1 is IBMX, which correlates well with previous literature citing that the cAMP /PKA pathway regulates the expression of NGFIB family through phosphorylation (Maria 2003). Maria et al demonstrated that stimulation of PKA activity augmented nur77 transactivation and DNA binding to the NurRE (Maria 2003). In addition, Tetradis et al showed that PTH, which is a potent stimulator of cAMP dependent signalling (Abou-Samra 1992), induced expression of Nurr1 through the cAMP protein kinase A (PKA) pathway in osteoblasts (Tetradis 2001). Recently, it has also been shown that elevation of intracellular cAMP levels in granulosa cells induced Nurr1 and nur77 expression, which were subsequently responsible for the down regulation of many FSH responsive genes including aromatase, which promotes transition from preovulatory follicles to corpora lutea (Wu 2005). There are also a number of other genes regulated by the NGFI-B family which are induced by the cAMP/PKA pathway. These include salivary-specific R15 gene (Lin 1996), steroidogenic P450c21 (21-hydroxylase) (Chang 1995), P450c17 (Zhang 1997) and a variety of thyrocyte-specific genes (Pichon 1999).

Addition of insulin to IBMX has a little effect on the induction of Nurr1 and has little effect on its own – a low level of induction is evident at 90mins. However, addition of dexamethasone to IBMX almost abolishes the induction of this gene. Dexamethasone alone elicits a small induction which may in part be in conjunction with the serum. It is known that dexamethasone and glucocorticoids in general antagonize the responsiveness of the NGFIB family of genes (Philips 1997b). Also nur77 and Nurr1 regulate POMC which in turn regulates ACTH, which is involved with the synthesis of glucocorticoids from the adrenal gland.

However, in this experiment dexamethasone would appear to somehow induce nur77 expression, but then most of the cocktail components seem to induce a hypersensitive induction of this gene, with no one constituent responsible for the expression of this gene in 3T3-L1's. Combinations of each component seem to result in an augmentative effect. This hypersensitivity of nur77 to the hormonal

cocktail constituents makes it very difficult to decipher how exactly the gene would be induced during the adipogenesis program, unlike its sister receptor Nurr1.

Induction of Nurr1 and nur77 in primary mouse pre-adipocytes and analysis of cocktail.

Nurr1 and nur77 are indeed induced in primary mouse pre-adipocytes (Fig 2.14 and 2.15). Nurr1 shows a sustained pattern of induction with BRL 49653. Cells induced without the BRL compound show a similar first phase of induction and then expression begins to drop off. However a small secondary induction is evident at 8 hours. This conflicts with the data obtained in the model system. nur77 expression appears to parallel that obtained in 3T3-L1 cells with an induction within 60mins followed by a rapid decrease in expression.

As for the 3T3-L1 cells, IBMX would appear to be the main inducer in this system for both genes. Dexamethasone and insulin have little or no effect on the induction of Nurr1. In the primary cells, the induction of nur77 would appear to be more reliant on IBMX, unlike 3T3-L1 cells where nur77 expression was sensitive to multiple induction components. Insulin and dexamethasone induce a small level of expression of nur77 (Fig 2.16 and 2.17).

In conclusion Nurr1 and nur77 are expressed in multiple mouse tissues, paralleling the results obtained in human tissue. NOR-1 expression could not be detected in either 3T3-L1 cells or primary pre-adipocyte cells. Nurr1 demonstrated more specific expression in the stromal vascular fraction of both adipose depots and coupled with the more specific induction of this gene in both 3T3-L1 cells and primary pre-adipocytes it was decided to focus on this family member to continue researching its role for in adipose tissue function and adipogenesis. In order to investigate the role of Nurr1 in adipogenesis a method of sustained expression is required. As the pharmacological tools are unavailable the alternative method is over-expression using adenovirus. As stated in Chapter

1, these receptors function as constitutive activators when expressed *in vitro*. These investigations will be described in Chapter 3.

Chapter 3: Effect of Nurr1 over-expression on adipogenesis and adipocyte function

Introduction

In vitro adipogenesis

The mechanisms of terminal adipocyte differentiation are well defined and clearly understood. A great deal of this work has been made possible through the use of the immortalised murine cell lines such as 3T3-L1, F442A and ob1771 preadipocytes that subsequently under-go differentiation upon addition of the appropriate hormone cocktail (Green 1974, Green 1975, Green 1976, Ailhaud 1992). These cells are morphologically similar to fibroblastic pre-adipose cells found in the stroma of adipose tissue and exhibit virtually all the same characteristics associated with adipocytes present within adipose tissue (Novikoff 1980). They also can form a fat pad when injected into nude mice (Green 1979).

Initiation of adipocyte differentiation results in a number of morphological and biochemical changes within the cell. These changes are brought about in the stages summarised below:

1. Pre-confluent proliferation
2. Growth arrest
3. Hormonal induction/Clonal expansion
4. Permanent growth arrest/terminal differentiation

Activation of this program is typically accomplished in 4-6 days.

One of the most common methods of differentiation involves the treatment of post-confluent cells with insulin, isobutylmethylxanthine (IBMX) and dexamethasone for two days, followed by an additional two days of insulin only (Cornelius 1994, Guller 1988, Hauner 1990, Schmidt 1990, MacDougald 1995). Within 72hrs the cells have adopted a rounded shape and deposition of lipids within the cytoplasm begins, until greater than 90% of the cytoplasm is filled with lipids.

Numerous proteins are expressed as a result of adipocyte differentiation (Cornelius 1994). Many of these are expressed as a direct result of the differentiation process, whilst others are pertinent to the process. Some are involved with lipid metabolism such as lipoprotein lipase and Glucose transporter IV (Vu 1996, Kaestner 1990). Others such as aP2 are adipocyte specific genes but are not necessary for the differentiation process itself. It is the study of genes such as aP2 which has brought about the identification of transcription factors and other proteins involved in the regulation of adipocyte differentiation (Cook 1988).

Regulation of adipogenesis

Three classes of transcription factors have been identified that directly influence fat cell development. The key players in this process are the CCAAT/enhancer binding proteins (C/EBP's), peroxisome proliferator-activated receptor (PPAR's) and the basic helix-loop-helix family (ADD1/SREBP1c).

PPARγ is now widely recognised as serving an important role in adipogenesis (Chawla 1994a, 1994b). Two isoforms of PPARγ exist, produced through the use of alternate promoters, PPARγ1 and PPARγ2, the latter containing an additional 28 amino acids at the N-terminus. PPARγ2 has been reported to be the form most associated with adipocyte differentiation and is highly expressed in adipose tissue. Evidence linking its role to adipogenesis includes the demonstration that over-expression of PPARγ2 in NIH3T3 cell line under adipogenic conditions results in induction of adipocyte differentiation (Tontonoz 1994). PPARγ heterodimerises with retinoid X receptors (RXR) alpha, beta and gamma which are also expressed in adipose tissue (Gearing 1993, Keller 1993).

Three members of the CCAAT/enhancer binding protein (α, β and δ) family of transcription factors were the first demonstrated to play a role in adipocyte differentiation (Cao 1991). C/EBPβ and C/EBPδ were initially proposed to be involved in the process based on observations that their expression is induced by

cAMP, insulin and dexamethasone and their induction profile is rapid and transient. The transient nature of expression is due to the fact that their sole purpose appears to be to initiate transcription of the PPARγ gene (Cao 1991, Yeh 1995, MacDougald 1994).

PPARγ contains within its promoter sequence a C/EBP binding site and therefore its expression is regulated by the C/EBP's. C/EBPα is also regulated by C/EBPβ and δ. Coincident with the rise in expression of PPARγ and C/EBPα, the levels of C/EBPβ and δ down regulated. Until recently, the model for the transcriptional cascade leading to adipogenesis was that C/EBPβ and δ induce low levels of PPARγ and C/EBPα, which are then able to induce each others expression through a positive feedback loop. However, Spiegelman's group have now shown that C/EBPα induces adipogenesis through PPARγ (See Fig. 3.1) (Rosen 2002).

It had been shown that PPARγ can promote adipogenesis in CEBPα deficient cells but to prove the converse had always been difficult as PPARγ knock-outs are embryonic lethal. Spiegelman's group created a fibroblastic cell line that lacked PPARγ and showed that C/EBPα has no ability to promote adipogenesis on its own. So it would appear that C/EBPα function is to induce expression of PPARγ (Rosen 2002). However, C/EBP does confer insulin sensitivity onto the adipocyte, via effects upon the transcription of IR and IRS-1 (Wu 1999). It also activates a number of adipocyte genes such as leptin and adipsin (Hollenberg 1997).

ADD1/SREBP1c, which plays a role in terminal differentiation, is a membrane bound basic helix-loop-helix leucine zipper transcription factor that upon transactivation is proteolytically cleaved with the soluble form translocating to the nucleus where it can bind two classes of response elements (Brown 1997). One of these response elements is an E-Box, a helix-loop-helix consensus sequence and the second is the unrelated sterol response element (Murre 1989, Yokoyama 1993). Upon cleavage, the activated form of ADD1/SREBP1c is responsible for

inducing the expression of genes involved with both lipid and cholesterol metabolism. There are several lines of evidence which suggest that ADD1/SREBP1c is involved in the regulation of adipogenesis. Expression of a dominant negative form inhibits the differentiation of 3T3-L1 cells (Kim 1996). Over-expression of ADD1/SREBP1c in NIH3T3 cells allows differentiation following addition of a PPARγ ligand. Furthermore the co-expression of ADD1/SREBP1c and PPARγ2 result in a synergistic effect on adipocyte differentiation (Kim 1996). It has also been postulated that ADD1/SREBP1c activated genes lead to the synthesis of endogenous ligands for PPARγ (Fajas 1998). ADD1/SREBP1c is responsible for stimulating the expression of many of the genes necessary for lipogenesis *in vivo,* including lipoprotein lipase, fatty acid synthase, resistin and glycerol phosphate acyltransferase (1996, Seo 2003, Ericsson 1997).

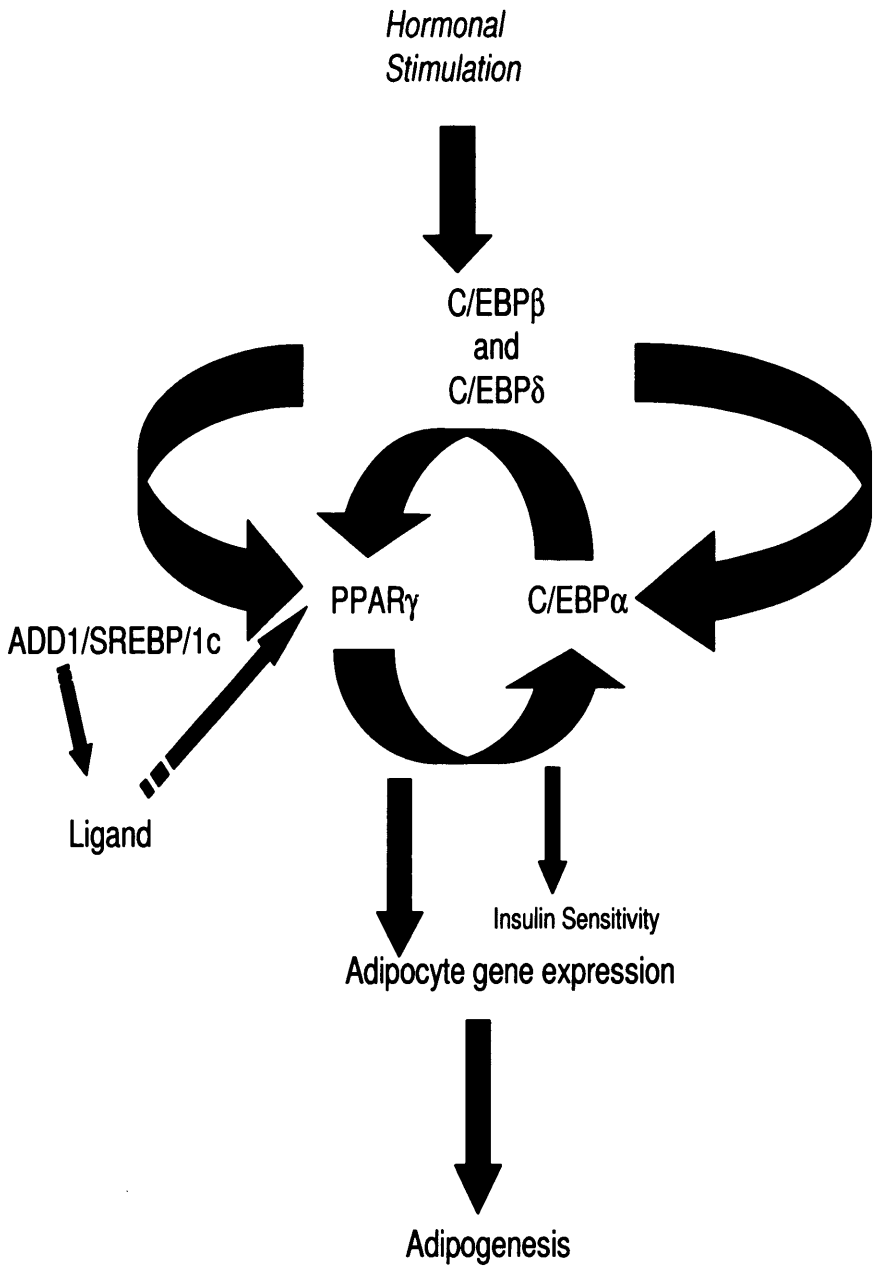


Fig. 3.1 Model of adipogenesis

Adapted from Rosen et al (2002) Genes & Dev. 16(1):22-26

Inhibitors of adipogenesis

Research efforts to date have largely been interested in the field of adipocyte differentiation and regulation. The subject of inhibition of adipogenesis has largely been ignored until recently. There is now a growing volume of literature examining many of the genes and signalling pathways proposed to be involved in the early events of adipogenesis and a growing number of putative genes involved in inhibition. Whilst a large number of mRNAs whose levels increase during adipocyte differentiation have been studied and characterised, very few have been identified that are down-regulated during adipocyte differentiation. Early studies focussed on the preadipocyte factor-1 (Pref-1) and retinoic acid mediated inhibition. However, more recently a new area of interest has opened up examining the Wnt signalling pathway. Other areas of study include increases of calcium concentration (and Calcineurin) (Neal 2002), arylhydrocarbon receptor (AhR) (Shimba 2001), PTHrP (Chan 2001), resistin-like molecule α (RELMα) (Blagoev 2002).

Pref-1 – Preadipocyte factor-1:

Pref-1 is a member of the epidermal growth factor –like (EGF) family (Smas 1993). It is solely found in the stromal vascular fraction and not the adipocyte fraction of adipose tissue. Proteolytic cleavage of Pref-1 results in two soluble forms, the larger of which inhibits adipocyte differentiation (Mei 2002). Pref-1 is highly expressed in 3T3-L1 pre-adipocytes at confluency and expression at both the mRNA and protein level is diminished upon differentiation. This down regulation is primarily at the transcription level (Smas 1998). *In vitro* differentiation of 3T3-L1 cells as described earlier involves the addition of a cocktail of dexamethasone, insulin and IBMX. Studies have shown that the reduction in pref-1 expression in these cells is comparable between the complete differentiation cocktail and dexamethasone alone, indicating a negative regulation by glucocorticoids (Smas 1999). It has also been shown that constitutive over-expression of pref-1 in pre-adipocytes blocks their conversion to adipocytes (Sul

2000). Although the decrease of pref-1 expression is only an early event and its expression may no longer be required for the differentiation process, from the data presented to date it is possible that pref-1 may have a role in maintaining the cells in an undifferentiated state. Xing *et al* suggested that pref-1 may be a non-reversible molecular checkpoint early in the differentiation process (Xing 1997).

Retinoic acid inhibition:

Retinoic acid (RA) is a known inducer of differentiation in many biological systems (Sporn 1983). However, it also acts an inhibitor of adipocyte differentiation (Kuri-Harcuch 1982, Sato 1980). The actions of RA are predominantly mediated by retinoic acid receptors (RARs) (Xue 1996). *In vitro*, adipocyte differentiation is efficiently inhibited when RA is added shortly after the exposure of pre-adipocytes to the differentiation cocktail (Kuri-Harcuch 1982, Stone 1990). There is convincing data to suggest that liganded RAR (alpha and gamma) prevents the transcriptional activity of C/EBP protein and that it is this mechanism which underlies the inhibitory effect of RA on differentiation. This mechanism would not seem to involve RAR binding to RARE's as a dominant negative RAR fails to prevent inhibition of adipogenesis (Schwarz 1997).

Wnt signalling:

Wnts are secreted signalling proteins that regulate many developmental processes (Cadigan 1997). They signal through binding to transmembrane receptors of the *frizzled* family. Initial *in vitro* experiments to assess the role of Wnt in adipogenesis were performed using Wnt-1. It was shown that 3T3-L1 cells expressing Wnt-1 failed to differentiate. Subcutaneous injections of 3T3-F442A cells expressing Wnt-1 into mice, resulted in mice with pads composed entirely of fibroblastic cells, indicating that Wnt signalling can repress adipocyte differentiation in a more physiological relevant model (Ross 2000).

Further work carried out to assess which of the Wnts were expressed in 3T3-L1 cells identified Wnt-5a and Wnt-10b. Only over-expression of Wnt-10b stabilised

β-catenin (regulator of Wnt target genes) and potently blocked adipogenesis. mRNA analysis of 3T3-L1 cells revealed that Wnt-10b expression is highest in growing and confluent cells and then decreases upon addition of differentiation cocktail (Ross 2000). Suppression of Wnt-10b can be brought about by the addition of IBMX alone and this suppression is not cell specific. This would suggest that elevation of intracellular levels of cAMP is responsible for the decrease in Wnt-10b expression during adipogenesis. Treatment of confluent preadipocytes with a cell permeable analogue of cAMP supported this conclusion (Bennett 2002).

Aims and objectives of this chapter

This chapter will investigate the effects of Nurr1 over-expression, using adenoviral expression system in differentiating 3T3-L1 cells. The effects of Nurr1 over expression will be investigated on:

(i) Growth cellular morphology by microscopy.

(ii) mRNA expression of adipogenic transcription factors and adipocyte marker genes.

(iii) Lipolysis and glucose uptake by adipocytes.

Experimental Procedures

Generation of Nurr1 adenovirus construct.

Nurr1 (Human) cDNA was PCR amplified from another plasmid containing the gene (GSK) with the addition of KpnI and XhoI restriction sites and a FLAG Tag.

Forward primer 5'-3' ggtaccatggattacaaagatgatgatgataagccttgtgttcagg

Reverse primer 5'-3' tctagattagaaaggtaaagtgtccag

The amplified product was cloned into the pCR2.1 Topo Cloning (Invitrogen) vector and then excised from this vector using the KpnI and XhoI (NEB, Herts. UK) restriction sites and ligated into pAdTrackCMV-GFP, which had also been digested with the same enzymes. Clones were checked for integrity by double strand sequencing and confirmed to be correct.

Generation of admid DNA: Nurr1pAdTrackCMV-GFP construct was linearised with PmeI (NEB), run on a 1% EtBr agarose gel and DNA extracted using Qiagen Gel Extraction Kit (as per manufacturer's instruction) (Qiagen, Sussex UK). BJ5183-Ad1 (Stratagene, Amsterdam Netherlands) cells were transformed with the Nurr1 construct. 40µl of electrocompetent cells were mixed with 2ul of Nurr1 and transferred to an electroporation cuvette. Electroporation was performed at 200Ω, 2.5kV, 25µF. 1ml LB was then added immediately and cells incubated with shaking for 1hr at 37°C. 100ul of transformed cells were plated on LB-Kanamycin plates and incubated over-night at 37°C. 12 of smallest colonies were picked and grown in 5ml of LB-Kanamycin overnight. DNA was prepared from 1.5ml of culture the following morning. Admid DNA was checked with a PacI (Promega) digest which should yield a large 30kb fragment plus a smaller one at 3 or 4.5kb.

Admid DNA which gave the correct banding pattern was re-transformed into XL10-Gold (Stratagene) cells for propagation. Diagnostic digests to check integrity of construct were also performed.

Generation of adenovirus: HEK293 cells are routinely cultured in EMEM, 10%FBS, 1% Non-Essential Amino Acids and 2mM L-Glutamine (all Invitrogen). T75 flasks were seeded with 2 x 10^6 HEK293 cells 24 hrs prior to

transfection, so that the confluency would be 50-70% at transfection. Cells were transfected with PacI linearised Nurr1 pAdTrackCMV-GFP (4μg), using Lipofectamine Plus (as per manufacturers instruction - Invitrogen) and incubated for 3hours, prior to washing the cells and replacing with fresh growth media. Cells were harvested approx. 5 days post-transfection. To harvest, cells were pelleted by centrifugation, resuspended in PBS and the virus released by 3 cycles of freezing-thawing, followed by pelleting of the cell debris. Supernatant was collected into a fresh tube and virus stored at -20°C.

Virus was then further amplified by 2 subsequent rounds of amplification in HEK293 cells. Cells were harvested 5-7 days post-infection and virus collected in the same fashion. Expression in mammalian cells was tracked using the GFP marker.

Transduction of 3T3-L1 cells with Nurr1pAdTrackCMV-GFP

3T3-L1 cells were cultured as described in Chapter 2. Prior to transduction, cells were seeded at density of $2x10^4$ cells per well in 24 well plates and left to attach of 4-24hrs.

Nurr1pAdTrackCMV-GFP virus (MOI ~100-200) was incubated with Poly-L-Lysine (0.5ug/ml) (Sigma) for 30 mins prior to the addition of the transduction mix to the cells. The transduction mixture on the cells was left to incubate over-night at 37°C 5%CO_2. Media was then removed the following morning and replaced with fresh growth media. Transduction efficiency was assessed by level of GFP fluorescence.

Western blot analysis of Nurr1 protein

3T3-L1 cells transduced with no virus, GFP virus or Nurr1 virus for 96hrs. Cells harvested in RIPA buffer (Sigma Aldrich) and snap frozen. Protein lysates runs on 4-20% Tris-Glycine gel (Invitrogen) before transferred to nitrocellulose membrane (Pierce Rockford, IL, USA) using Semi-Dry Blotter following manufacturer's conditions (Bio-Rad UK). Membrane blocked with 2.5% blocking buffer for 1hr before incubation with monoclonal FLAG antibody (Sigma Aldrich F3165) for 1hr and subsequently mouse secondary antibody,

Alexa Fluor 680 allophycocyanin (Invitrogen/Molecular Probes A21000). Membrane finally washed in PBS-tween before visualisation on Licor Odyssey system (Licor BioSystems, Cambridge UK).

Differentiation of 3T3-L1 cells

Following virus removal, cells were allowed to reach confluency. Post confluent cells were then treated with the adipocyte differentiation induction medium – dexamethasone 2.5nM (Sigma Aldrich), isobutylmethylxanthine 0.5mM (IBMX Sigma Aldrich) and insulin 5mg/ml (Sigma Aldrich). Wild type (WT) 3T3-L1 cells treated in same manner. After 48 hours post-induction media was removed and replaced with DMEM + 10% FBS and insulin only (5mg/ml) for the duration of the experiment and changed every 2-3 days. RNA samples were isolated at T0, T24, T48, T72, T96, T168 and T216 hours post-induction.

Isolation of RNA from differentiating 3T3-L1 cells

Media was removed and cells washed briefly with 1ml of PBS, followed by the addition of 500µl Trizol (Sigma Aldrich) per well to lyse the cells. All samples were stored at -80°C and batch processed. Samples were thawed on ice ready for RNA extraction. To each sample 100µl of chloroform was added and sample tubes mixed thoroughly. Tubes were then placed on ice for 15-20mins to allow separation of the phases. Samples were then centrifuged at room temperature for 10mins at 12000 x g. Following centrifugation, the upper aqueous phase was carefully removed to a new RNAse/DNAse free tube. 0.7 volume of isopropanol was then added to each. To facilitate a better precipitation of RNA, tubes were incubated for 1-2 hours at -20°C. Samples were then centrifuged at 12000 x g for 10 mins to pellet the RNA. Supernatants were carefully removed and washed with 100µl of 70% ethanol. Following another centrifugation step (5mins 12000 x g), supernatant was removed and the pellet air-dried. Finally the RNA pellet was resuspended in 25µl DEPC water.

cDNA synthesis

Prior to cDNA synthesis, all RNA samples were subjected to DNAse treatment to remove any contaminating DNA in the sample. To each RNA sample, 1 unit of DNAse (Promega) 3µl of 10x Buffer and then made up to a final volume of 35µl with DEPC water and incubated at room temperature for 30mins. The reaction was stopped by the addition of 1µl Stop reagent and incubated at 65°C for 5 mins. Tubes were then returned to ice.

The concentration of RNA was measured by optical density at 260nm; an OD of 1 is equivalent to 40µg/ml RNA in an undiluted sample. All readings were performed on a NanoDrop Spectrophotometer. 1µl of undiluted sample is placed on the machine's aperture and an OD 260/280 performed, calculating the concentration per µl and also the quality of RNA. Pure RNA would have and A260/A280 ratio of approximately 2. Deviations from this indicate protein or RNA contaminants.

1µg of RNA was mixed with 1µl (0.5ug) of oligo dT (Invitrogen) and made up to 12µl with DEPC water and annealed at 72°C for 15 mins. The samples were then cooled slowly on ice for 15mins. To this 5 x First Strand Buffer, 2µl DTT (100mM), 1 µl dNTP's (10mM.) and 1µl of SuperScript II Reverse Transcriptase (Invitrogen) were added giving a total volume of 20µl. Samples were then incubated at 42°C for 45 mins, followed by a heat inactivation step at 70°C for 15 mins and then cooled at 4°C. To each reaction 80µl of DEPC water was added, giving a final concentration of 10ng/µl.

SybrGreen PCR analysis

Semi-quantitative polymerase chain reaction was carried out using Sybrgreen and specific primers for β-Actin, Nurr1, PPARγ, C/EBPα, Perilipin, HSL and CD36 (Sigma Genosys). Master Mix was prepared containing 12.5µl of 2x SyBrgreen Master Mix (Applied Biosystems, Warrington UK), 1µl of forward primer (10mM), 1µl of reverse primer (10mM), 5.5µl of dH_2O and 5µl of cDNA. Semi-quantitative PCR was performed on the ABI 7700 using standard ABI7700 PCR

conditions. Genomic standards were run in parallel to facilitate relative quantification.

Primer sequences:

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
β-Actin	gagctatgagctgcctgacg	agtttcatggatgccacagga
Nurr1	tcgacatttctgccttctcc	ccactctcttgggttccttg
nur77	ggtatccgaaagtgggcaga	cggaggatgaagagttccag
PPARγ	gagcccaagtttgagtttgc	ggcggtctccactgagaata
C/EBPα	ggaacagctgagccgtgaac	gcgacccgaaaccatcct
CD36	ccattggtgatgaaaagca	atcaccactccaatcccaag
HSL	tggtgcagagagacacttcg	ggcttgcgtccacttagttc
Perilipin	aaggatcctgcacctcacac	gtaacacccttcagggcatc

Fig. 3.2. Primer sequences for SyBrGreen PCR analysis

Analysis of lipid content in differentiated 3T3-L1 cells

Cells were seeded into the appropriate vessel for downstream analysis. For confocal studies cells were seeded at density of $3x10^4$ cells on to a confocal slide in a 60mm tissue culture dish (Nunc). Cells were allowed to attach and grow to confleuncy. Post confluent cells were then treated with the adipocyte differentiation induction medium – DMEM/FBS containing - dexamethasone 2.5nM (Sigma Aldrich), isobutylmethylxanthine 0.5mM (IBMX Sigma Aldrich) and insulin 5mg/ml (Sigma Aldrich). 48hours post-induction, the hormone

cocktail was removed and replaced with DMEM/FBS + insulin (5mg/ml) for the duration of the experiment, changing media every 2-3 days.

Oil Red O Staining:

Media was removed from differentiating cells and cells washed with PBS. Cells were then fixed with 3.7% formaldehyde for 1hour. Fixed cells were then washed twice with PBS, before addition of Oil Red O for 1 hour. Cells were then rinsed with PBS and images viewed on an inverted microscope and photographed.

Oil Red O (4.2g) was dissolved in 1200ml of absolute isopropanol and left without stirring over-night at room temperature. This solution was then vacuum filtered and diluted with 900ml of distilled water and left over-night without stirring and then filtered twice.

Confocal analysis - Zeiss LSM 510:

$\Delta\Psi_m$: For measurement of lipid content and $\Delta\Psi_m$, adipocytes were loaded with 1 μM Bodipy 493/503, a neutral lipid dye, and 25 nM tetramethyl rhodamine methyl ester (TMRE) (Invitrogen/Molecular Probes D3922), which were allowed to equilibrate for 30 minutes. Cells were maintained in a HEPES buffered physiological salt solution containing 156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose and 10 mM Hepes, pH adjusted to 7.4 with NaOH. TMRE is a lipophilic cationic dye that equilibrates between compartments in response to potential differences. TMRE was used at very low concentrations (30 nM), when the signal is proportional to dye concentration, which in turn depends on the potential differences between compartments, so that comparative estimates of $\Delta\Psi$ may be obtained from confocal images (Mojet 2001). TMRE fluorescence was excited using the 543 nm HeNe laser line and measured at >590 nm using a Zeiss 510 CLSM confocal microscope and measurements were made from projections of Z-stack data to avoid sampling bias from a restricted optical section. A decrease in TMRE signal indicates mitochondrial membrane depolarisation (Loew 1993; Boitier 1999). $\Delta\Psi_m$ was related to lipid content by coloading with Bodipy 493/ 503 (excitation at 488 nm, measured at 505-550 nm). The images were then binarised above a threshold, so

that all pixels containing signal (lipid droplets) were set to unity and all background to zero, again using a projection of a z-stack to include the full cell volume. The mean signal per cell was then expressed simply as a fractional value – total lipid engorgement could give a signal of one, no lipid at all a value of zero.

Lipolysis analysis

Primary adipocytes (+/-Nurr1 adenovirus) were differentiated as described previously using insulin, dexamethasone, IBMX and BRL 49653 until >90% cells contained lipid (as assessed by light microscopy). Cells then incubated over-night in Cell-Gro (VWR). Following this pre-incubation, cells were then incubated for 5hrs in Cell-Gro containing one of the following β-adrenergic agonists - 1μM dobutamine (Sigma Aldrich), 1μM clenbutarol (Sigma Aldrich), 1μM CL316243 (gift from Gokhan Hotamisligil, Harvard University, USA) or with no agonist addition. Following the incubation period, supernatants were moved and assayed for NEFA release using a NEFA C kit (Wako VA USA), measuring at OD550nm.

Glucose uptake

3T3-L1 cells were differentiated as described previously using dexamethasone, insulin and IBMX. Once cells were assessed to be >80% differentiated they were treated with Nurr1 adenovirus or media alone for 24 hrs, then incubated for a further 72-96 hrs with appropriate media changes, and then 24hrs prior to the analysis media was replaced with Cell-Gro without Insulin. Cells were washed with Krebs Ringer Phosphate Buffer, pH7.4 (Sigma Aldrich), at 37°C for 60mins. Cells were then incubated +/- insulin (1μg/ml) for 30 mins. After 10mins, the glucose uptake inhibitor cytochalasin B (5μM) (Sigma Aldrich) was added to the appropriate wells. After a subsequent 10mins incubation 1μl tritiated deoxyglucose (^{3}H-DOG Perkin Elmer 37MBq/ml, Bucks. UK) was added per well and incubated for a further 10mins. Cells were then placed on ice and washed twice with cold PBS (Invitrogen), then lysed with 850μl of 0.1% SDS by

repeat pipetting. 800μl was then added to 10ml of scintillation fluid and the amount of ^{3}H-DOG uptake measured by liquid scintillation counting.

Statistical analysis

Results are expressed as mean±SD or SEM for normally distributed values or median (interquartile range) for skewed data. Comparisons between groups were made by two-tailed Student's *t*-test, Mann-Whitney U test or Wilcoxon rank test, as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Over-expression of Nurr1 in differentiating 3T3-L1 cells

Over-expression of Nurr1 was successfully achieved in 3T3-L1 cells, with an average of 40-50% transduction efficiency achieved in all experiments. This equated to approximately a 60 fold over-expression (assessed by mRNA analysis). Although there would seem to be a bi-phasic response, this is bourne out by the SEM values and expression would appear to be relatively consistent with expression levels dropping at the later time point. Expression was easily tracked by the use of the GFP marker (Fig 3.3 and 3.4). Transduction efficiency with the GFP null virus was similar (Fig 3.5). Over-expression of Nurr1 is also evident from the Nurr1 western blot, using FLAG antibody, which shows a band of the correct size (68kD) (Fig 3.6). There is also non-specific bands evident in the other lanes but these are above that of the Nurr1 protein.

Analysis of markers of adipogenesis

PPARγ and C/EBPα were tracked as markers of adipogenesis. PPARγ expression was induced within the first 24 hours in control WT cells (non-infected) and continued to increase until 96 hours, after which expression was more or less maintained. In the Nurr1 over-expressing cells, expression also increased at 24 hours but did not increase any further after 48 hours and was sustained at levels which were about 50% of that of the control WT cells (Fig 3.7).

In contrast to PPARγ, little or no difference was seen in C/EBPα expression, with levels increasing in the first 24 hours and peaking at 48 hours (Fig 3.8). At 48 hours the levels of C/EBPα in Nurr1 overexpressing cells was about 2 fold higher that of control WT cells, but levels returned to similar thence after. This difference in expression between the control WT and Nurr1 over-expressing cells at 48hrs is insignificant. A decrease in expression is evident in the Nurr1 over-expressing cells at the later time points.

The difference between control WT and Nurr1 over-expressing cells was most apparent in the accumulation of lipid within the cells (see next result section), which was also supported when genes involved in lipid accumulation were

analysed. Perilipin expression increased in parallel to control WT cells for the first 72 hours. However in Nurr1 over-expressing cells, levels fell to practically nothing in comparison with control WT cells (Fig 3.9). Hormone Sensitive Lipase (HSL) only reached expression levels which were 50% of that found in control WT cells, but the pattern of expression was mirrored (Fig 3.10). Finally, the level of CD36 expression achieved was only 20% that observed in control WT cells (Fig 3.11).

Data is expressed as % expression, as opposed to % induction, as the length of time of expression is longer than that observed in Chapter 2. % expression is calculated from gene of interest/β-actin from a number of experiments.

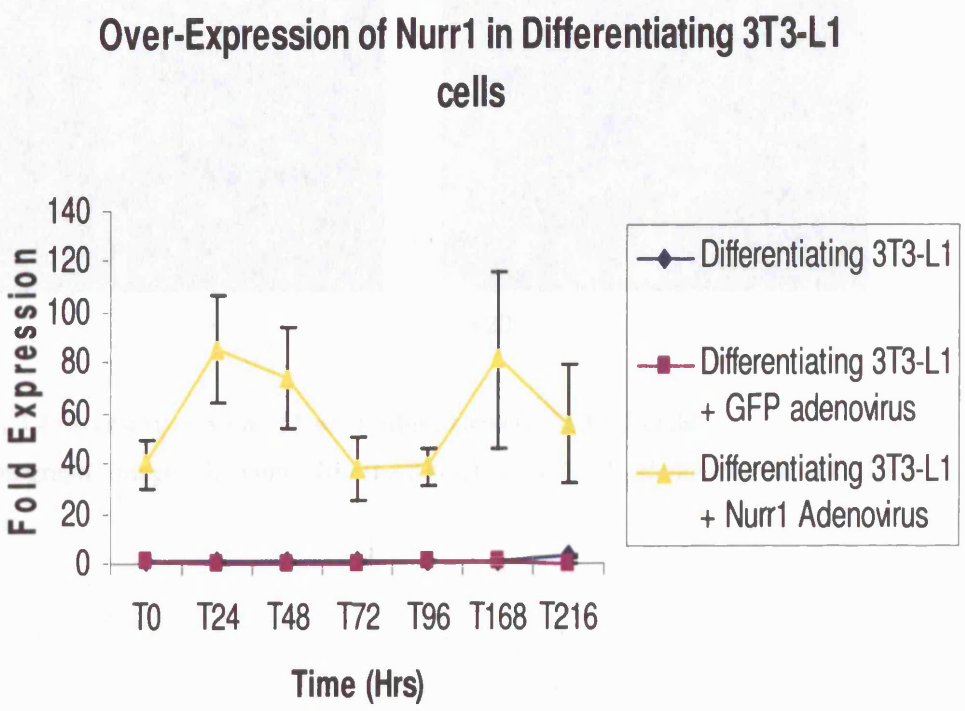


Fig. 3.3 Over-expression of Nurr1 adenovirus in 3T3-L1 cells

Fold expression, over and above basal levels of Nurr1 in differentiating 3T3-L1 cells. Graph depicts Nurr1 over-expression in the 3T3-L1 cells infected with either Nurr1 adenovirus, GFP adenovirus or no virus. Data expressed as mean fold expression compared to T0 of differentiating 3T3-L1 cells +/- SEM, n=5, as assessed by SybrGreen analysis. No statistically significant differences in Nurr1 expression between GFP (pink) and normally differentiating 3T3-L1 cells (blue) at any time-point (all $p>0.05$), but in the cells with Nurr1 adenovirus significant elevation in Nurr1 expression is apparent at all time-points (all $p<0.001$).

x10 x20

Fig. 3.4 Over-expression of Nurr1 adenovirus in 3T3-L1 cells

Photograph image showing Nurr1pAdTrackCMV-GFP adenovirus in 3T3-L1 cells.

a) b)

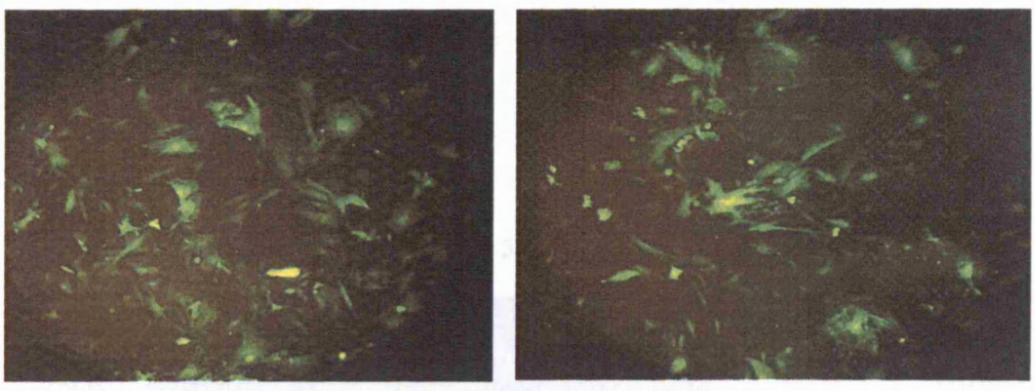


Fig. 3.5 Comparison of Nurr1 and GFP null adenovirus

Photographs showing GFP expression in a) Nurr1pAdTrackCMV-GFP vs b) pAdTrackCMV-GFP null adenovirus.

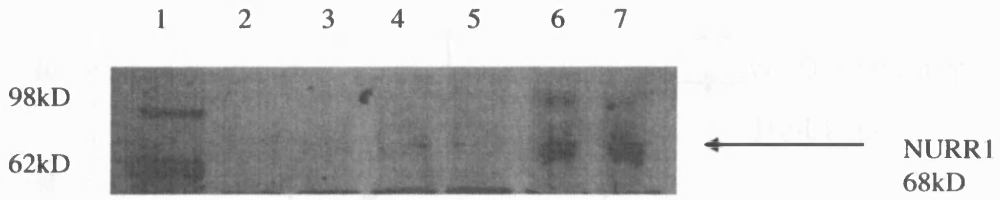


Fig. 3.6 Western blot analysis of Nurr1 over-expression using anti-FLAG antibody.

Membrane blot showing control non-infected 3T3-L1 cells (lane 2+3), GFP adenovirus (lane 4+5), Nurr1 adenovirus (lane 6+7). Primary antibody, mouse anti FLAG monoclonal, secondary antibody, goat anti-mouse Alexa Fluor 680 allophycocyanin

Image viewed using Licor Odyssey system.

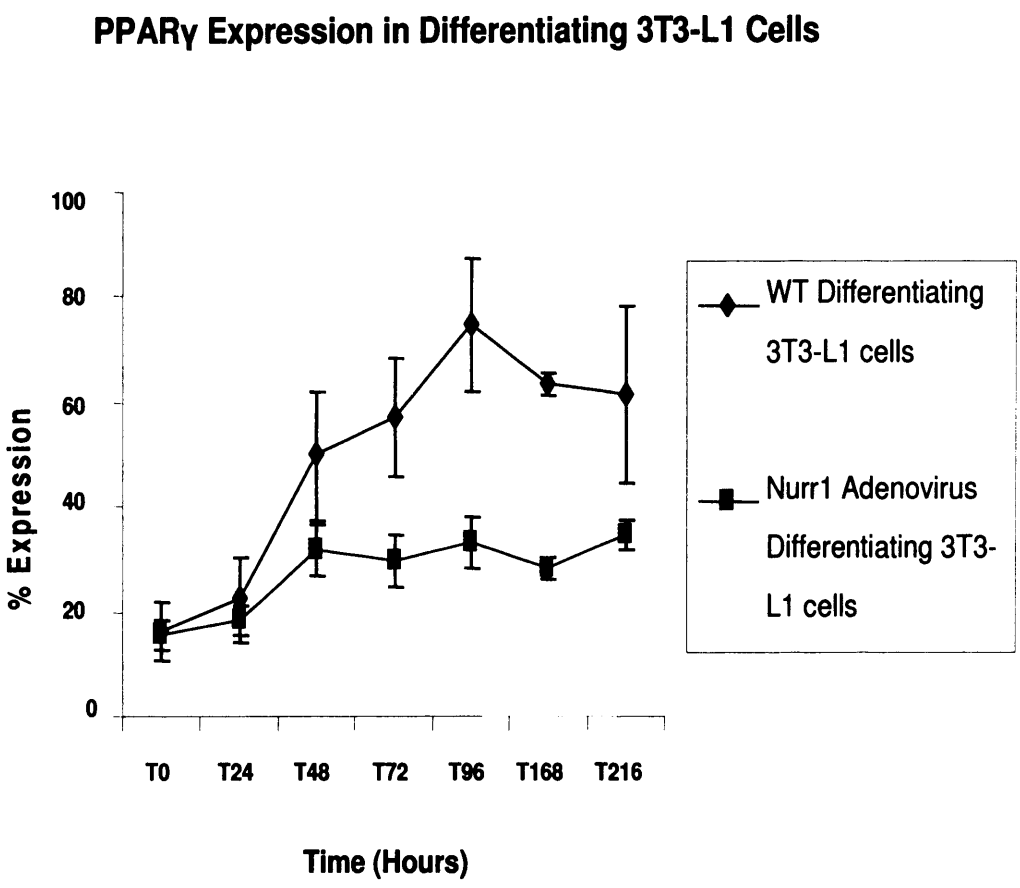


Fig. 3.7 Expression of regulatory genes during adipogenesis in 3T3-L1 cells
Expression of PPARγ in control non-infected WT and Nurr1 over-expressing 3T3-L1 cells. Data expressed as mean % expression of T0 of WT, after normalisation to β-actin, +/- SEM, n=4, as assessed by SybrGreen analysis. T0 values are expression of PPARγ in pre-adipocytes compared to those that showed no differentiation. There was significant elevation in PPARγ expression after T48 in all cells ($p<0.01$), however this elevation was suppressed in Nurr1 cells compared to WT at T48-T216 (all $p<0.001$).

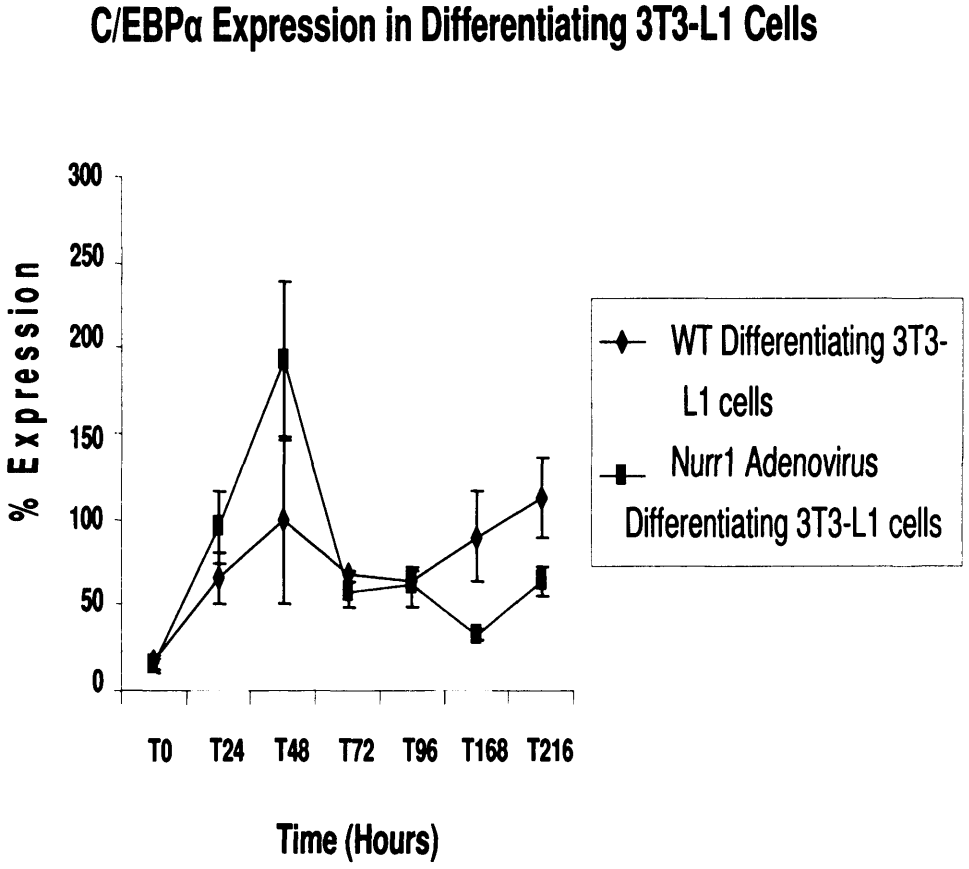


Fig. 3.8 Expression of regulatory genes during adipogenesis in 3T3-L1 cells
Expression of C/EBPα in control non-infected WT and Nurr1 over-expressing 3T3-L1 cells. Data expressed as mean % expression, following normalisation to β-actin, +/- SEM, n=4, as assessed by SybrGreen analysis. T0 values are mean expression of C/EBPα in pre-adipocytes capable of differentiating compared to those that showed no differentiation. There was significant supression in C/EBPα expression in only T168 and T216 (both $p<0.01$) in Nurr1 compared to WT cells.

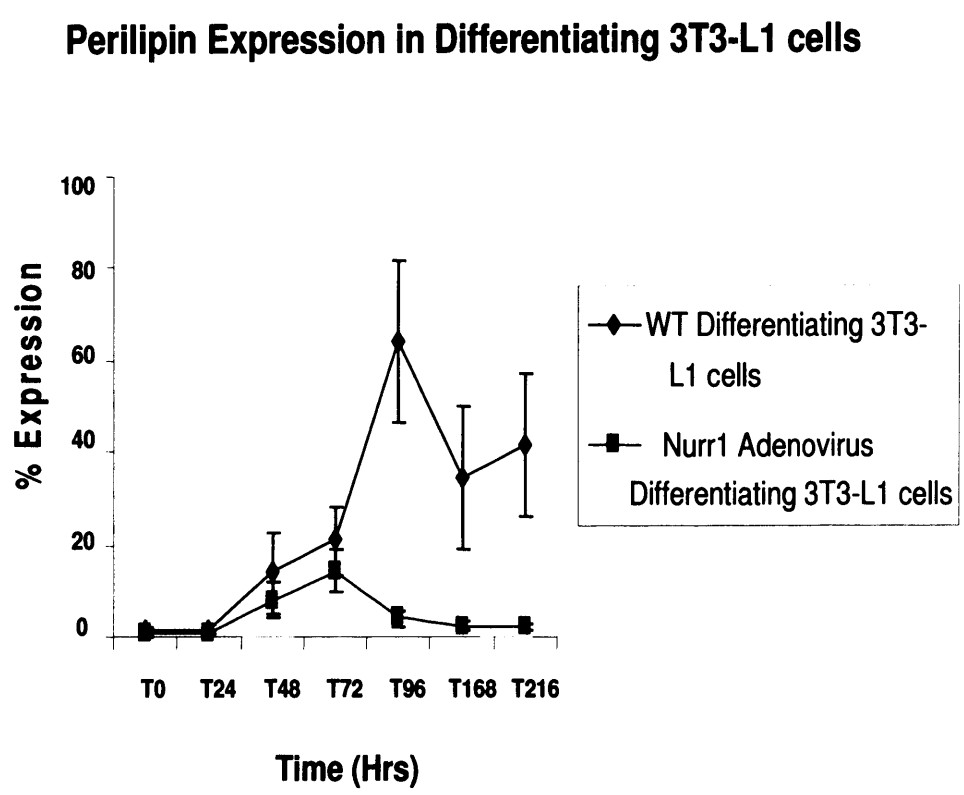


Fig. 3.9 Expression of adipocyte genes during adipogenesis in 3T3-L1 cells.
Expression of perilipin in control non-infected WT and Nurr1 over-expressing 3T3-L1 cells. Data expressed as mean % expression of T0 of WT cells, following normalisation to β-actin, +/- SEM, n=4, as assessed by SybrGreen analysis. Compared to T0, expression was significantly elevated in WT cells at T72 to T216 (all $p<0.001$), however, while in Nurr1 cells expression increased at T48 and T72 compared to T0 ($p<0.01$) all other levels were not different from that seen T0 and were significantly lower than those in WT cell ($p<0.001$).

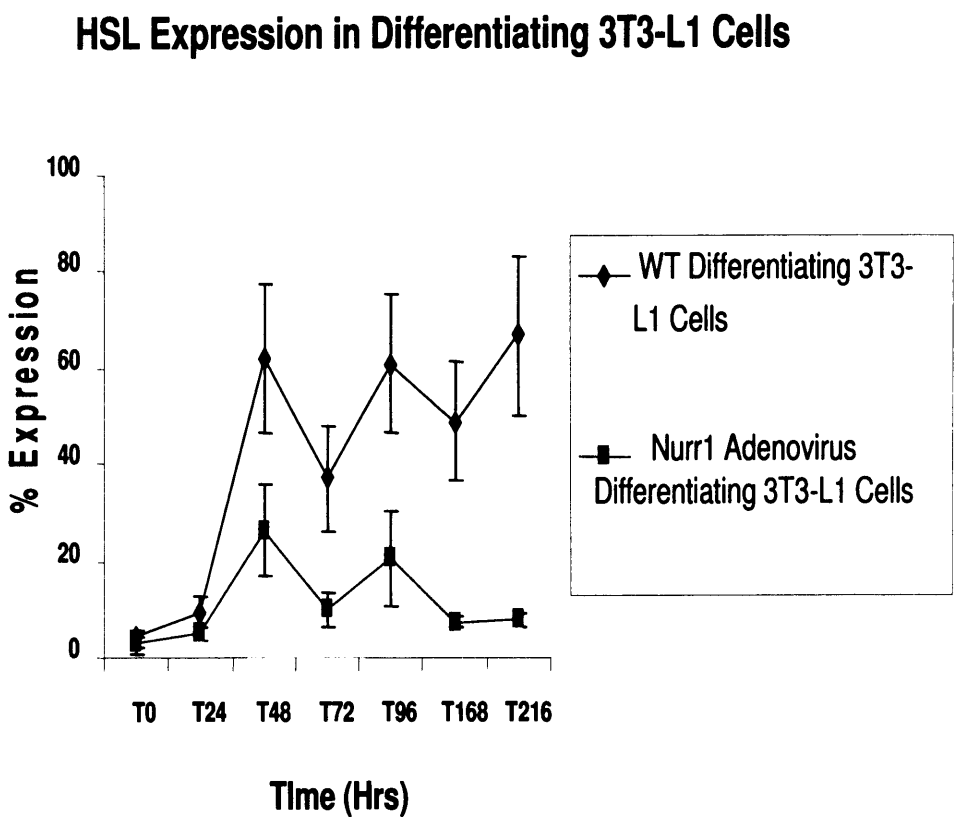


Fig. 3.10 Expression of adipocyte genes during adipogenesis in 3T3-L1 cells.
Expression of Hormone Sensitive Lipase in control non-infected WT and Nurr1 over-expressing 3T3-L1 cells. Data expressed as mean % expression of T0, following normalisation to β-actin, +/- SEM, n=4, as assessed by SybrGreen analysis. Levels significantly elevated following induction of differentiation (T48 - T216) in WT cells but remained suppressed in Nurr1 cells (all $p<0.001$).

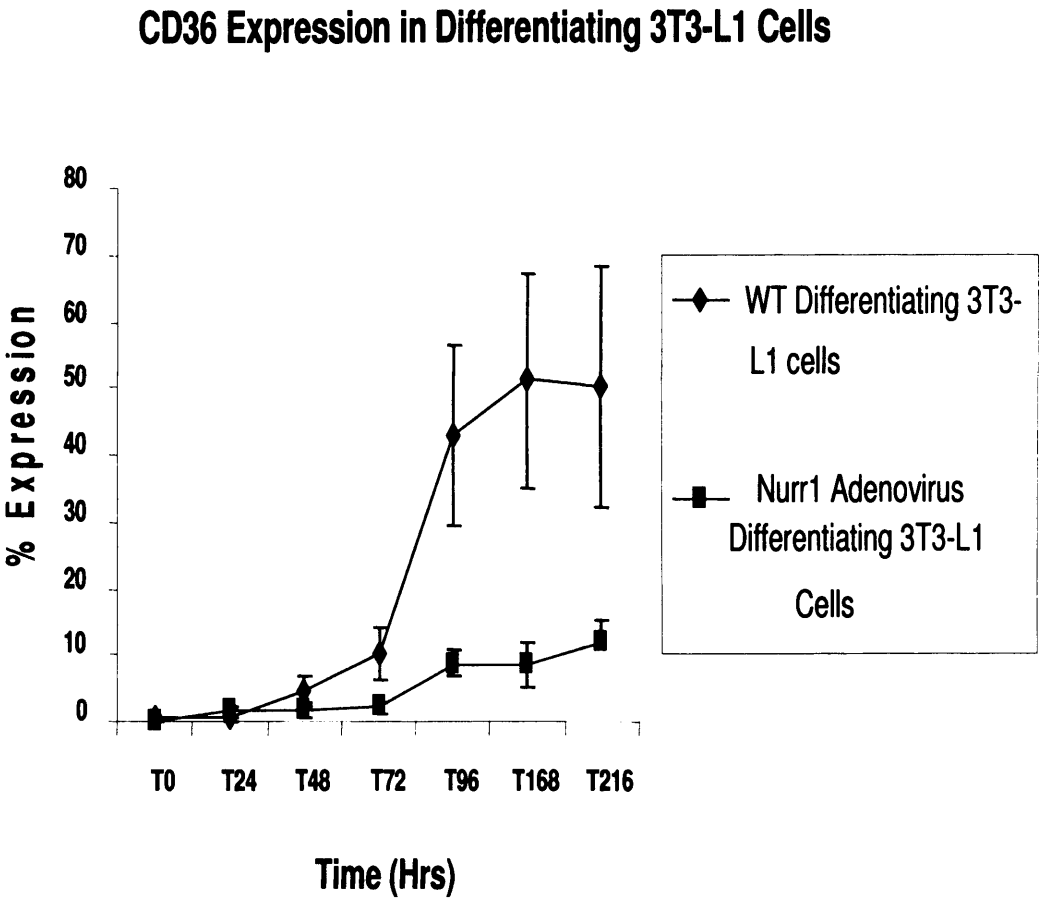


Fig. 3.11 Expression of adipogenic genes during adipogenesis in 3T3-L1 cells.

Expression of CD36 in control non-infected WT and Nurr1 over-expressing 3T3-L1 cells. Data expressed as mean % expression compared to T0, following normalisation to β-actin, +/- SEM, n=4, as assessed by SybrGreen analysis. Levels significantly elevated following induction of differentiation (T72 - T216) in WT cells but remained suppressed in Nurr1 cells (all $p<0.001$).

Analysis of lipid content in differentiated 3T3-L1 cells

Analysis of the cells by Oil Red O (ORO) staining showed a greatly reduced stained area in the Nurr1 over-expressing cells compared to control (non-infected) differentiating 3T3-L1 cells. That which is stained appears as very small lipid droplet (Fig. 3.12). This is also paralleled with the Bodipy staining on the confocal microscope (Fig. 3.13 and Fig. 3.14).

Lipolysis in differentiated primary mouse adipocytes +/- Nurr1 adenovirus

Lipolysis in the absence and presence of Nurr1 actually produced the same results, which is unusual because the Nurr1 population of cells had on average at least 50% less adipocytes than that of the control WT differentiating 3T3-L1 cells. For that reason the results are expressed as the average number of cells containing lipid (Fig. 3.15). Expressed in this manner, it is apparent that although the percentage of lipid containing cells in the Nurr1 population is lower, they have a higher rate of lipolysis as witnessed by the NEFA release from these cells.

Glucose uptake in differentiated 3T3-L1 cells +/- Nurr1 adenovirus

It was apparent from the lipolysis results that it would be difficult to really interpret lipolysis and glucose uptake in 3T3-L1 cells as the Nurr1 overexpressing cells are not fully differentiated adipocytes. The ideal way would be to differentiate the cells, so that >90% of them are lipid laden and then add Nurr1 adenovirus to the adipocytes and observe if lipid accumulation could be reversed. This provided a number of hurdles to overcome first:

- adipocytes are very fragile so any interventions could dislodge the cells.
- because of this fragility and the nature of the adipocytes they are more difficult to transduce with adenovirus.

Over-expression of Nurr1 in differentiated 3T3-L1 cells has no effect on glucose uptake (Fig. 3.16).

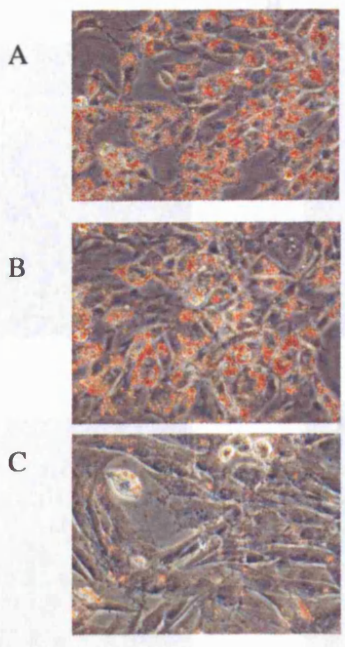


Fig. 3.12 Oil Red O images of differentiating 3T3-L1 cells at day 8

(A) GFP Virus (B) Control WT (C) Nurr1 Virus

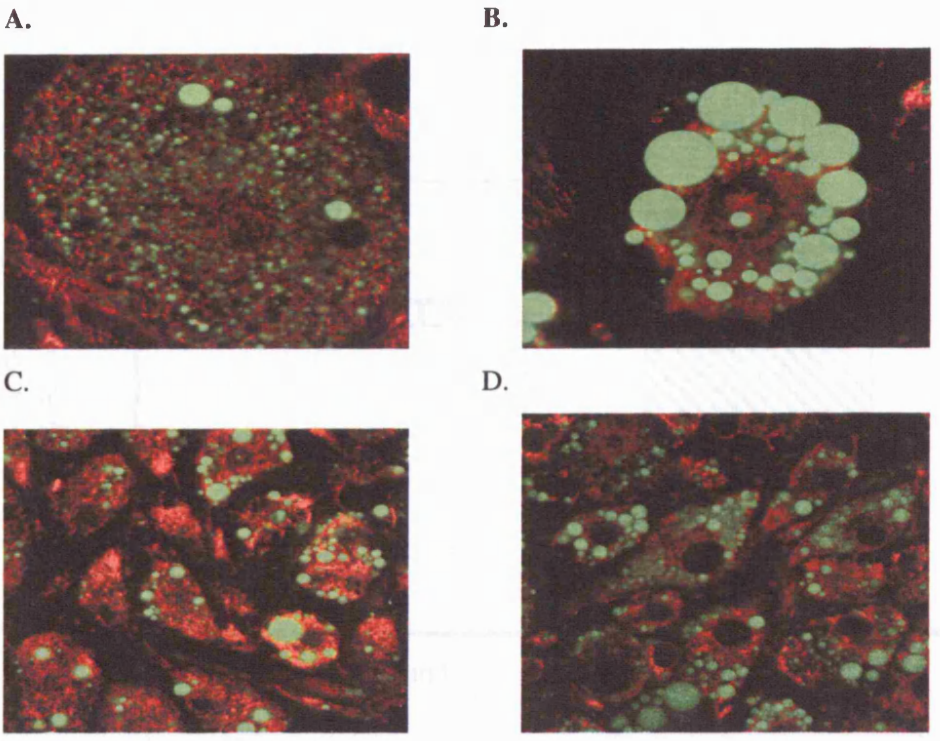


Fig. 3.13 Confocal images of differentiating 3T3-L1 cells

(A), (B) Single cell images showing Nurr1 adenovirus and control non-infected WT 3T3-L1 cells.

(C), (D) Multi cell images showing Nurr1 adenovirus and control non-infected WT 3T3-L1 cells.

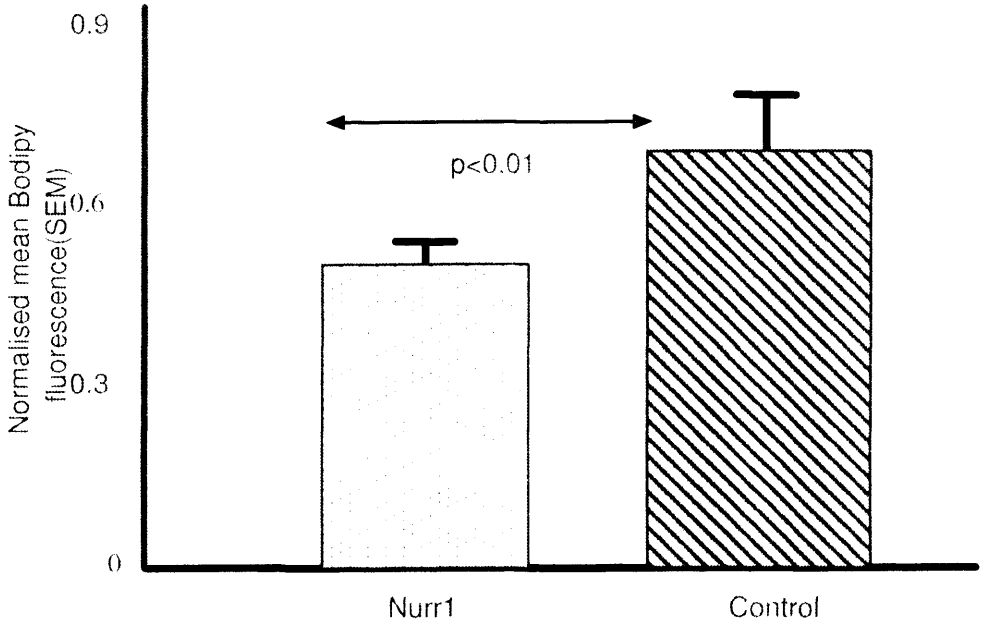


Fig. 3.14 Index of Bodipy 493/503 staining of 3T3-L1 cells +/-Nurr1

The index of Bodipy 493/503 staining, representing cellular lipid content, of 3T3-L1 adipocytes differentiated using dexamethasone, IBMX and insulin in the absence and presence of Nurr1 adenovirus (mean +/- SEM, n=40). Statistical analysis using 2-tailed student t-test.

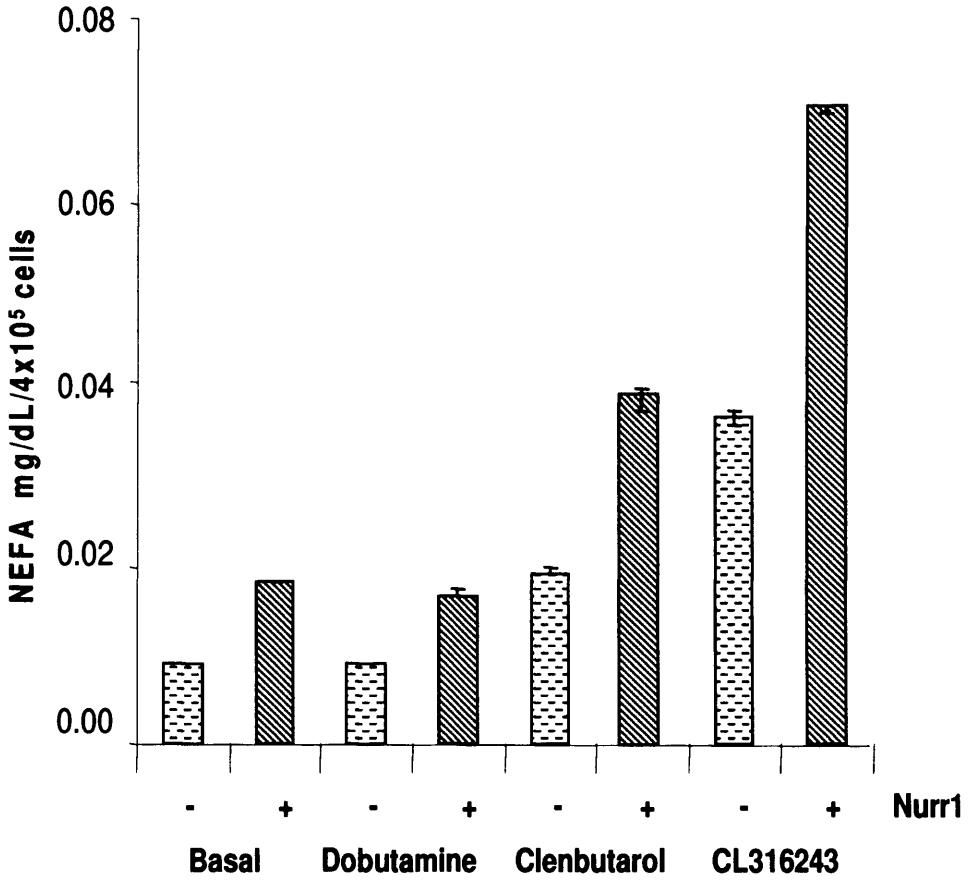


Fig. 3.15 NEFA release from primary adipocytes +/- Nurr1

The release of Nurr1 from primary adipocytes +/- Nurr1 post treatment with a β1- (dobutamine), β 2- (clenbutarol), β 3- (CL316243) adrenoceptor agonists or no stimulant. Data presented as mean and standard deviation of NEFA release mg/dL/4x10^5 cells. Cells differentiated using dexamethasone, insulin, IBMX and BRL49653 (n=3). NEFA release is significantly elevated in adipocytes without Nurr1 following induction with clenbutarol or CL316243 ($p<0.01$). However, in adipocytes with Nurr1 compared to those without, both basal and stimulated NEFA release was significantly elevated (all $p<0.001$).

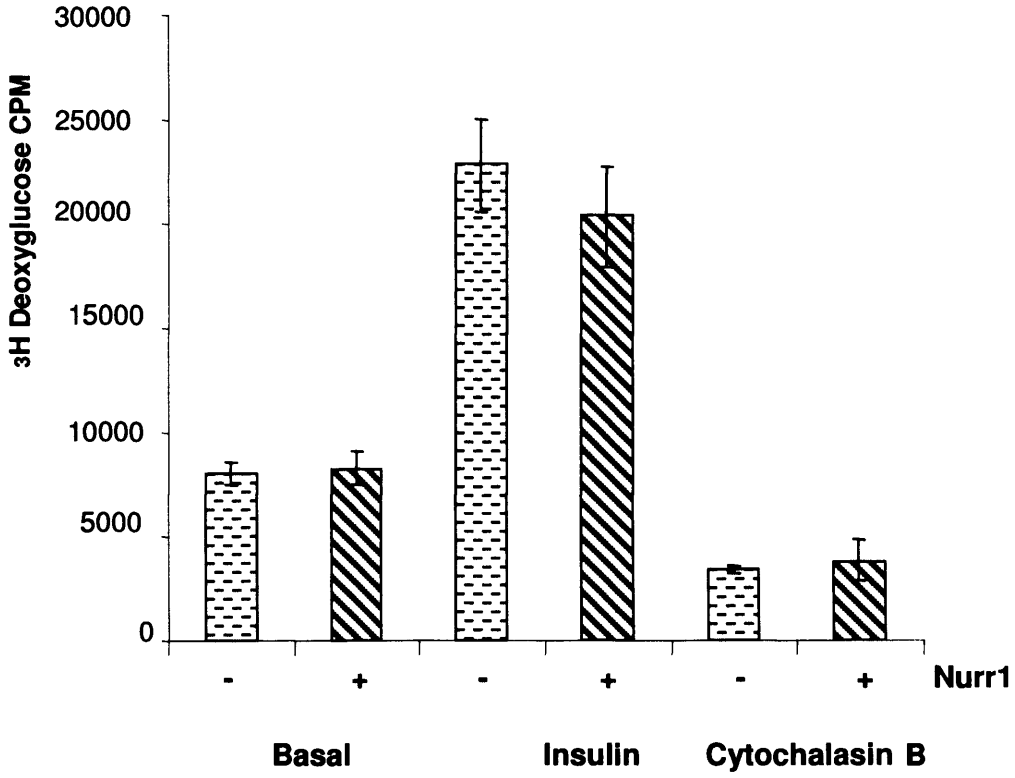


Fig. 3.16 Glucose uptake in 3T3-L1 cells +/- Nurr1

The effect of Nurr1 over-expression on insulin stimulated glucose uptake in 3T3-L1 adipocytes, shown as mean CPM per 10^5 adipocytes with standard deviations (n=4). Cells have been differentiated with insulin, dexamethsone and IBMX. Virus added to the cells upon 90% differentiation and experiment performed 72-96 hrs post virus.

Discussion

Over-expression analysis of Nurr1 in differentiating 3T3-L1 cells

3T3-L1 cells are an excellent system for studying the differentiation and biochemistry/biology of fat cells. However, these cells are notoriously difficult to transfect using naked DNA. Virally mediated introduction of genes permits a vastly enhanced level of gene delivery and adenovirus has become a popular and powerful tool for the introduction of DNA into many cell types. In a number of cell lines, adenovirus vectors which have been made replication deficient can be used to deliver a gene of interest to 100% of the cells in culture with little or no adverse effect on the transduced cells. However, even adenovirus is not 100% efficient for 3T3-L1 cells. To improve transduction Orlicky & Schaack have shown that incubating the virus with a poly –lysine mix increased the efficiency dramatically (Orlicky 2001). Adenovirus transduction as a means to deliver DNA to cells has been used by many researchers working in the adipogenesis field (Bérériat 2005, Shimba 2004, 2005, Seo 2004). In the set of experiments in this study, on average a transduction efficiency of 40-50% was consistently achieved, which was sufficient, to assess the affects of Nurr1 on the 3T3-L1 adipogenesis program (Fig. 3.3 and 3.4). Nurr1 over-expression was assessed both at the mRNA level and the protein level.

Nurr1 and adipogenesis

From experiments in the previous chapter we know that Nurr1 is expressed in mouse and human adipose tissue and that it can be induced transiently in 3T3-L1 and primary mouse pre-adipocytes. This would suggest a putative role for Nurr1 at some stage in the adipogenic program.

To identify and elucidate the role of Nurr1 in adipogenesis, the effect of its over-expression in the model 3T3-L1 system was assessed. Genes which have previously been identified as integral to the adipogenic program were assessed

as well as other genes which have been characterised in adipogenesis and adipose tissue.

As discussed in the introduction of this chapter, PPARγ and C/EBPα are widely recognised as being key players in adipogenesis (Chawla 1994a, Chawla 1994b, Cao 1991). Many groups have shown the induction of these genes early in the process and that they are key for the expression of many of the later adipogenic proteins. In the Nurr1 over-expression studies, a reduction of approximately 50 % is evident for PPARγ expression, in Nurr1 transduced 3T3-L1 cells. At this stage it cannot be stated whether or not this reduction in PPARγ expression is responsible for the effects on perilipin, HSL or CD36, or if this effect is mediated by Nurr1 directly on these genes. In contrast to PPARγ, C/EBPα does not show the same difference in expression – expression levels are comparable across the time course studied (Figs. 3.7-3.11).

However, there is a reduction in lipid accumulation as assessed by Oil Red O and confocal microscopy, which is consistent with the expression levels detected for the lipid associated genes perilipin, HSL and CD36 (Figs. 3.12-3.14).

Perilipins are highly phosphorylated proteins in adipocytes that are not secreted and are localised at the surface of the lipid droplet (Greenburg 1991). It has been postulated that perilipin coats the lipid droplet and thereby interferes with HSL access to the lipid (Blanchette-Mackie 1995, Londos 1996, Souza 1998). Phosphorylation is brought about activation of protein kinase A by cAMP, which results in translocation of perilipin away from the surface of the lipid allowing hormone sensitive lipase to hydrolyse the lipid core (Londos 1996). This is discussed in more detail below. It has been shown that by inducing lipolysis, in 3T3-L1 cells, with TNFα, a decrease in perilipin expression was evident; however this lipolysis could be rescued by the over-expression of perilipin (Souza 1998). Perilipin knock-out mice are much leaner and more muscular than controls, have 62% smaller white adipocytes and have elevated basal lipolysis that is resistant to β-adrenergic agonist stimulation. These mice

are also resistant to diet-induced obesity and by cross-breeding with $Lepr^{db/db}$, obesity is reversed by increasing the metabolic rate (Martinez-Botas 2000).

Hormone sensitive lipase, the rate limiting enzyme of intracellular triglyceride hydrolysis, is a major determinant of fatty acid mobilisation in adipose tissue and other tissues. Expression of this gene has been detected in a wide variety of tissues including white and brown adipose tissue, muscle, steroidogenic tissues, mammary gland, macrophages and pancreas (Holm 1988, Khoo 1993, Holst 1996, Mulder 1999). Phosphorylation of HSL is required to stimulate lipolysis, brought about by increases in intracellular cAMP concentrations and activation of PKA. HSL -/- mice are resistant to diet induced obesity. These mice show a decrease in weight and triglyceride levels in WAT but an increase in both BAT and liver triglycerides (Harada 2003). Also by crossing the HSL null mice with the $Lepr^{ob/ob}$ mice, obesity and adipogenesis can be inhibited (Sekiya 2004). It has also been suggested that the reduced expression of PPARγ in the WAT of the knock-out has an anti-inflammatory effect. And that this might some how allow up regulation of TNFα (Daynes 2002) which results in lipolysis.

CD36 is a fatty acid translocase protein which is expressed in tissues involved in fatty acid metabolism, such as adipose tissue, skeletal and cardiac muscle (Greenwalt 1995, Motojima 1998, Goudriaan 2002). CD36 has been shown to increase in expression during the process of adipogenesis following induction of 3T3-F442A's and Ob1771 cells (Abumrad 1993, Greenwalt 1995). In mice, mRNA expression of this gene is induced by PPARα in the liver and PPARγ in adipose tissue (Motojima 1998).

The decrease in markers of adipogenesis, adipocyte specific genes and the reduction in lipid accumulation all advocate a role for Nurr1 in adipose tissue function.

Nurr1 effects on lipolysis and glucose uptake

Lipolysis is the hydrolysis of triglycerides into its components – glycerol and fatty acids. The rate limiting step in the process is catalysed by HSL, which has been discussed above. The stimulation of lipolysis is brought about by multiple effectors including TNFα, IL-1, IFNα, β and γ, prostaglandins and catecholamines (Kawakami 1987, Ogawa 1989, Feingold 1992). Catecholamines act through β-adrenergic receptors resulting in an increase in cAMP concentrations, activating the PKA pathway which in turn phosphorylates the enzymes involved in lipolysis such as HSL. However, HSL is not the only enzyme to mediate lipolysis and other triglyceride lipases may work in the absence of HSL, responding to similar stimuli (Okasaki 2002).

Lipogenesis is the formation of triglycerides and the major mechanism by which this occurs is through digestion of triglycerides in the blood stream by LPL into fatty acids, thereby allowing them to diffuse into the adipocyte. The fatty acids are then converted to triacylglycerol by fatty acyl-CoA synthase, preventing diffusion back out of the cell, thereby facilitating storage (Voet 1990). Lipolysis in adipocytes can be stimulated *in vitro* by the addition of β-adrenergic agonists and then NEFA or glycerol release measured.

Alternatively, triglycerides can be formed from the *de novo* synthesis of acetyl-CoA and malonyl –CoA precursors, which involves glucose as the base substrate. Unlike fatty acids, glucose cannot passively diffuse through the cell membrane, but instead uptake is mediated by a family of transporters known as the glucose transporters (GLUTs). GLUT1 and 4 are the predominant transporters in adipocytes (Jung 1998). In unstimulated adipocytes, the majority of GLUT4 (95%) and a GLUT1 to lesser degree (30-40%) is located in intracellular pools. Stimulation with insulin redistributes these proteins to the plasma membrane, enabling glucose translocation to provide the substrate for lipogenesis. However, chronic insulin exposure downregulates GLUT4 expression and upregulates GLUT1 expression; resulting in an increase in

constitutive glucose uptake and a reduction in insulin-stimulated uptake. This is the basis of the glucose uptake assay.

The data generated from lipolysis and glucose uptake experiments were difficult to interpret, even when performed in a "reverse" mode, where Nurr1 virus was added following differentiation of 3T3-L1 cells into adipocytes. The lipolysis results suggested that the cells had a higher rate of lipolysis compared to the control cells, despite the fact there was less lipid in the first place (Fig. 3.15). Previous results from the Nurr1 over-expression studies suggested that HSL mRNA was much reduced in this population of cells possibly due to the fact that fewer cells were lipid laden. However, as mentioned above, HSL is not the only lipase to be expressed in adipocytes and there is evidence demonstrating that other lipases can regulate lipolysis in the absence of HSL. In addition atrial naturetic peptides have been shown to have potent lipolytic effects (Lafontan 2005). Interestingly, a naturetic peptide showed the largest fold increase at T72 hrs (94 fold) and significant fold increase at T192hrs (4 fold) in the microarray data described in Chapter 4 (Table 10).

The glucose uptake experiment indicated that over-expression of Nurr1 had no effect on lipogenesis (Fig 3.16). However as stated earlier the basis of these experiments assume that the cells all have differentiated, which is not the case in the Nurr1 cells which still have very much the appearance of an pre-adipocyte cell.

It can be concluded that over-expression of Nurr1 in differentiating 3T3-L1 cells decreases the expression of regulatory as well as adipocyte specific genes, either by a direct or an indirect mechanism. This in turn results in a dramatic reduction of lipid accumulation in the over-expressing population of cells. The rate of glucose uptake appears to be unaffected by Nurr1, however the basal level of lipolysis in differentiating primary adipocytes is elevated compared to control WT cells. In order to explore fully the effect of Nurr1 over-expression on differentiating 3T3-L1 cells a much higher through-put technique would

need to be employed allowing analysis of many different pathways. Chapter 4 discusses the effect of Nurr1 over-expression as assessed by microarray analysis.

Chapter 4 – Effect of Nurr1 over-expression on adipocyte gene expression by microarray analysis

Introduction

To understand the complex mechanisms and networks involved in biological pathways and diseases, it is no longer sufficient to focus on isolated pathways or single genetic events. Although quantitative and semi-quantitative PCR are excellent means by which to analyse changes in mRNA gene expression, they assume that the scientist knows exactly which pathways and genes are affected. The high throughput format and semi-quantitative analysis of microarray technology provides a very attractive platform for screening large numbers of gene expression changes, thereby allowing the scientist to adopt a broad based approach to acquiring knowledge of intricate regulatory networks and complex pathways.

Various types of microarrays exist based on their target material – cDNA, mRNA, protein, small molecules, tissues, or any other material that allows quantitative gene analysis.

The Affymetrix system employs an oligonucleotide based system prepared by light directed DNA synthesis to deposit 22 nucleotides and two types of probes – a Perfect Match (PM) and a MisMatch (MM), using a combination of two techniques – photolithography and solid phase DNA synthesis. The PM probe matches the target sequence exactly, whilst the MM probe has a single nucleotide change in the middle of the sequence and together these are known as the probe pair. A number of probe pairs constitute a probe set and are used to investigate different regions within the same sequence. The intensity information obtained from the values of each probe is combined and the average expression value obtained as the measured value. Following mRNA extraction, RNA for the 2 treatments under comparison is reverse transcribed using an oligo dT primer. This is followed by an RNaseH mediated second strand synthesis. This double stranded cDNA then serves as a template for *in vitro* transcription which is carried out in the presence of a biotinlyated nucleotide analog/ribonuclotide mix and T7 RNA polymerase which generates biotin labelled amplified cRNA. The biotinlyated cRNA fragments are then purified and fragmented before hybridising

to the GeneChip expression array. The GeneChip array is them loaded onto the Fluidics station for washing and staining, with strepavidin phycoerythrin. The probe array is then scanned in either the Agilent Gene Array Scanner or the Affymetrix Gene Chip Scanner (Figure 4.1 shows a summarised overview of the procedure). The data produced is a series of black /white/grey dots, which is then analysed by the scanner to yield intensity values. An example of the type of result recorded is illustrated in Fig 4.2. All catalog GeneChip® expression arrays are designed to have a minimum sensitivity of 1:100,000. This concentration ratio corresponds roughly to a few copies of transcript per cell, or an approximate 1.5 pM concentration.

Large scale genomic approaches have become increasingly powerful tools for probing molecular mechanisms. Microarray analysis has been used as a technique to investigate the time-dependent regulation of gene expression during adipocyte differentiation (Burton 2002, Guo 2000, Ross 2002). And the hugely successful use of differential gene expression has been used to dissect the biology of nuclear receptors and the pathways and genes which they transcriptionally regulate (Ross 2002, Wang 2005). Microarray has been used to investigate the role of Nurr1 in regulating inflammatory processes in synovial cells (Davies 2005).

In this chapter the effect of Nurr1 over-expression on differentiating 3T3-L1 cells was investigated using microarray analysis to identify changes in adipogenesis related genes and other pathways/regulatory networks which were modulated (directly or indirectly) by Nurr1. Subsequently, expression changes identified in a number of cytokines were confirmed using ELISA's.

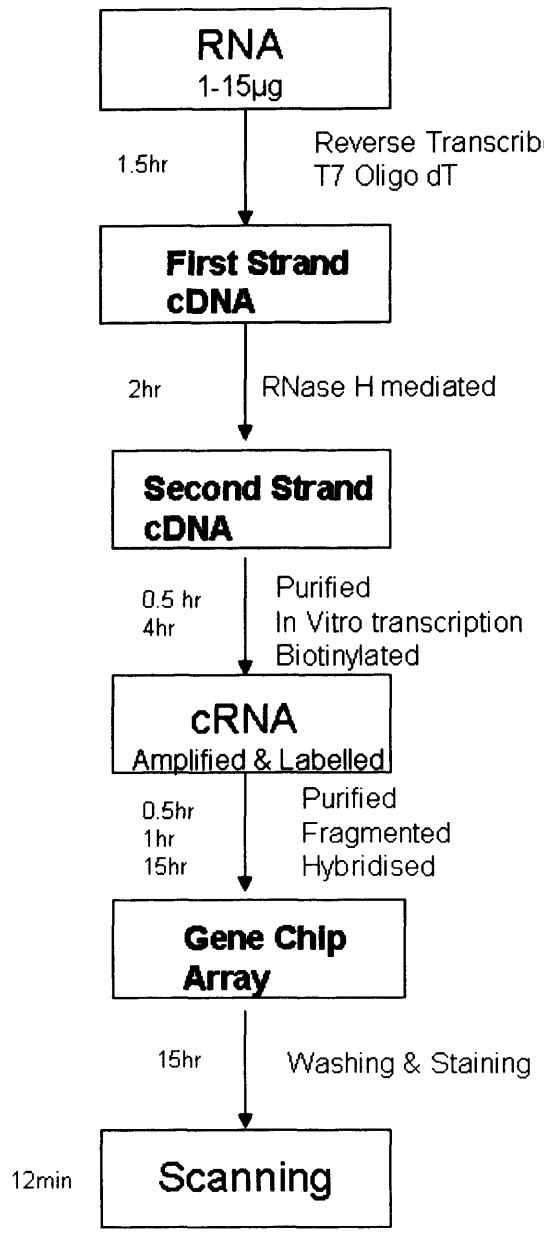


Fig. 4.1 Overview of microarray procedure

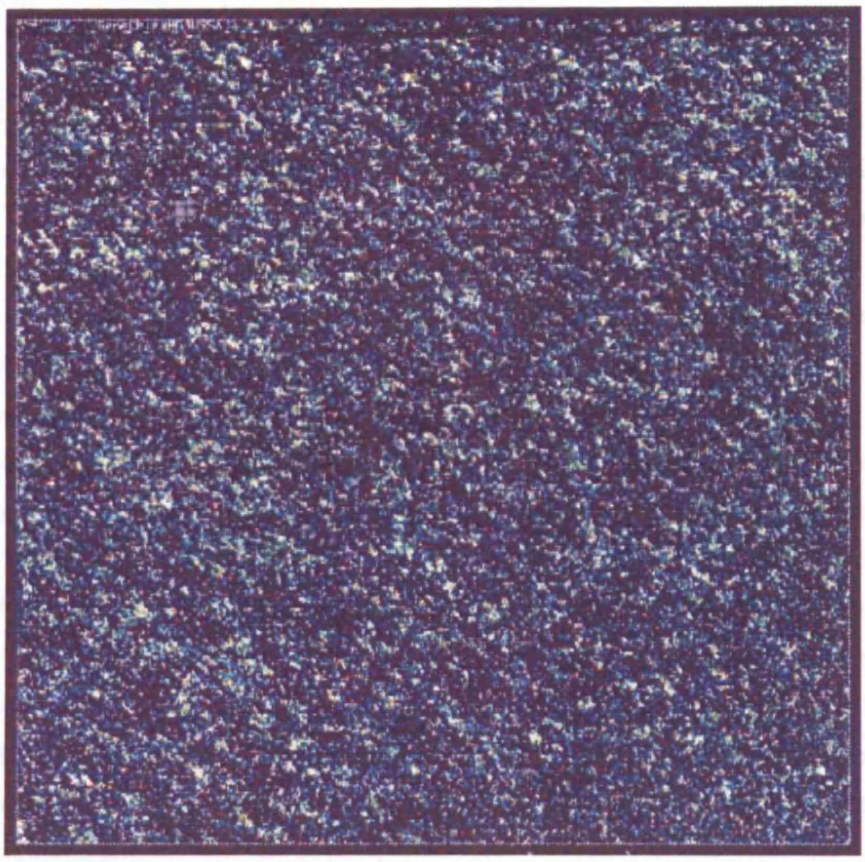


Fig. 4.2 Image of an Affymetrix GeneChip Array

Data from an experiment showing the expression of thousands of genes on a single GeneChip® probe array. Image courtesy of Affymetrix.

Experimental Procedures

Transduction of 3T3-L1 cells with Nurr1pAdTrackCMV-GFP

3T3-L1 cells were cultured as described in Chapter 2. Prior to transduction, cells were seeded at density of $5x10^4$ cells per well in 6-well plates and left to attach over-night. Nurr1pAdTrackCMV-GFP adenovirus was added to the cells and left to incubate over-night at 37°C 5%CO_2. Media was removed the following morning and replaced with fresh growth media. Transduction efficiency was assessed by the level of GFP fluorescence. Control 3T3-L1 cells were non-infected.

Differentiation of 3T3-L1 cells

Following virus removal, cells were allowed to reach confluency. Confluent cells were then treated with the adipocyte differentiation induction medium – dexamethasone 2.5nM (Sigma Aldrich, Dorset UK), isobutylmethylxanthine 0.5mM (IBMX Sigma Aldrich) and insulin 5mg/ml (Sigma Aldrich). Control wild type 3T3-L1 cells (non-infected) were treated in same manner. Forty eight hours post-induction the media was removed and replaced with DMEM + 10% FBS and insulin only (5mg/ml) for the duration of the experiment and replaced every 2 days. RNA samples were isolated at 72 (T72) and 192 (T192) hours post-differentiation.

Isolation of RNA from differentiating 3T3-L1 cells

The media was removed and cells washed briefly with 1ml of PBS followed by the addition of 1ml Trizol (Sigma Aldrich) per well to lyse the cells. All samples were stored at -80°C and batch processed. Samples were then thawed on ice ready for RNA extraction. To each sample 200µl of chloroform was added and sample tubes mixed thoroughly. Tubes were then placed on ice for 15-20mins to allow separation of the phases and then centrifuged at room temperature for 10mins at 12000 x g. Following centrifugation, the upper aqueous phase was carefully removed to a new RNAse/DNAse free tube and 0.7 volumes of isopropanol added to each. To facilitate efficient precipitation of RNA tubes were incubated for 1-2 hours at -20°C. Samples were then centrifuged at 12000 x g for

10 mins to pellet the RNA, then the supernatant solution was carefully removed and the pellet washed with 100µl of 70% ethanol.

Following another centrifugation step (5mins 12000 x g), the supernatant was removed and the pellet air-dried. Finally the pellet was resuspended in 25µl DEPC- water.

RNA quantification and QC

The concentration and integrity of the RNA was assessed using the RNA 6000 Nano Assay on the Agilent 2100 (Agilent, West Lothian UK). The RNA quantification assay was set up according to manufacturerer's protocol. Briefly, a gel dye mix was prepared using RNA gel matrix and an RNA dye, and then loaded onto an RNA chip, using a chip priming station. The RNA 6000 Nano marker is then loaded into all wells, except those with gel-dye mix. 1µl of RNA 6000 ladder was added to the corner well and 1µl of RNA sample added to each well, vortexed and run on the Agilent 2000 for an average run time of 45 mins.

See Figs. 4.3 and 4.4 for a view of results.

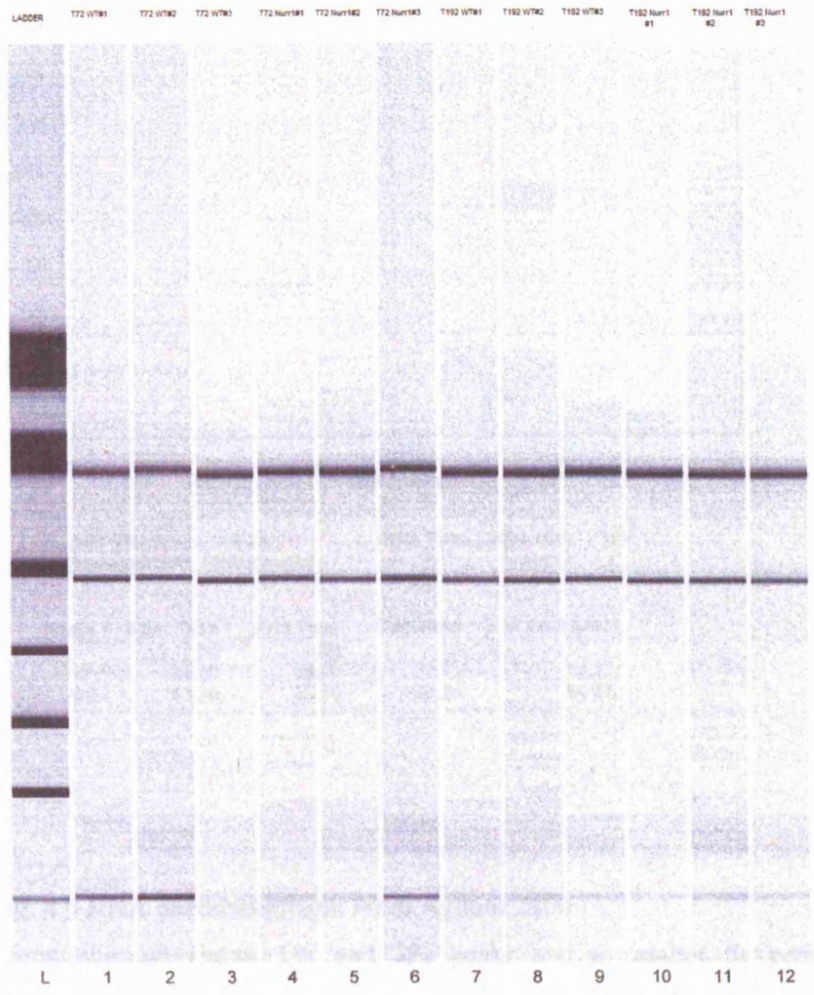
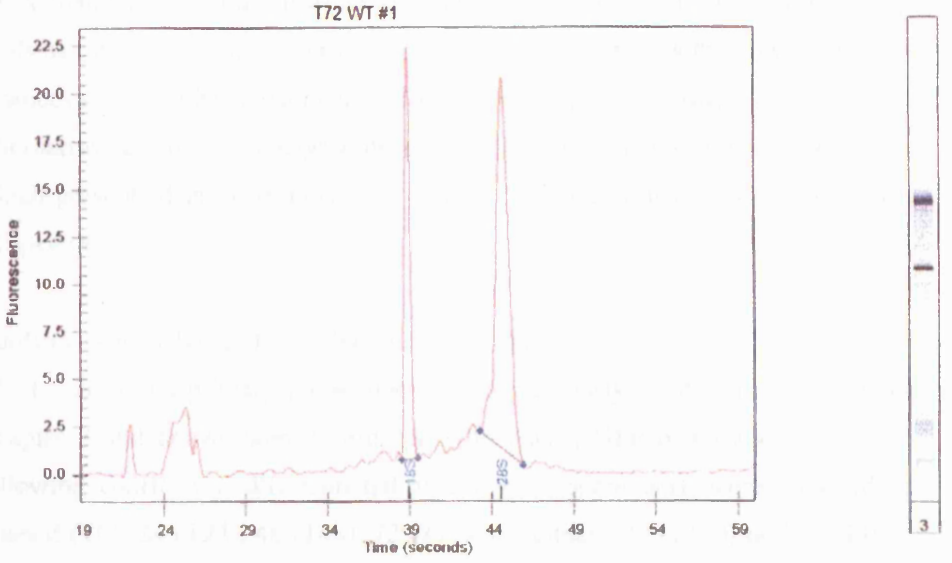


Fig. 4.3 In silico gel image of RNA from Agilent 2100

Gel generated from RNA 6000 Nano Assay on Agilent 2100. In silico gel generated by the Agilent 2100, showing 18s and 28s bands.



Corrected RNA Area: 118.23 rRNA Ratio [28S / 18S]: 1.97
RNA Concentration: 266.42 ng/ul

#	Name	Start Time (s)	End Time (s)	Corr.Area	% of total Area
1	18S	38.50	39.50	21.71	18.37
2	28S	43.30	45.90	40.28	34.07

Fig. 4.4 RNA chromatograph from Agilent 2100

Chromatograph shows 18s and 28s peaks and a smaller 5s peak, with calculated RNA concentration and quality for one of the control 3T3-L1 samples.

Microarray analysis

RNA samples sent to the Institute of Child Health Labs. at UCL for microarray analysis and QC. RNA processed according to Affymetrix recommended protocol and hybridised to a mouse 430-2 GeneChip® probe array.

Microarray results were analysed using a GSK bioinformatics in-house package – which presents data as fold changes. (A >2-fold change is deemed significant). All $p<0.001$.

Confirmation analyses of cytokine microarray data:

3T3-L1 cells and primary pre-adipocytes (as previously cited in the methods in Chapter 3 and above) were transduced with Nurr1, GFP or no adenovirus and following confluency, differentiated as above. Supernatants were removed at times 0 (T0), 24 (T24), 48 (T48), 72 (T72) and either 120 (T120) or 192 (T192) hours after induction of adipocyte differentiation and stored at -80°C until ready for assay. Two-site microplate ELISA assays (R&D Systems, UK) for mouse IL-6, MCP-1 and RANTES were performed following manufacturer's protocol and plates read at 450nm. Results were expressed as pg/ml. The results obtained from assays with <10% inter and intra-assay coefficients of variation (CVs) were accepted.

Statistical analysis

Results are expressed as mean±SD or SEM for normally distributed values or median (interquartile range) for skewed data. Comparisons between groups were made by two-tailed Student's *t*-test, Mann-Whitney U test or Wilcoxon rank test, as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Microarray analysis

As adipogenesis induces a vast array of known gene changes these were initially analysed in the control cells, between the early (T72) and late (T192) time-points. In these cells, as expected, there was an increase in the adipogenic transcription factors, PPARγ and C/EBPα, as well as elevation in all the adipocyte specific genes such as HSL, adipsin etc. Morphologically these cells were adipocyte like and accumulated lipid. These results confirmed that the control cells were differentiating as normal. The data were then analysed to look at the effect of Nurr1 specifically on the various adipocyte genes and the pathways that may be regulated by Nurr1.

Upon receiving the microarray data, an in-house GSK bioinformatics program was employed to annotate and analyse the data and express changes as fold change compared to control cells with associated p-values. Results were expressed as a fold increase or decrease compared to control cells at the same time point. The data represented in Fig. 4.5 shows a Venn diagram of the overall changes across the time points. A change of at least 2-fold was considered significant. All genes under discussion had p-values <0.001. Tables 1-10 highlight a number of gene expression changes.

Of note is the fact that, Nurr1 exhibited a 70-fold expression increase, reflecting the adenovirus over-expression, over control cells at the earlier 72 hour timepoint (T72), which then decreased with time to about 4-fold at the later 192 hour timepoint (T192) (Table 8).

Previous data already showed a number of genes that were differently expressed during the differentiation process upon Nurr1 over-expression – namely PPARγ, HSL, CD36 and perilipin. As expected these changes are mirrored in the data obtained here. The microarray result showed many more adipogenic genes exhibiting a decrease in expression and also the up-regulation of many genes that are typically down regulated during adipogenesis. The results obtained for changes in mRNA expression at the early time point (T72) are more likely to

point to a direct Nurr1 effect while the later point (T192) could reflect secondary effects or the lower adipogenic rate. Therefore, a reduction in adipocyte specific genes is more likely to be a reflection of less adipocyte number and reduced lipid accumulation.

Many of the pro-inflammatory adipose factors were significantly elevated in the T72 Nurr1 over-expressing adipocytes and these results were followed up by determining changes in their protein levels, by ELISA, namely those of IL-6, MCP-1 and RANTES.

The results for IL-6 protein secretion confirm those seen in the gene expression obtained in the microarray results, in that IL-6 protein release was significantly higher in the Nurr1 over-expressing cells compared to either control untransfected 3T3-L1 cells or cells expressing GFP (Fig. 4.6). GFP was used as a control in this experiment to ensure the adenovirus itself was not responsible for the up-regulation of the cytokines in the study.

In 3T3-L1 adipocytes, an increase in MCP-1 secretion whilst not as dramatic as those obtained for IL-6, was also statistically significant in Nurr1 cells (Fig. 4.7). The microarray data showed a 5-fold increase in MCP-1 mRNA, which was a much smaller increase than that obtained for IL-6 mRNA. These results were also confirmed in primary adipocytes (Fig. 4.9-4.10).

RANTES release from 3T3-L1 cells in response to Nurr1 over-expression was striking, and this is the first reported evidence of RANTES release in adipocytes. Again the results at the protein level are analogous to that seen at the mRNA level (Fig. 4.8) and were confirmed in primary murine adipocytes (Fig. 4.10).

A)

Time Point	Up-Regulated Genes	Down-Regulated Genes
T72 Unique	1467	718
T192 Unique	224	895
T72 & T192 in Common	925	978

B)

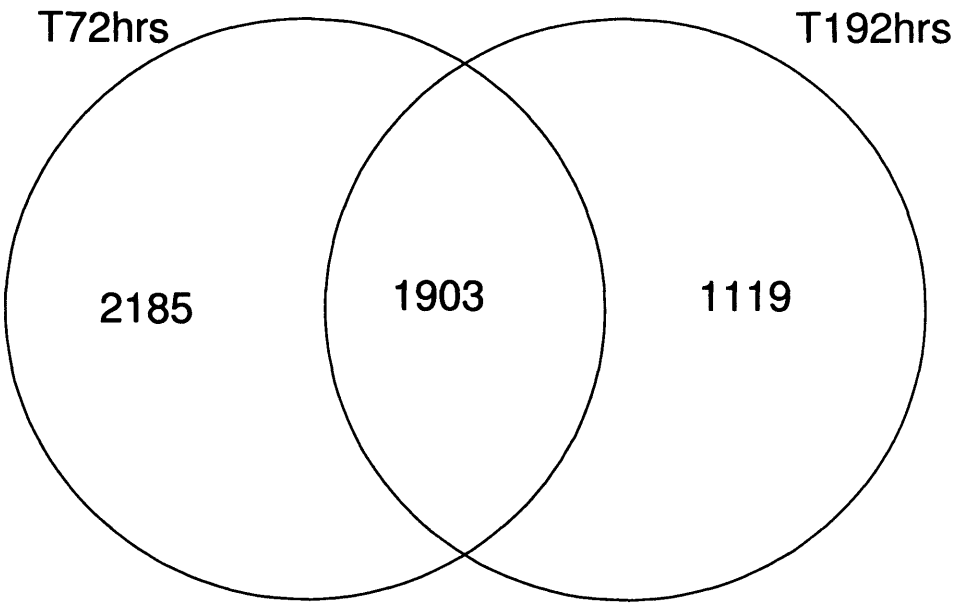


Fig. 4.5 Numerical representation of mRNA expression changes during differentiation between Nurr1 over-expressing cells and control cells.

a) No. of genes up and down regulated at each time point

b) Venn diagram representing genes in common and unique to each time point

Adipogenesis transcription factors and related genes:				
Gene no.	**Gene name**	**Function**	**Fold at 72h**	**Fold at 192h**
1418982_at	C/EBPα	Master regulator of adipogenesis	8.25 ↓	5.9↓
1420715_a_at	PPARγ	Master regulator of adipogenesis	1.8↓	2.8↓
1425262	C/EBPγ	May act as inhibitor of other C/EBP isoforms. Lacks transactivation domain. Defective form?	3.9↓	1.9↓
1460336_at	PGC1α	Adaptive thermogenesis, fatty acid oxidation, gluconeogenesis	2.1↓	-
1449945_at	PGC1β	Constitutive adrenergic independent mitochondrial biogenesis	4.8↓	14↓
1450402_at	PPARBP	Involved in transcriptional activation of NR's including PPARγ	3.4↑	1.5↑

Table 1 Regulators of adipogenesis

Lipid metabolism related genes:				
Gene no.	**Gene name**	**Function**	**Fold at 72h**	**Fold at 192h**
1422820_at	Hormone sensitive lipase	Rate limiting enzyme of intracellular triglyceride hydrolysis	3.3↓	30↓
1423828_at	Fatty acid synthase	Key enzyme in de novo lipogenesis	2.6↓	2.5↓
1425809_at	FABP4	FABP4 binds fatty acids and transports them to the nucleus where the FABP4/fatty acid complex activates PPARγ	-	9↓
1422811_at	Fatty Acid Transporter (SLC27a1)	Membrane protein that facilitates LCFA transport across the plasma membrane	3↓	-
1423166_at	CD36	Fatty Acid Translocase involved in FA metabolism	1.4↓	5.8↓
1435281_at	CPT1	Energy balance; mitochondrial oxidation of LCFA	4.7↑	2.5↑

Table 2 Lipid metabolism related genes

Secreted factors/adipokines:				
Gene no.	**Gene name**	**Function**	**Fold at 72h**	**Fold at 192h**
1450826_a_at	Serum amyloid A3	Inflammatory biomarker; Up regulated by IL-6 and TNFα	4.2↑	-
1449182_at	Resistin	Pro-inflammatory cytokine. Inhibits adipogenesis. Putative role in insulin resistance	5.4↓	49↓
1450297_at	IL-6	Elevated in obesity, is associated with metabolic syndrome, proinflammatory cytokine	15↑	-
1420380_at	MCP-1	Chemokine,	5.5↑	-
1418126_at	RANTES	Chemokine	20↑	-
1417867_at	Adipsin	Early adipocyte marker	5.4↓	17↓
1422651_at	Adiponectin	Endogenous insulin sensitiser, anti-inflammatory, elevated in healthy, lean subjects	2.8↓	7↓
1436900_x_at	Leptin	Produced by adipocyte	-	-

Table 3 Secreted factors/adipokines

Other adipogenesis associated genes:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1426516_at	Lipin 1	Modulation causes dramatic shifts in adiposity	2.7↓	5↓
1460290_at	Lipin 2	Related to Lipin1	-	2.8↓
1419015_at	WISP2	Inhibits proliferation in VSMC's	20↑	2.2↑
1448700_at	GOS2	Up regulation specific for adipogenesis; associated with growth arrest	4.2↓	8.1↓
1450403_at	STAT2	Induced by cytokines, no known function in adipose	9.6↑	1.4↑
1450259_a_at	STAT5	Increased during adipogenesis, possible regulation of preadipocyte proliferation and differentiation	4↓	-
1449038_at	11βHSD1	Conversion of inactive cortisone to active cortisol. Inhibitors lower cholesterol and triglycerides	2.5↓	7.5↓
1450010_at	17βHSD 12	Steroid metabolism	-	2.1↓
1417369_at	17βHSD4	Steroid metabolism	2.2↓	2↓
1448865_at	17βHSD7	Steroid metabolism	-	2.7↓
1416968_a_at	HSD3β7	Steroid metabolism	-	2.3↓

Table 4 Adipogenesis associated genes

Nitric oxide pathway related genes:				
Gene no.	**Gene name**	**Function**	**Fold at 72h**	**Fold at 192h**
1438879_at	DDAH1	Enzyme involved in the metabolism of ADMA	4.1↑	-
1416457_at	DDAH2	As above	2.8↑	-
1416239_at	ASS	Catalyses the condensation of citrulline and aspartate to form arginosuccinate, precursor of arginine.	9 ↑	2.4↑
1428195_at	SAHH	Adenosylhomocysteinase	-	4.4↓

Table 5 Genes associated with the Nitric oxide pathway.

Matrix Metalloproteinase's and associated genes:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1417234_at	MMP11	Involved in insulin resistance. -/- higher body weight.	13↑	1.7↑
1418945_at	MMP3	Impairs adipose tissue development	-	11↑
1448383_at	MMP14	Unknown function in adipose tissue	5.8↑	-
1417256_at	MMP13	Bone Resorption	5.5↑	3↑
1460287_at	TIMP2	Unknown function in adipose tissue	4.4↑	3.4↑
1460227_at	TIMP1	Unknown function in adipose tissue	3.5↑	2.5↑
1457058_at	ADAMTS2	procollagen N-proteinase; increased in osteoarthritis	3↑	-
1416871_at	ADAM8	Osteoclast stimulating factor	2.8↑	-
1422561_at	ADAMTS5	aggrecanase in mouse cartilage	2.4↑	-
1452595_at	ADAMTS4	Degradation of aggrecan in arthritic cartilage.	2.2↑	-
1421859_at	ADAM17	modulate cell migration; unregulated by angII	-	2↓
1425170_a_at	ADAM15	Cartilage remodelling	-	2.2↓
1421171_at	ADAM12	Involved in induction of adipogenesis	2↓	-

Table 6 Matrix Metalloproteinases and associated genes

KLF transcription factors:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1448890_at	KLF2	Negatively regulates adipogenesis	3↑	3↓
1417395_at	KLF4	Pro-inflammatory signal. Down regulated in adipogenesis	5.4↑	2.1↑
1451739_at	KLF5	Down regulated in adipogenesis	3.25↑	-
1419355_at	KLF7	As above	2.75↑	-
1422264_s_at	KLF9	Up regulated in adipogenesis	-	2.3↓
1427786_at	KLF14	Unknown function in adipose tissue	3.4↑	-
1448181_at	KLF15	Up-regulated in adipogenesis	3.7↓	24↓

Table 7 KLF transcription factors

Other nuclear receptors:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1450749_a_at	Nurr1	Putative role in adipogenesis	70↑	3.7↑
1443952_at	RevErB alpha	Potential role in glucose homeostasis and/or inflammation. Increased during adipogenesis	5↓	-
1418157_at	Coup-TF1	Putative role in inhibiting differentiation through heterodimerisation with RAR	3↑	-
1425792_a_at	RORγ	Induced during differentiation of 3T3-L1 cells	5.8↓	7.8↓
1450444_a_at	LXRα	Regulate cholesterol and fatty acid metabolism in liver tissue and in macrophages. Putative role in the execution of adipocyte differentiation by regulation of lipogenesis and adipocyte-specific gene expression	4.7↓	16.5↓
1426464_at	TRα	Metabolically active.	4.3↓	3.2↓
1420583_at	RORα	Induced during differentiation of 3T3-L1 cells	2.8↓	2.3↓
1421747_at	ERRγ	Not known to be expressed in 3T3-L1 cells	4.1↑	-
1418175_at	VDR	Evidence to show inhibition of differentiation of preadipocytes	3.2↑	-
1416505_at	nurr77	Induces lipolysis in skeletal muscle	2.4↑	-

Table 8 Nuclear receptor expression changes

GPCRs:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1419721_at	HM74a	Anti-lipolytic	8.6↓	10.8↓
1425216_at	GPR43	Free fatty acid receptor	7.2↓	11.7↓
1457745_at	GPR4	Lysophospholipid receptor	8.3↑	-
1418603_at	Arg Vaso R	Role in hypertension and regulation of HPA	7.6↑	-
1423571_at	EDG1	sphingosine-1-phosphate/ chemotactic Role in atherogenesis Endothelial cell functions	6.4↑	-
1460661_at	EDG3	As above	5↑	-
1415832_at 1423396_at	AngiotensinII Angiotensinogen	Production of inflammatory cytokines Hypertension	3.3↑ -	- 2.5↓
1421667_at	GPR66	Neuromedin U Receptor (NMU) Type 1 for the anorexigenic hormone NMU	3.2↑	0
1421073_a_at	EP4	Prostaglandin E4 receptor, signals via elevation in intracellular cAMP levels. Pro-inflammatory	2.8↑	0
1417625_s_at	RDC1	Chemokine orphan receptor 1 Similar to CXCR2. Involved in angiogenesis and endothelial cell migration	19↑	2.8↑
1450260_at	GRPbombesin R	Involved in Satiety	8.8↑	2.8↑
1421471_at	NPY1	Role in regulating energy homeostasis	6.1↑	2.7↑

Table 9 G-protein coupled receptor expression changes

Other genes of interest:				
Gene No	**Gene Name**	**Function**	**T72**	**T192**
1450791_at	Natriuretic peptide precursor B	Proposed to have lipolytic effects	95↑	4↑
1449015_at	Resistin Like α	Putative role in insulin resistance	7↓	-
1423954_at	Complement component 3	Precursor to acylation-stimulating protein. Increases efficiency of triacylglycerol synthesis. KO – reduced fat, obesity resistant & improved insulin sensitivity	4↓	4↓
1450652_at	Cat K	Role in bone degradation	17↑	2.6↑
1450383_at	LDLR	Lipoprotein transport. Some evidence over-expression can stimulate adipogenesis	2.8↓	10.2↓
1422631_at	AHR	Down regulated during adipogenesis. Can inhibit adipogenesis if over-expressed	13↑	-
1450110_at	ADH3	Alcohol Dehydrogenase putative NBRE??	4.4↑	-
1418848_at	Aquaporin 7	Adipose glycerol transporter	-	15↓
1448201_at	SFRP2	Modulator of wnt signalling –wnt inhibitor	10↑	2.8↑

Table 10 Genes associated with adipose tissue

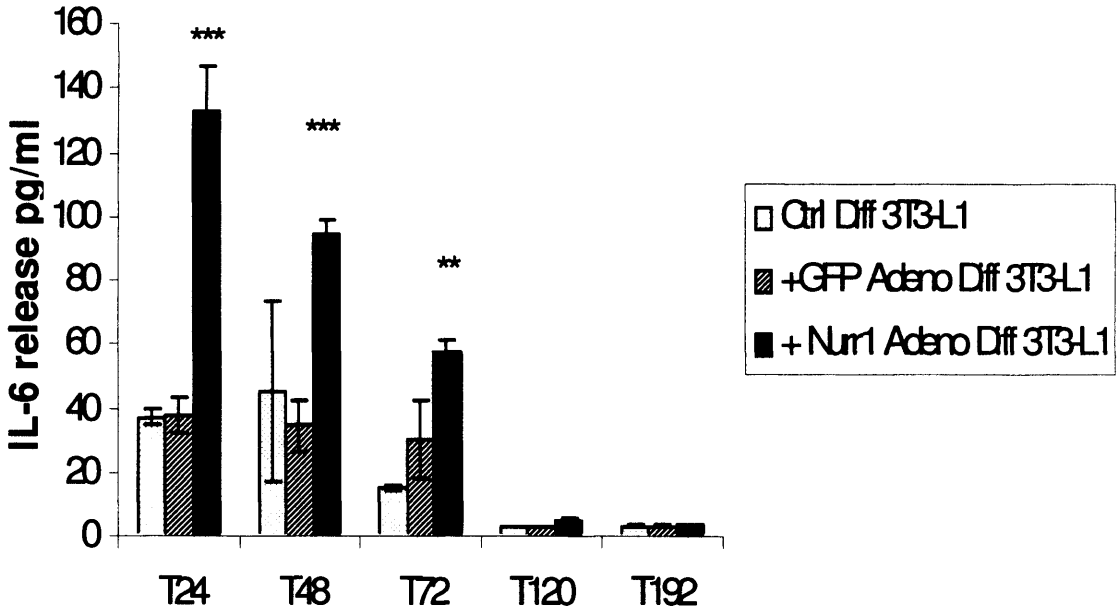


Fig. 4.6 Mouse IL-6 release in differentiating 3T3-L1 cells.

The release of IL-6 into supernatant of 3T3-L1 cells during differentiation in untransduced, GFP transduced or Nurr1 transduced cells. Results presented as mean with standard deviations (n=5)

** $p = 0.004$; *** $p < 0.001$

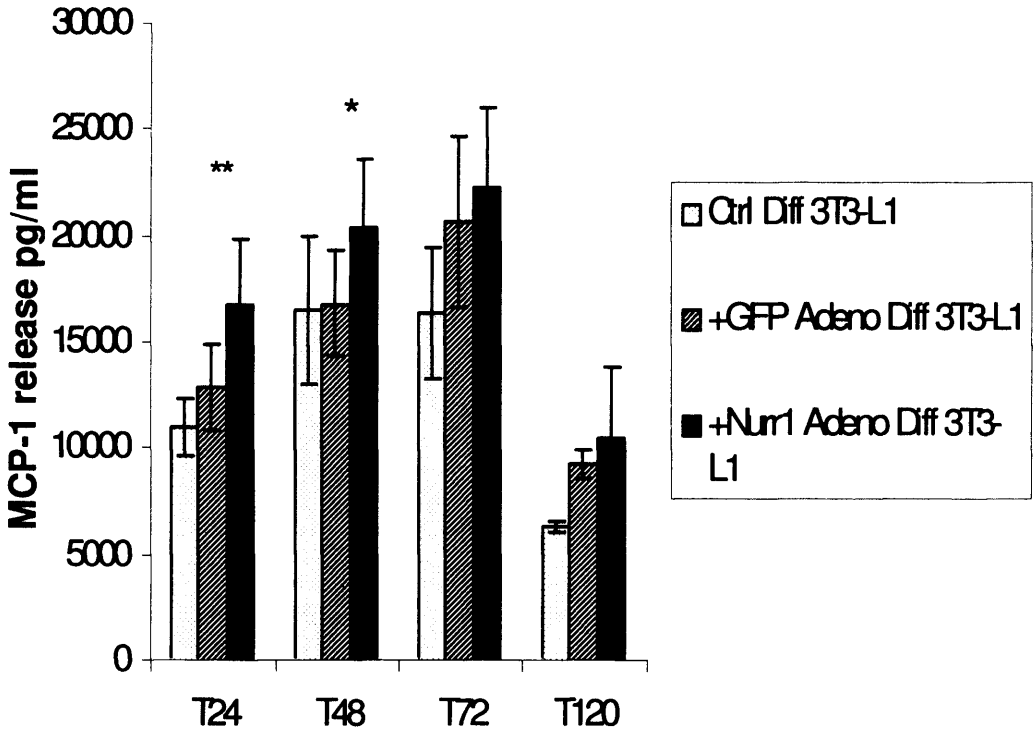


Fig. 4.7 MCP-1 release from differentiating 3T3-L1 cells.

The release of MCP-1 into supernatant of 3T3-L1 cells during differentiation in untransduced, GFP transduced or Nurr1 transduced cells. Results presented as mean with standard deviations (n=5)

** p =0.002 * p =0.02

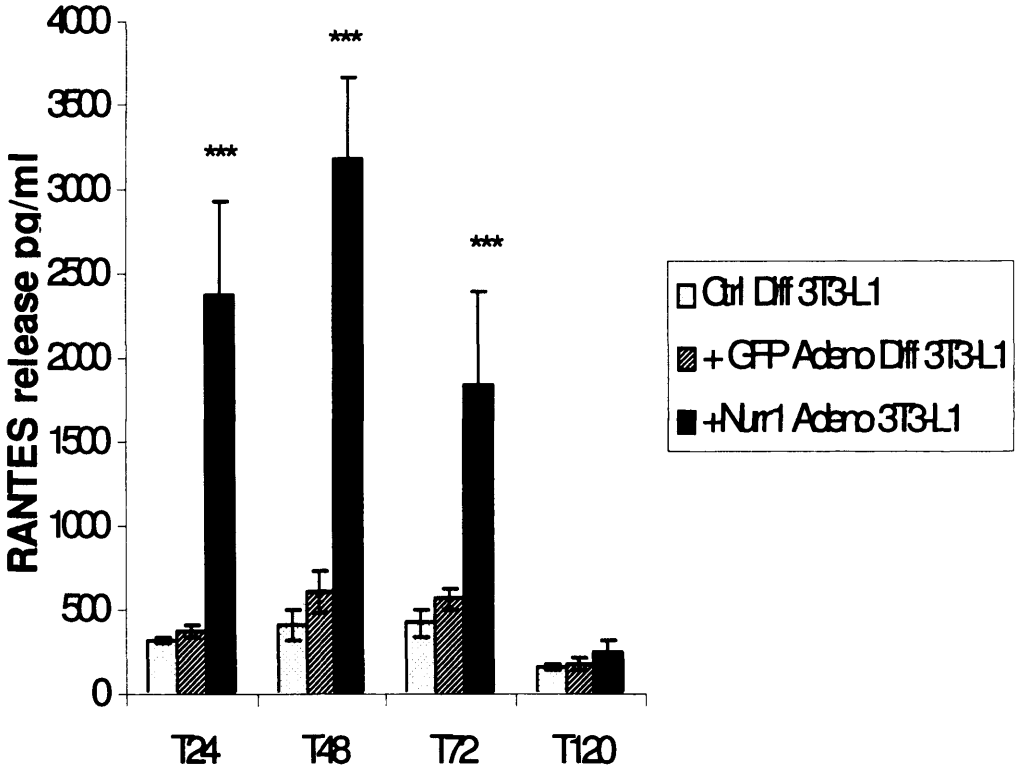


Fig. 4.8 RANTES release from differentiating 3T3-L1 cells.

The release of RANTES into supernatant of 3T3-L1 cells during differentiation in untransduced, GFP transduced or Nurr1 transduced cells. Results presented as mean with standard deviations (n=5)

*** p <0.001

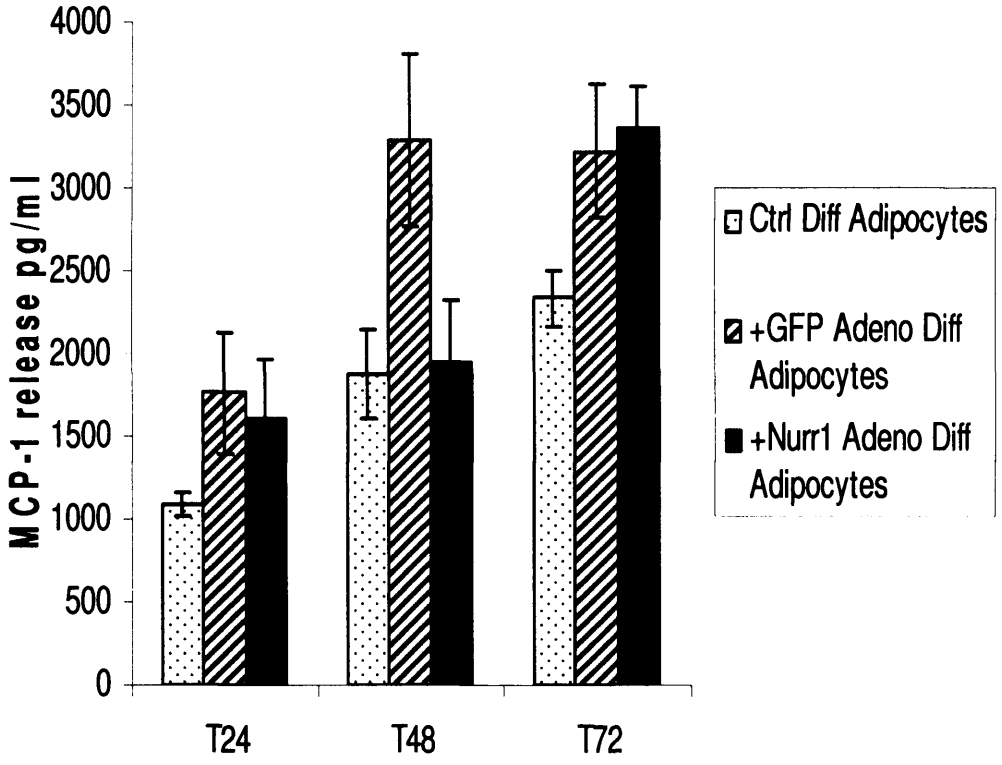


Fig. 4.9 MCP-1 release from differentiating mouse primary adipocytes

The release of MCP1 into supernatant of murine primary adipocytes during differentiation in untransduced, GFP transduced or Nurr1 transduced cells. Results presented as mean with standard deviations (n=5)

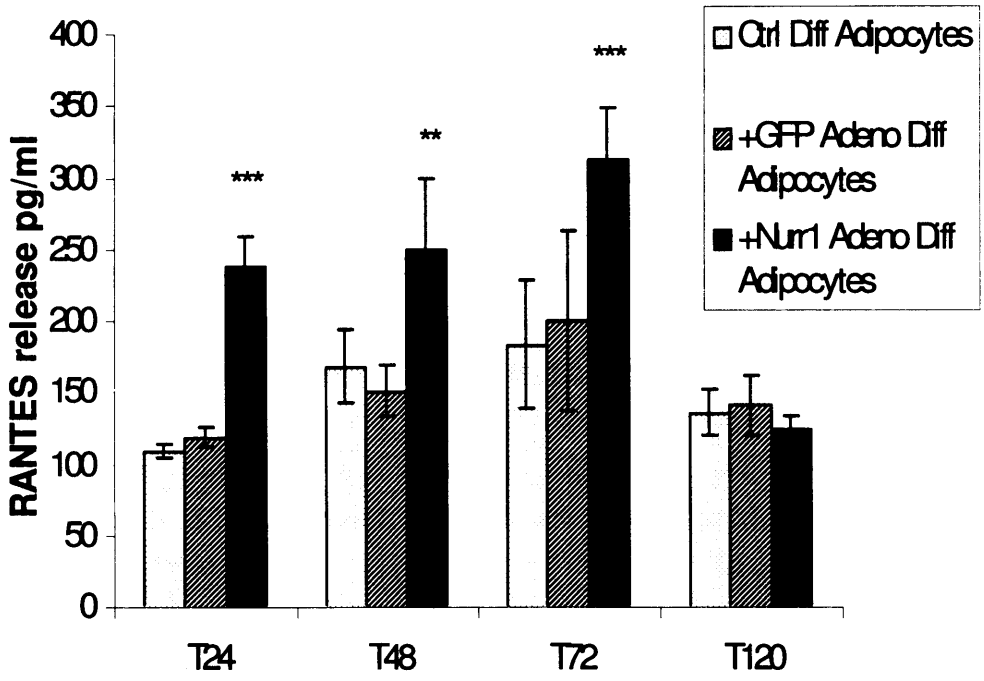


Fig. 4.10 RANTES release from differentiating mouse primary adipocytes

The release of RANTES into supernatant of murine primary adipocytes during differentiation in untransduced, GFP transduced or Nurr1 transduced cells. Results presented as mean with standard deviations (n=5)

**** $p<0.001$ ** $p=0.005$*

Discussion

Microarray analysis is an incredibly powerful means of investigating changes in gene expression, as witnessed by the tremendous number of changes observed in this particular experiment. As a tool, most scientists use it as an investigative means at the beginning of experiments to validate targets and to examine compound effects on a cell-line or animal tissue. In this study, a lot of the investigative work was performed before the microarray analysis and it was used to substantiate the SybrGreen analysis and of course also to probe further gene expression changes occurring due to the over-expression of Nurr1 in 3T3-L1 adipocytes. The wealth of data obtained from such an experiment is overwhelming and deep scrutiny of the results is required to try and elucidate the reasons behind these gene expression changes. What is paramount to remember is that this, along with SybrGreen analysis, is all at the mRNA level and although the differences between the control or GFP adenovirus and Nurr1 adenovirus cells is phenotypically evident, further experiments are required to corroborate these findings at the protein level.

Potent effects were evident on several of the adipogenic transcription factors belonging to the PPAR and C/EBP familys (Table 1). Previously PPARγ was identified (Chapter 3) as being expressed at much lower levels in the Nurr1 over-expressing cells compared to wild type, a result which has been confirmed here. However, whilst the fold reduction of PPARγ was not as marked as witnessed before, the trend was similar and as stated the results are all semi-quantitative so expression levels are only indicative. Surprisingly C/EBPα showed a dramatic reduction in expression which was not evident in the previous SybrGreen studies. This anomaly could be due to design of primers employed in the SybrGreen studies and further primer sets would be required to confirm this finding. As PPARγ and C/EBPα have been discussed at length in Chapter 3 they will not be discussed further here. Nurr1 also significantly reduced C/EBPγ mRNA levels, which encodes a small protein belonging to the C/EBP family lacking transactivation domains. Therefore dimerisation of this molecule with the other C/EBP isoforms may produce inactive heterodimers and is thought to be a defective form or an inhibitor of the other forms of C/EBPs.

Two co-factors, PGC1α, β as well as the PPAR binding protein, PBP (Table 1) were identified as being down regulated and up regulated respectively. The PGC1 co-factors interact with and co-activate PPARγ as well as a number of other nuclear receptors (Kamei 2003). PGC1α is expressed in several tissues and is cold inducible in brown fat and skeletal muscle (Larrouy 1999). PGC1β is expressed in brown fat and heart (Lin J 2002). Ectopic expression of PGC1 in white fat or skeletal muscle cells induces a broad program of thermogenesis, including increased respiration, mitochondrial biogenesis and increased expression of UCPs (Spiegelman 2000). Although decreased levels of PGC-1 expression have been reported in morbidly obese individuals, the lower levels of PGC1 evident from the microarray data may be a consequence of lower levels of PPARγ (Semple 2004) or it could be indicative of insulin resistance (Hammarstedt 2003).

PPAR binding protein (PBP) was originally cloned as a co-activator for PPAR (Zhu 1997) as well as other nuclear receptors (Torra 2004, Savkur 2004). PBP is an integral component of a multiprotein thyroid hormone receptor-associated protein (TRAP)/vitamin D(3) receptor-interacting protein (DRIP)/activator-recruited cofactor (ARC) complex required for transcriptional activity. Its elevated level in this study could be due to the fact that this protein co-activates a number of other nuclear receptors, one of which is also up-regulated in this study, VDR.

HSL and CD36, 2 genes identified with altered expression patterns in Chapter 3; showed the same reduced expression levels in the microarray study (Table 2). HSL is greatly reduced by T192 with CD36 showing lower, but still significant, levels of decrease. It is possible that this is a consequence of reduced adipogenesis which decreases the expression of these genes and hence resulting in lower lipid accumulation. All the other fatty acid metabolism genes identified have lower levels of expression, except for CPT1 – carnitine palmitoyltransferase (Table 2). CPT1 is located in the outer mitochondrial membrane. Although not normally expressed in adipose tissue it may be suggestive of its activity and kinetic properties with changing physiological state.

Some the most intriguing and exciting data to emerge from the microarray study was that obtained for the adipokines (Table 3). Gene expression studies in adipose tissue have revealed an increased expression of inflammatory markers in obese animals (Soukas 2000, Moraes 2003, Weisberg 2003) and conversely a decrease in their expression following weight loss (Clement 2004). As well as adipocytes,

adipose tissue contains a number of other cells types, contained in the stromal vascular fraction. A study comparing the adipocyte and stromal vascular fraction (SVF) revealed that the majority of adipose cytokines are in the non fat cells (Bouloumie 2005). It is also suggested that an integral part of the inflammatory response might evolve from progressive macrophage infiltration into the adipose tissue during weight gain (Xu 2003, Weisberg 2003).

Resistin: Resistin belongs to a family of cysteine rich proteins termed RELMs (resistin like molecules), each having unique tissue expression (Steppan 2001a). The principal source of resistin is the adipocyte in the mouse; its release was increased in obese mice and accompanied by insulin resistance. Adipocyte derived resistin was thought to be the link between obesity and diabetes (Steppan 2001b). However, human studies show an entirely different picture. Unlike the situation in mice, human fat cells do not produce resistin, although there is some evidence of resistin derived from non–fat cells (Nagaev 2001, Fain 2003). There are some findings which suggest both resistin and resistin-like molecules can inhibit preadipocyte differentiation (Kim 2001, Blagoev 2002). However, in the results obtained in the microarray study of Nurr1 over-expressing cells, it is possible that the observed down-regulation of resistin expression may be more of a character of the reduced lipid accumulation in the 3T3-L1 cells (Table 3).

IL6: IL-6, a cytokine, is secreted by many tissues and cell types including adipose tissue (Papanicolaou 2000, Mohamed-Ali 1997, Fried 1998). It decreases expression of lipoprotein lipase and its activity, in the 3T3-L1 cell line, (Greenberg 1992) as well as increasing lipolysis (Feingold 1992, Hardardottir 1994), possibly through a down regulation of triglyceride deposition and an increase in fuel mobilisation (Greenberg 1992). This cytokine can be induced by a number of molecules including TNFα, IL-1β and also by β-adrenergic agonists (Stephans 1992, Flower 2003, Mohamed-Ali 2000). IL-6 deficient mice have reduced glucose tolerance and females have increased circulating triglycerides and leptin insensitivity. These mice develop mature onset obesity with an increase in sub-cutaneous adipose tissue that can be partly reversed by IL-6 injections (Wallenius 2002). Interestingly IL-6 also has stimulatory effects on the HPA axis, increasing hypothalamic secretion of CRH and release of ACTH and cortisol (Mastorakos

1993) – note that the NGFIB family also play a role in the HPA axis as discussed in Chapter one. The microarray data shows a 15-fold increase in IL-6 mRNA (Table 3) and the ELISA results also demonstrate an increase in this cytokine in response to Nurr1 overexpression (Fig. 4.6). We know that IL-6 is expressed at higher levels in the pre-adipocyte than the adipocyte; however the results show approximately 3-fold increase in IL-6 release compared to the wild type control differentiating cells. We also know that glucocorticoids suppress the induction of IL-6, and in general antagonize the responsiveness of the NGFIB family of genes (Philips 1997). And as alluded to above, nur77 and Nurr1 regulate POMC which in turn produces ACTH, which is involved with the synthesis of glucocorticoids from the adrenal gland.

Is it possible that Nurr1 induces IL-6 and then there is some kind of feedback system that can re-induce Nurr1 or keep it constitutively active? Prostaglandin E2 and PGI2 both elevate intracellular cAMP and are constitutively produced by adipocytes and this may mediate an increase in Nurr1 which then induces IL-6.

MCP1: Monocyte chemoattractant-1 is a pro-inflammatory chemokine, mainly produced by macrophages and endothelial cells, and suggested to be involved in atherosclerosis in vitro (Inoue 2002, Tabata 2003). Circulating MCP-1 has been found to be elevated in obese animal models and reduced after weight loss (Sartipy 2003, Takahashi 2003). As with the other cytokines, expression is higher in the pre-adipocyte stromal vascular fraction as compared to the adipocyte fraction (Christiansen 2005). There is contrasting evidence which suggest that IL-6 can induce MCP-1, which may be due to the model systems used (Murao 1999, Fasshauer 2004). Like with IL-6, lipid accumulation is inhibited in the presence of MCP-1, down-regulating LPL expression and a number of other adipogenic genes expressed in mature adipocytes (Gerhardt 2001, Greenberg 1992). Again MCP-1 was another cytokine with elevated expression levels in the Nurr1 population of cells (Table 3). There would appear to be a significant increase in MCP-1 release in 3T3-L1 cells paralleling that found at the mRNA level, but the results were variable for the primary cells, with GFP giving wildly erratic release, making confirmation of this result difficult to verify (Figs. 4.7 and 4.9). GFP adenovirus has had no influence on either IL-6 or RANTES release or mRNA expression, although there is some previous reports to suggest that adenoviral vectors can have

some effect on the release/expression of both MCP-1 and RANTES (Zhang 2003). At this stage it is hard to deduce whether the MCP-1 effect is real and further work will be required.

RANTES: RANTES, an abbreviation for "regulated upon activation normal T cell expressed and secreted" (Appay 2001), is induced in leukocyte migration by RANTES binding to a GPCR – CCR1, 3, 4 or 5 and mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes, but also acts on basophils, eosinophils, natural killer cells, dendritic and mast cells (Schall 1991). To date there is no evidence of expression or release of RANTES from adipose tissue. Here is the first evidence that basal levels of RANTES expression can be detected in both 3T3-L1 cells and primary differentiated adipocytes (Table 3). In 3T3-L1 cells, expression in the Nurr1 population is approximately 5 fold higher than that of either control or GFP infected cells, peaking 2 days post-induction and returning to basal levels by day 5. In the primary cells, although approximately 10 fold less release of RANTES is observed, there is still an increase in release observed in the Nurr1 cells, up to day 3 and levels have returned to baseline by day 7 (Figs. 4.8 and 4.10). Any concern that this is an adenoviral effect is borne out by the absence of induction in the GFP cells.

Adiponectin: Adiponectin is secreted exclusively from the adipocyte (Scherer 1995). In contrast to leptin, adiponectin is reduced in obesity and increased in response to weight loss. It has also been shown to decrease in diabetes but improves with insulin sensitising agents (Chandran 2003). It has been described as an endogenous insulin sensitiser and an anti-inflammatory factor. Adiponectin knock-out mice develop insulin resistance, glucose intolerance, dyslipidemia and susceptibility to vascular injury and atherosclerosis. Restoration of adiponectin reverses these effects (Maeda 2002, Kubota 2002). The microarray data shows a 3-7 fold decrease in adiponectin levels, which maybe solely due to the reduction in number of adipocytes, compared to the control cells – this would correlate with the data which suggests that this adipokine is expressed and secreted by adipocytes (Table 3).

Adipsin: Adipsin is another adipokine which is secreted from the adipocyte (Wang 2004) and is the enzyme responsible, along with C3 and factor B, for the generation of another adipose tissue protein – acylation-stimulating protein (ASP). An early observation was made that expression was reduced in both genetic and acquired models of obesity; however it is no longer regarded as a signalling molecule in energy balance but more of an early adipocyte marker gene (Flier 1987). Again reduction of adipsin mRNA expression (5-17 fold) in the microarray data maybe more indicative of a decreased number of adipocytes (Table 3). The decrease in expression of complement component 3 could also be due to fewer mature adipocytes. Mice lacking this factor have a greater caloric intake with normal fat absorption but are significantly leaner (Faraj 2004).

SAA3: Expression of Serum Amyloid A (SAA), as with other acute phase reactants, is predominantly in the liver. However, expression in tissues other than the liver has been reported (Norkina 2004). Adipocytes are highly responsive to inflammatory stimuli and treatment with TNFα and IL-6 induces serum amyloid A3 protein in 3T3-L1 cells (Lin 2001). It could be speculated that the increase in SAA3 expression (4-fold) apparent from the microarray data is probably due to the up-regulation and release of IL-6 (Table 3).

Matrix Metalloproteinases: Adipocyte differentiation is associated with an extensive reorganisation and remodelling of the extra cellular matrix (ECM). Two major families of proteases have been involved in the process – serine proteinases and matrix metalloproteinases (MMPs) (Carmeliet 1998). MMPs are highly regulated enzymes with expression, secretion and activity levels under very precise control. They are synthesised in a precursor format and must be proteolytically cleaved to generate the mature enzyme. Their activity is further modulated by Tissue Inhibitors of MMPs (TIMPs) (Gomez 1997). Several MMPs have intriguing and opposing effects in the adipogenic process suggesting that they have distinct roles and substrates during fate decisions and/or terminal differentiation. Discussions will centre on those MMPs and related genes that were highlighted by the microarray data. The microarray data indicates an increase in expression of MMP3, 11, 13 and 14 (Table 6). MMP3 and 11 knock out mice both exhibit an increase in body weight, compared to WT (Maquoi 2003, Lijnen 2002), suggesting

roles in adipose tissue development. It was also suggested that MMP3, among other MMPs, may have implications for angiogenesis (Stetler-Stevenson 1999), which is central to the differentiation of adipose tissue (Crandall 1997). So an increase in expression of these particular MMPs would have a negative role in adipogenesis.

TIMP 1 and 2 also showed an increase in expression (Table 6). It would appear expression of both these TIMPS is highest in the pre-adipocyte with expression decreasing during differentiation (Maquoi 2002). The other metalloproteinase associated family of proteins – the ADAM and ADAMTS (A Disintegrin And Metalloproteinase and ADAM proteins with a thrombospondin (TS)), have also had minor implications in adipogenesis, with most information available on ADAM 12 and ADAMTS1. Data on ADAM 12 suggests an involvement in adipogenesis as over-expression of this gene in mice leads to an increase in body fat compared to WT, through an unknown mechanism (Kawaguchi 2002). ADAMTS1 KO shows a leaner phenotype (Shindo 2000), although in this microarray study no apparent changes were found in this gene. There does not appear to be any evidence relating to the other members of the ADAMs family highlighted in this study, but changes in their gene expression could be related to changes in cellular differentiation, due to the over-expression of Nurr1 (Table 6).

Krüppel-Like Factors (KLF): These are members of a family of transcription factors, which are important regulators of development, cellular differentiation and growth, as well as pathogenesis of atherosclerosis and tumor development (Kaczynski 2003). Members of this family are characterised by multiple zinc fingers containing regions with conserved sequences (Bieker 2001). A role for this family has been demonstrated in adipocyte biology. One study has shown KLF2 (LKLF) can decrease expression of PPARγ as the promoter contains a consensus KLF binding site but also possibly through inhibtion of C/EBPα (Banerjee 2003). Others have shown a down-regulation of its expression during differentiation (Soukas 2001, Burton 2002). More recently, it has been established that KLF2 does not actually affect the differentiation process *per se*, but actually maintains the cells in a preadipocyte form (Wu 2005). Another member, for which a putative role has been postulated, is KLF15. KLF15 is induced late in 3T3-L1 cell differentiation and positively regulates GLUT4 (Gray 2002). It is also suggested

that it promotes the maintenance of the morphological and biochemical characteristics of mature adipocytes through direct induction of PPARγ expression (Mori 2005). The majority of the other family members have also been shown to up or down-regulated during differentiation of 3T3-L1 cells (Inuzuka 1999, Mori 2005). A number of the KLF family members were conversely regulated in the microarray study. KLF2, the negative regulator of adipogenesis, was up-regulated at the early time point and the surprisingly down-regulated at the later time point, for reasons unknown. KLF4, 5 and 7 have previously been shown to be down-regulated in adipogenesis and are upregulated in this study at the earlier time–point, in the Nurr1 over-expressing cells, with KLF4 still exhibiting an increase at the later time point. KLF4 has been implicated in pro-inflammatory signalling and can be induced by IFNγ, LPS and TNFα (Feinberg 2005). KLF9, which has been shown in the Mori paper to up regulated during differentiation, is down-regulated at the later time point. These results, along with what is already known about these factors would suggest multiple roles for this family in adipogenesis (Table 7).

Nuclear receptors: Quite a few nuclear receptors with implications in adipogenesis appear to be differentially expressed in this experiment (Table 8). It cannot be categorically stated that these changes are due to Nurr1 direct action on their expression, but perhaps it could be due to the non-differentiation of the cells and lack of lipid accumulation. Further work would be required to characterise whether the changes are directly mediated by Nurr1.

Rev-Erb alpha, encoded on the opposite strand of the thyroid receptor alpha gene, has been shown to be dramatically induced during the differentiation of 3T3-L1 cells (Chawla 1993). It is possible that it acts as an enhancer of adipogenesis by acting downstream of PPARγ (Fontaine 2003). In contrast to this, its expression is down-regulated in C2C12 mouse myoblastoma cell line during differentiation into multinucleated myotubes (Downes 1995). Its down regulation in this study could be due to the down regulation of PPARγ. Because it is encoded on the opposite strand to TRα, this could explain the down –regulation of this gene as well. TRα is involved in the modulation of fatty acid synthesis via regulation of expression of lipogenic enzymes. SPOT14, a thyroid responsive gene, is also hugely down-regulated in this study.

Another family of nuclear receptors which have been reported to be up-regulated during differentiation of 3T3-L1 cells are the ROR's – retinoid-like orphan receptor. ROR alpha and gamma were upregulated in 3T3-L1 cells while ROR beta could not be detected (Austin 1998). ROR alpha and Rev-ErbA alpha have been reported to have opposing effects on inflammatory responses in VSMC, whether there is evidence in adipose tissue remains unknown (Laitinen 2005). However, in this study both ROR alpha and ROR gamma are down-regulated, again possibly due to the down regulation of PPARγ and subsequent reduction in lipids.

Liver X receptors have a well established role in regulating cholesterol homeostasis (Tontonoz 2003). They are also potent stimulators of fatty acid and triglyceride synthesis through the regulation of SREBP1c and its downstream targets (Yoshikawa 2001). It has also been reported that LXR knock-outs have defective hepatic lipid metabolism and are resistant to diet induced obesity (Kalaany 2005). There is also some evidence that LXR's can regulate the activity of PPARγ during adipogenesis and knock-down of LXR expression using siRNA inhibited differentiation (Seo 2004). LXR expression is markedly reduced in this study and this reduction of LXR could be responsible for the reduced lipogenesis and subsequent adipocyte gene expression and lipid accumulation.

Another nuclear receptor which recently has been shown to be implicated in differentiation is COUP-TF1, which was induced upon the addition of an RAR agonist (TTNBP), known to inhibit adipogenesis (Brandebourg 2005). It has also been shown that mutations of the fly homolog, Svp, impair body fat (Hoshizaki DK 1994). Interestingly we observe an increase in COUP-TF1 expression at the earlier time point. As a role for this receptor has not been fully elucidated in adipogenesis, any conclusions as to why there is an increase would be difficult to reach until further study.

VDR another receptor in which an increase in expression from the microarray experiment is evident also has some association with adipogenesis and obesity. Recently, it has been demonstrated that VDR expression is one of the earliest nuclear receptors, excluding the NGFIB's, to be expressed during the differentiation of 3T3-L1's, with levels peaking at 4hrs and then returning to basal levels by 24hours (Fu 2005). Polymorphisms in VDR have been associated with obesity in type 2 diabetes with early age of onset (Ye 2001). Also, serum

concentrations of 1,25-dihydroxy vitamin D are negatively correlated with obesity (Arunabh 2003). And finally, there are studies to suggest that *in vitro* VDR activation might inhibit adipogenesis (Sato 1988).

To date, ERRγ has not been shown to be expressed in 3T3-L1 cells. Even the recent paper examining the expression of all nuclear receptors in 3T3-L1 differentiation was unable to detect expression (Fu 2005). Therefore, as of yet there is an unknown role for this receptor in adipose.

A number of GPCR's were shown to be differentially regulated upon over-expression of Nurr1, with a number of these known to be involved in energy homeostasis, satiety and hypertension. The activity of these GPCR's may be regulated by nuclear receptors and their ligands, so their differential regulation may be a consequence of up/down regulation of these. However, it is worthy to note, that the anti-lipolytic receptor HM74a and the free fatty acid receptor GPR43 are greatly down-regulated at both time points (Table 9).

Steroids and adipose tissue: Sex steroid hormones are involved in the metabolism, accumulation and distribution of adipose tissue. The distribution of fat is greatly different for men and women, with men having a more central fat deposition, whilst women a more gluteal/femoral accumulation (Bjorntorp 1996). LPL and leptin, two key adipose proteins, appear to be regulated by sex steroid hormones in a classic genomic mechanism. Normal amounts of steroids such as estrogen and androgens favour homeostasis of adipose tissue, with decreases in either leading to some form of obesity, normally central (Mayes 2004). It is also known that glucocorticoids, as mentioned earlier in Chapter 2, and the HPA axis have much influence on adipose tissue and so may be involved in the pathogenesis of obesity. The enzyme 11β-HSD (1 and 2) inter-converts the active glucocorticoid, cortisol, and inactive cortisone. 11β-HSD1 is the only isoform present in adipose tissue, where it acts predominantly as an oxoreductase to generate cortisol (Ricketts 1998). Given the direct regulation of the steroid synthesis pathway by members of the NGFIB family (Fig. 4.11) and the intricate involvement of steroids in adipose tissue, the results obtained by the microarray are very encouraging in the supposition of this family in adipogenesis (Table 4). A number of steroid metabolising enzymes are all down-regulated, especially 11β-HSD1, inhibitors of which have been cited as potential obesity pharmacological

agents. Although specific isoform 1 inhibitors have yet to be developed for metabolic syndrome some success has been met as a diabetes therapy (Barf 2002).

Nitric Oxide pathway: Specific amino acids, particularly L-arginine, can modify insulin action, perhaps through both NO-dependent and NO-independent pathways (Tong 2004). L-arginine either acts as a precursor in the formation of NO, catalysed by a family of NO synthases (NOS), or is metabolised to L-ornithine, urea and polyamines. Intracellular arginine is maintained by three important processes: transport from extracellular sources via the cationic amino acid transporters, intra-cellular protein degradation and biosynthesis in the 'citrulline–NO cycle', where it is regenerated from citrulline via successive actions of ASS and ASL (Fig. 4.12) (Husson 2003).

Another enzyme that may participate in the cellular regeneration of L-arginine by providing cytosolic citrulline is DDAH (Fig. 4.12). This enzyme catalyses the catabolism of asymmetric dimethyl arginine (ADMA) to form citrulline and dimethlyamine. ADMA is released during the hydrolysis of proteins that contain arginine residues which are methylated by protein arginine methyltransferases (PRMTs). It competitively inhibits all forms of NOS and therefore causes endothelial dysfunction (Vallance 2002). Thus, inhibition of DDAH activity could potentially depress cytosolic citrulline, the substrate for ASS, as well as elevate levels of an endogenous NOS inhibitor, and *vice versa*.

Both extracellular transport and intracellular regeneration contribute significantly to the levels of cellular arginine. However their relative contributions may vary depending on the prevailing hormonal and metabolic conditions. It has been discovered that a number of the enzymes shown in Figure 4.12 are expressed in adipocytes, however, ASS, the rate-limiting step in the citrulline-NO pathway, and both isoforms of DDAH are particularly abundant. The tissue generates significant amounts of ADMA, and DDAH activity is modifiable by changes in weight and insulin sensitizers (Mohamed-Ali 2005). A number of the enzymes that regulate cellular NO are identified here, DDAH1/2 and ASS, as being expressed in 3T3-L1 cells and interestingly would appear to be increased upon the over-expression of Nurr1, which would lead to a decrease in circulating ADMA levels (Table 5). Putative PPAR response elements have been identified in the DDAH2 gene (Achan 2002) which could possibly also function as NBRE's. Increases in ADMA levels

are implicated in cardiovascular disease and diabetes, therefore therapies which modulate its expression would be advantageous. Thus, ASS and DDAH may be good targets to regulate the production of NO.

As can be seen there are large number of gene changes brought about by the over-expression of Nurr1 in differentiating 3T3-L1 cells. It is more than likely the changes at the earlier time point are due to Nurr1, whilst the later changes may reflect the pre-adipocyte like nature of the cell. In this thesis, only a smaller number of genes have been discussed and the comprehensive involvement of Nurr1 in regulating these pathways still needs to be further elucidated. However, what is evident is the number of different cellular adipose functions which have been affected, some of which have not been recognised before eg NO pathway.

To date, most of the work in the thesis has concentrated on the over-expression of Nurr1 in differentiating 3T3-L1 cells. Initial data in Chapter 2 demonstrated that the adipogenic cocktail induced a rapid transient induction of expression whereas the subsequent chapters concentrated on the overexpression of Nurr1 in differentiating cells. What is unknown at this stage is whether or not the transient induction of Nurr1 is required for adipogenesis, by either switching off the genes which maintain the cells in the preadipocyte state, or for inducing very early adipogenesis events. The next chapter demonstrates preliminary data into the knock-down of Nurr1 and nur77 in differentiating 3T3-L1 cells, to begin to decipher which phenomenon the NGFIB's are responsible for in adipogenesis.

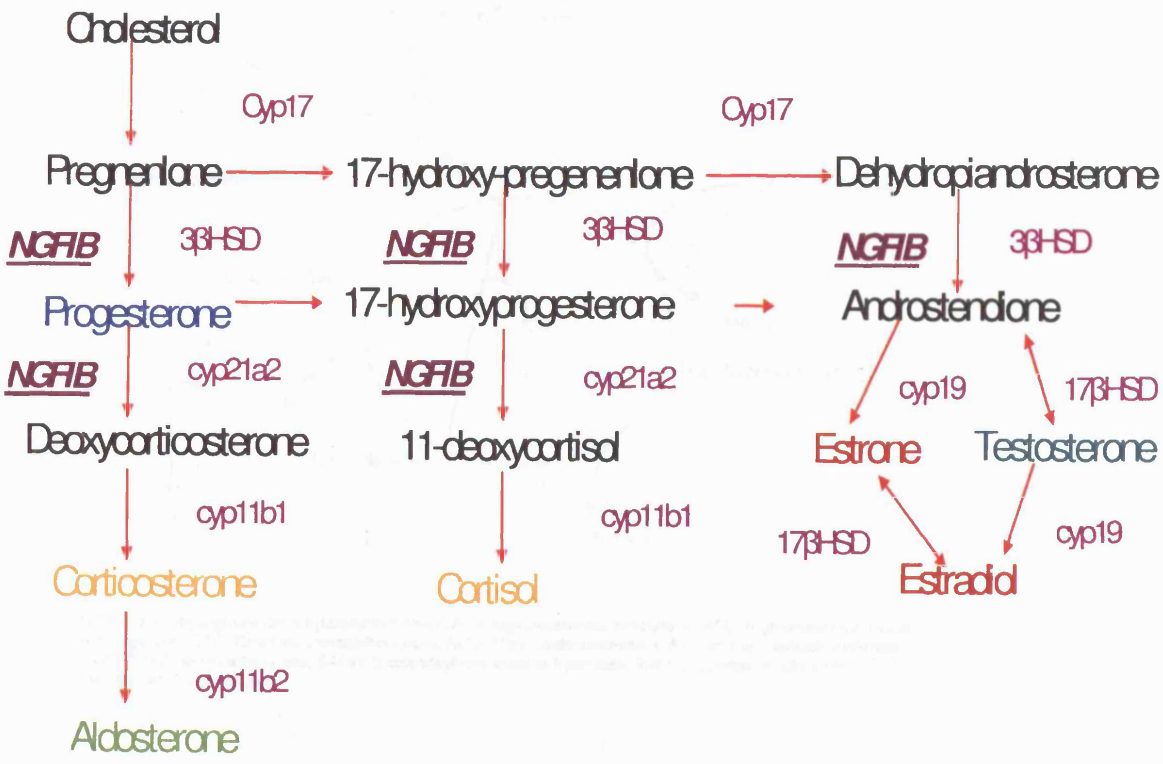
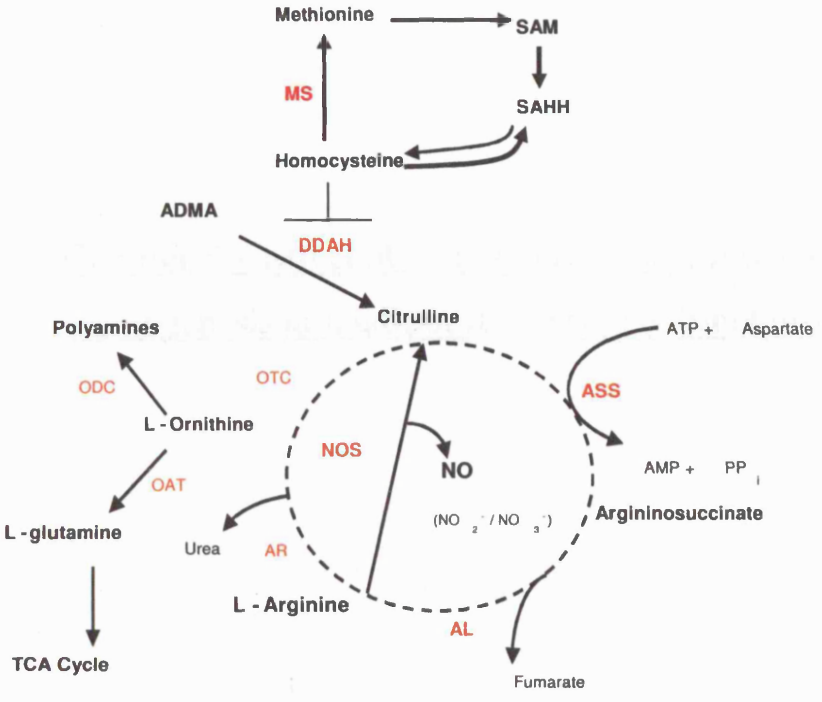


Fig. 4.11 Steroid synthesis and NGFIB involvement



DDAH: dimethylarginine dimethylaminohydrolase; ASS:Arginosuccinate synthetase; ASL: Arginosuccinate Lyase; AR: Arginase; OTC: Ornithine transcarbomylase; NOS: Nitric oxide synthase; OAT: Ornithine aminotransferase; ODC: Ornithine decarboxylase; SAHH: S-adenosylhomocyteine hydrolase; SAM: S-adenosylmethionine; MS: Methionine synthase

Fig. 4.12 Interrelationship between the NO-citrulline pathway and methionine/homocysteine metabolism.

Chapter 5 – Effect of Nurr1 gene knockdown on adipogenesis and adipocyte secretory functions

Introduction

A number of techniques have been used in an attempt to down-regulate gene expression e.g. antisense oligonucleotides and ribozymes (Loke 1989, Funato 1992). However, they were relatively unsuccessful in producing effective silencing of the gene in question in complex mammalian systems. Recent developments in the RNA interference methodologies have seen siRNAs become the method of choice for the down regulation of target genes.

RNAi was first used in gene knock down studies in *C.elegans*, drosophila and plants (Hamilton 1999, Tuschl 1999, Sijen 2001). It was discovered that long double-stranded RNA can specifically silence genes through an evolutionary conserved mechanism to protect against viral infections. Introduction of long dsRNA inhibits gene expression by sequence specific degradation of mRNA. The long dsRNA is digested into smaller 21-23mer active intermediates by a member of the RNase III family of dsRNA-specific endonucleases, Dicer (Bernstein 2001). These small interfering RNAs (siRNAs) act as functional intermediates and induce target mRNA cleavage by being incorporated and unwound by the RNA inducing silencing complex (RISC) (Hammond 2000). RISC then utilises the unwound antisense siRNA to bind to a complementary sequence on the mRNA and cleaves between nucleotide 10 and 11 of the siRNA, initiating degradation and inhibiting further gene expression (Elbashir 2001).

However, as a method, its use in mammalian systems is limited due to long dsRNA's eliciting an interferon response, resulting in general inhibition of protein synthesis, often leading to cell death (Baglioni 1979). It was discovered that in contrast to the long dsRNA. siRNAs (21-23nucleotides) generally do not elicit this interferon response (Elbashir 2001, Caplen 2001) Over the past few years a number of methods have been developed to create these siRNAs including chemical synthesis (Elbashir 2001), in vitro transcription (Donze 2002) or a vector based system (Miyagishi 2004). The efficiency of the siRNA is more dependent on the design of these siRNA, rather than the method of transfection itself. Advances in the understanding of the actual biochemical mechanisms of action of siRNA (Schwarz 2003), statistical analysis of experimentally verified siRNA (Mittal 2004) and sequence characteristics of functional siRNA (Reynolds 2004, Jagla 2005) have revolutionised target validation, drug discovery and therapeutic development.

Over-expression as a method of target validation is also an extremely powerful tool of target validation, but the ability to be able to down-regulate the gene and analyse functional and phenotypic changes is incredible. The use of both approaches, over-expression and siRNA, to complement each other, should allow elucidation of the role of Nurr1 in adipogenesis.

As a target, the NGFIB family have been subjected to siRNA studies but mainly in the fields already described in Chapter 1. Nurr1 has been knocked down *in vivo* to investigate the expression of genes related to the dopaminergic system of the ventral mesencephalon (Backman 2003). Both Nurr1 and nur77 down regulation in the human granulosa-like cell line, KGN, significantly decreased aromatase expression (Wu 2005). And finally knock down of Nurr1 and nur77 in HeLa cells, reduced the anchorage dependent growth and promoted intrinsic apoptosis, similar to that observed in other experimental cancer cell lines. This work showed that both Nurr1 and nur77 exhibit oncogenic functionalities with regards to cell proliferation and apoptosis, suggesting evaluation of these targets as potential therapeutic targets for cancer (Ke 2004).

To date all the experimental work in this thesis has concentrated on the over-expression of Nurr1 in 3T3-L1 adipocyte cells. This chapter focuses on very preliminary work on knock-down of Nurr1 and nur77 in 3T3-L1 cells using siRNA.

Experimental Procedures

Nurr1 and nur77 siRNAs

Mouse Nurr1 SMARTpool siRNA, nur77 SMARTpool siRNA and transfection control siGlo were obtained from Dharmacon (Perbio, Northumberland UK). SMARTpool combines four SMARTselection-designed siRNAs with a high probability of knock down. Sequences for these were not available. Reagents were resuspended as recommended by the manufacturer.

Transfection efficiency

3T3-L1 cells were seeded in 6-well plates at a density of $4x10^5$ cells per well. Transfections were performed when cells were 60-70% confluency. siGlo (25nM and 50nM) reagent was used to assess transfection efficiency into 3T3-L1 cells and also the best delivery agent – Fugene (Roche Diagnostics, East Sussex UK) or Lipofectamine 2000 (Invitrogen, Paisley UK).

Fugene method: 2µl of Fugene was diluted in 300µl of Optimem (Invitrogen). 25nM/50nM of siGlo was diluted in 300µl Optimem, mixed and the complex incubated for 15 mins. A further 400µl of Optimem was added to the complex, before adding to the cells. Cells were returned to incubator and incubated at 37°C 5% CO_2 for 6 hrs. Transfection mix was removed after 6hrs and replaced with fresh growth media (as described in Chapter 2).

Lipofectamine 2000 Method: 1µl of Lipofectamine 2000 was diluted in 300µl of Optimem. Then followed as per Fugene method above.

24 hrs post-transfection the cells were assessed for transfection efficiency by fluorescence light microscopy (Abs Max 550nm, Emission Max 570nm).

Nurr1 and nur77 siRNA Transfections

Following the Lipofectamine 2000 protocol, 3T3-L1 cells were transfected as before with 1nM, 10nM or 50nM of Nurr1/nur77 or 25nM of siGlo control or mock transfected. Post confluent cells were treated with the differentiation cocktail as described in Chapter 1 (dexamethasone, IBMX and insulin). 2 hrs post-induction cells were lysed for RNA extraction, cDNA synthesis and SybrGreen analysis as described before.

Functional analysis of Nurr1 and nur77 siRNA in 3T3-L1 cells

As outlined in the transfection protocol above, 3T3-L1 cells were transfected with 10nM of Nurr1, nur77 or siGlo Control. Cells were then grown to confluency and differentiated with IBMX alone. 14 days post-differentiation cells were analysed for differentiation efficiency by Oil Red O staining.

Statistical analysis

Results are expressed as mean±SD or SEM for normally distributed values or median (interquartile range) for skewed data. Comparisons between groups were made by two-tailed Student's *t*-test, Mann-Whitney U test or Wilcoxon rank test, as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

3T3-L1 cells transfection efficiency with siGLO

3T3-L1 cells were assessed for their transfection efficiency using siGlo siRNA control with either Fugene or Lipofectamine 2000. siGlo siRNAs are labelled with the fluorophore Cy3, which produces a strong, punctate, perinuclear staining pattern that is sustained in cultured cells for at least 7 days. These are a non-targeting, non-functional siRNA, which can be used to assess transfection efficiency or as a negative control. The fluorescence observed 24-48hrs post-transfections is most likely associated with the intact molecule.

The 3T3-L1 cells proved to be very susceptible to toxicity issues, resulting in cell death. Initial experiments performed using 100nM siGlo proved toxic to the cells, so 25nM and 50nM were chosen for subsequent transfections. Upon examination under a fluorescent microscope, Fugene transfected cells displayed no fluorescent signal at either concentration. Lipofectamine transfected cells displayed transfection efficiency in the region of 75% at the lower concentration of siGlo which also demonstrated the least toxicity issues. There was some evidence of toxicity visible at the higher concentration used. The appearance of the cells was less fibroblast-like.

Nurr1 and nur77 siRNA in 3T3-L1 cells

Using the optimised siGlo protocol, 3T3-L1 cells were transfected with a range of concentrations of either Nurr1 or nur77 (1nM, 10nM, 50nM), siGlo (25nM) and mock transfected cells (Lipofectamine 2000 only). Following transfection the cells were grown to confluency before the addition of the differentiation cocktail (dexamethasone, insulin, IBMX) and then 2hrs post-induction the cells were lysed for RNA extraction and analysis by SybrGreen for both Nurr1 expression and nur77 expression (Fig 5.1 – 5.4). Between 70-80% knockdown at the mRNA level was evident for both Nurr1 and nur77. No effect was evident on nur77 expression in the Nurr1 siRNA experiment, whilst some evidence of compensation was evident in the nur77 siRNA experiment.

14 Days post IBMX differentiation the cells were analysed for lipid accumulation by Oil Red O staining. It was difficult to draw any conclusions from this experiment.

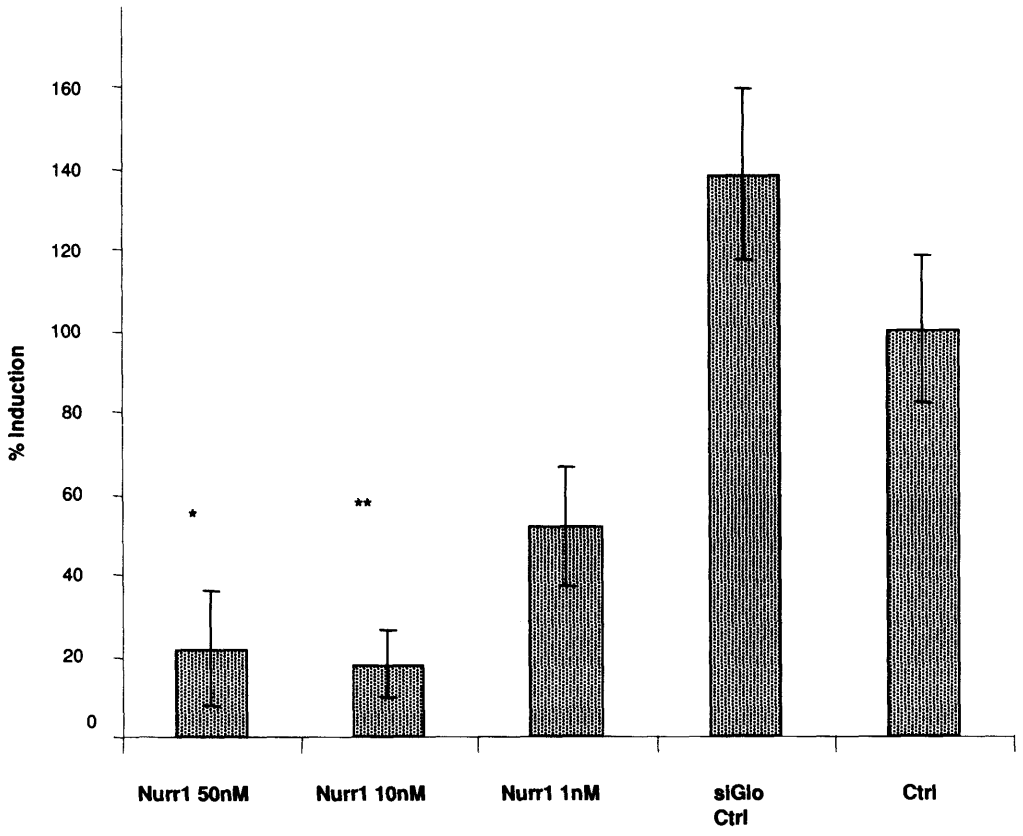


Fig. 5.1 Nurr1 expression in 3T3-L1 cells expressing Nurr1 siRNA

Nurr1 expression as assessed by SybrGreen Analysis and expressed as %age induction compared to the control mock-transfected cells, with SEM, n=3. Data normalised to β-actin.

** p=0.04, **p=0.003*

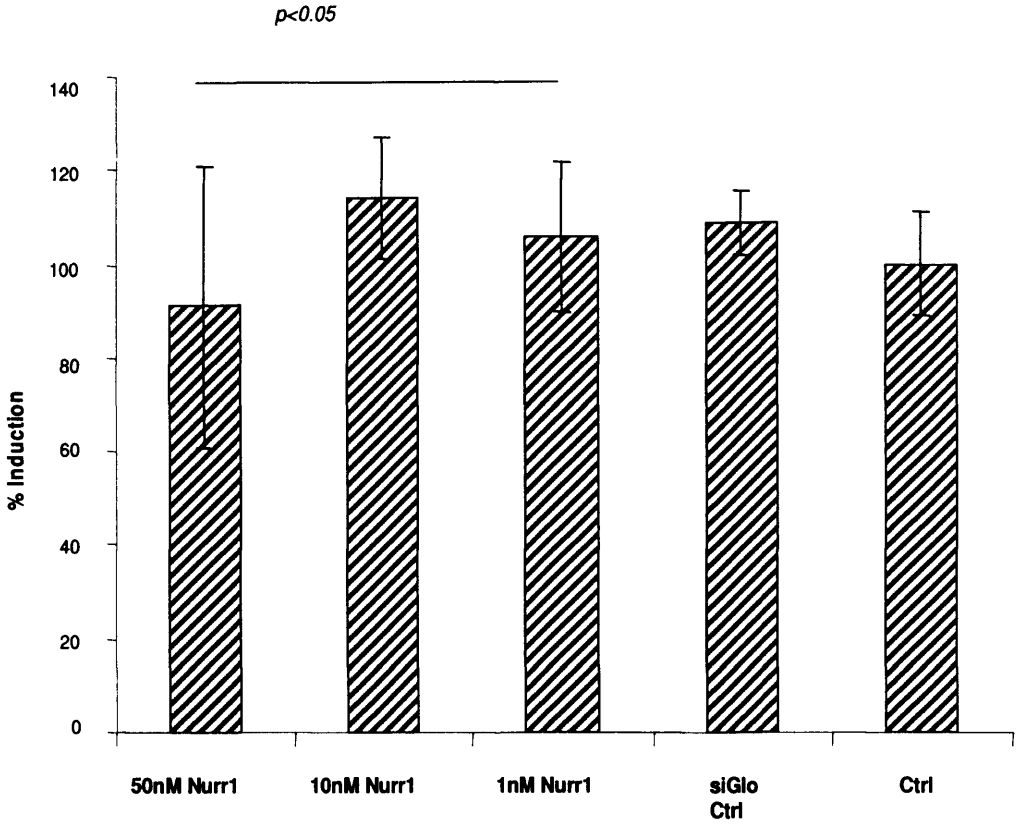


Fig. 5.2 nur77 expression in 3T3-L1 cells expressing Nurr1 siRNA

nur77 expression as assessed by SybrGreen Analysis and expressed as %age induction compared to the control mock-transfected cells, with SEM. Data normalised to β-actin.

** p=0.05 = ns.*

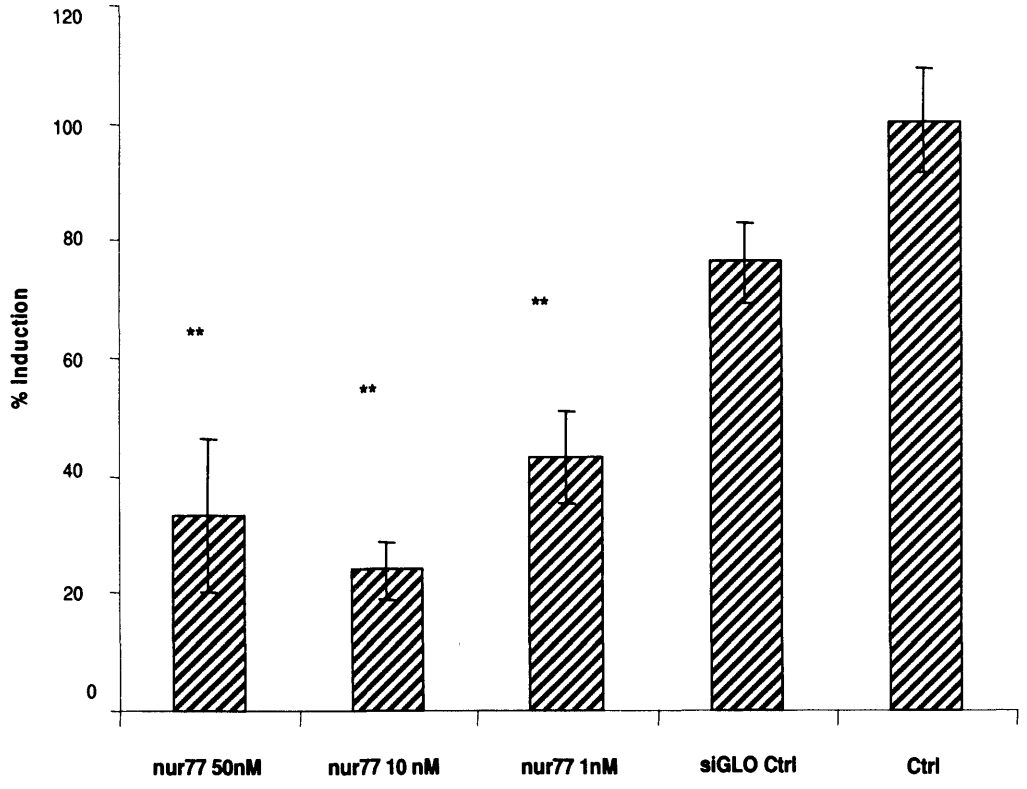


Fig. 5.3 nur77 expression in 3T3-L1 cells expressing nur77 siRNA

nur77 expression as assessed by SybrGreen Analysis and expressed as mean %age induction compared to the control mock-transfected cells, with SEM, n=3. Data normalised to β-actin.

** *p=<0.01*

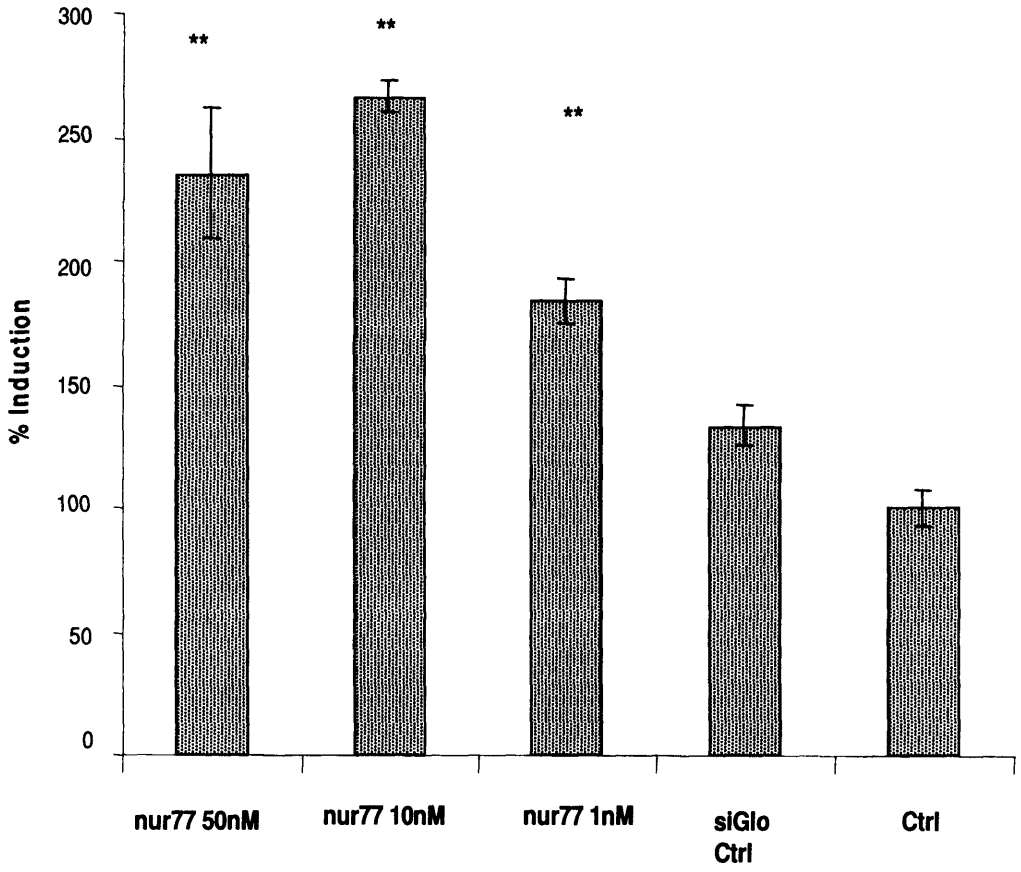


Fig. 5.4 Nurr1 expression in 3T3-L1 cells expressing nur77 siRNA

Nurr1 expression as assessed by SybrGreen Analysis and expressed as mean %age induction compared to the control mock-transfected cells, with SEM, n=3. Data normalised to β-actin.

***p<0.01*

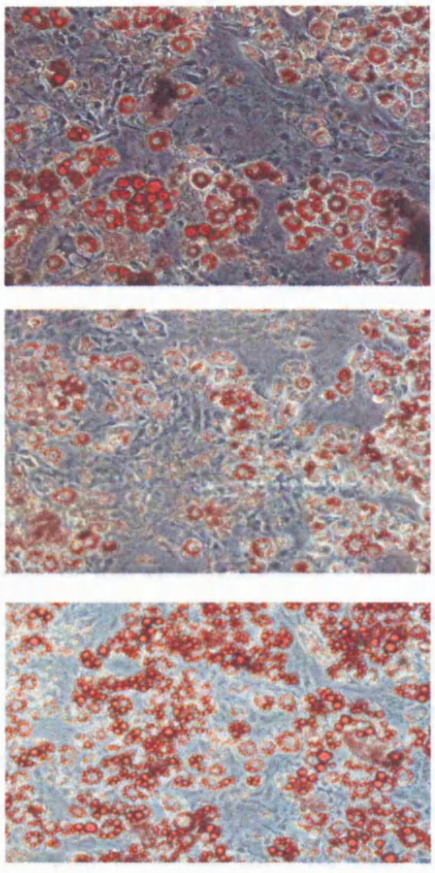


Fig. 5.5 Oil red O staining of differentiated 3T3-L1 cells following transfection of siRNA

3T3-L1 cells were transfected with siRNA for a) siGlo control, b) Nurr1, c) nur77 and then differentiated with IBMX alone. Cells assessed for lipid accumulation by Oil Red O staining 14 days post differentiation.

Discussion

Dharmacon's SMARTpool technology is based on 4 non-identical siRNAs directed at different regions of the target sequence ensuring efficient knock-down of the gene. The scientist can then perform further experiments to identify which siRNA produces the best down regulation of the target. Unfortunately at the early stage of optimisation the sequence for each duplex is unknown to the scientist. Based on statistically significant analysis and specific algorithms, Dharmacon guarantee at least 75% knock-down, when "transfected under optimal cell culture conditions".

3T3-L1 cells are, as discussed in chapter 3, notoriously difficult to transfect. However, advances have been made with the cationic lipids available for use today. Surprisingly Lipofectamine 2000 exhibited the most efficient transfection (75%) of the siGlo reagent and Fugene which is marketed as being able to transfect even the most difficult cell lines failed. Another means which would be a potential way to introduce the siRNA to the cells is Amaxa Nucleofector technology. This is a technology which is especially designed for primary cells and hard-to-transfect cell lines. It is a non-viral method based on electroporation and cell specific solutions. The DNA is delivered directly to the cell nucleus, thereby allowing detection of expression a few hours later.

Both Nurr1 and nur77 siRNAs were used in the experiment as it is known that these family members can play redundant roles for each other (Crawford 1995, Cheng 1997). Both siRNAs appear to work well in 3T3-L1 cells in that, on average a 75% knock-down of both genes is evident. In the Nurr1 down-regulated cells, nur77 expression appears to be unaffected. However, in the nur77 down-regulated cells Nurr1 expression is at least 2 fold higher than the control cells. This result correlates well with literature reports on the nur77 knock-out mouse – which demonstrates an increase in expression of Nurr1 in the absence of nur77 (Crawford 1995) (Fig. 5.1-5.4).

A limited number of functional studies have been performed on the siRNA studies. An initial study to assess the ability of 3T3-L1 cells to undergo adipogenesis and differentiate into adipocytes was carried out using an IBMX only protocol. IBMX alone has the ability to differentiate about 60% of the population of cells. The reason IBMX was chosen as the sole differentiation inducer in this particular experiment is because it is known from Chapter 2 (Fig. 2.10) that IBMX is the sole inducer of Nurr1, in the differentiation cocktail. nur77 can also be induced but to a lesser extent

that Nurr1. The preliminary results are at this stage quite inconclusive as there is no SybrGreen mRNA data on each of the adipogenesis markers that have been analysed before (Chapter 3). It could be postulated that perhaps nur77 knock down cells have differentiated to a slightly greater degree than either the control or Nurr1 knock-down cells (Fig. 5.5). However, at this stage it is unknown how stable the silencing of each siRNA species is. Following the three day incubation in IBMX the cells are then replenished with medium containing insulin, which is known to induce nur77 and induce Nurr1 to a very small degree (Fig. 2.8). This may have an effect on the experiment.

The unprovoked release of IL-6 from 3T3-L1 cells post-differentiation has also been examined. However, the data from this experiment was unreproducible due to a high degree of variability and the level of sensitivity to measure IL-6 is at the lower end of detection of the ELISA kit. This would need to be repeated in primary adipocytes which have a higher basal concentration of IL-6.

The results obtained in this chapter are all very preliminary and further work would be required to optimise the transfection of the siRNA and to analyse the effect that knock-down of Nurr1 and nur77 will have on 3T3-L1 cells. It is likely that the down-regulation of both NGFIB species will be required to assess their function in adipocytes and adipogenesis. It will also be very important to assess the knock-down of the mRNA also results in knock-down at the protein level. As the available antibodies appear not to be very specific for family members further work will be required to assess/design a more specific antibody (see supplementary data).

Chapter 6: Conclusions and Future Plans

Interest in the NGFIB family has escalated dramatically in the last 2-3 years with a wealth of data published on their implications in a number of disease areas from Parkinson's disease (Le 2003) to inflammation (Crofford 1993, McEvoy 2001, McEvoy 2003, Pei 2005). Pharmaceutical interest has waned slightly as data would suggest that this family may be intractable as drug targets in the more traditional approach. However, as a family of nuclear receptors they remain some of the most fascinating and interesting molecules. The data presented herein is some of the first data on their involvement and implications in the secretory and metabolic adipocyte functions, as well as in adipocyte differentiation.

Initial findings showed that this family were highly expressed in human adipose tissue, with evidence that Nurr1 could be induced during differentiation (Chapter 1). The results presented in Chapter 2 formed the basis of the project as they follow up and substantiate this initial data. In these studies, it was found that NOR-1 was not basally expressed in adipocytes and recent evidence seems to suggest that its expression may need to be induced by β-adrenergic agonists (Muscat 2005). This family member appears to be predominantly associated with extraskeletal myxoid chondrosarcoma (EMC), through fusions and translocations (Ohkura 2002) and at this stage it because of its lack of involvement in adipogenesis it was decided to not study this further in this project. Murine adipocyte Nurr1 and nur77 exhibited similar expression patterns during adipogenesis to those obtained from the human cells, in that they are both rapidly and transiently elevated very early in the adipogenic process. This was then further confirmed by the expression data in adipose tissue fractions and found that the Nurr1 and nur77 receptors diverge in their expression profiles. Following collagenase digestion of adipose tissue nur77, the more abundant of the two receptors, appears to be equally abundant in both the adipocyte and stromal vascular fractions, whilst Nurr1 is expressed 10-fold higher in the stromal vascular fraction. Both are significantly induced during differentiation, but are induced by different stimuli. Nurr1 is preferentially and potently induced by the cAMP inducer, IBMX. cAMP is an important regulator of both adipogenesis and lipolysis. It has also been shown that Nurr1 has a cAMP response element present its promoter, whereas the nur77 promoter does not appear to contain one (Ohkubo 2000, McEvoy 2002). This combination of data suggested that Nurr1 is the family member which is most likely to be implicated in the adipogenic program.

The transient nature of induction of Nurr1 makes studying the function of its expression a little more challenging; however there are many over-expression methods available for use, although not all are amenable to all cell lines eg 3T3-L1 cells. In Chapter 3, adenovirus over-expression of Nurr1 is described in order to investigate the function in the mouse 3T3-L1 cell line. Despite the transient induction of Nurr1 expression it must be remembered that it is possible that *in vivo* constitutive expression of this receptor may exist, as adipose tissue reveals high expression of Nurr1. Astonishingly, dramatic results were achieved, with a significant reduction in PPARγ expression and substantial decrease in expression in several adipocyte specific genes. In addition, at the phenotypic level, a significant decrease in lipid accumulation was also evident. Glucose uptake and lipolysis experiments were challenging to conduct and interpret. Both experiments work on the premise of lipid laden cells and as discovered Nurr1 dramatically reduced the lipid accumulation. Numerous attempts were made to perform the experiments including addition of the virus following differentiation. But as already described this produces another set of challenges. The methods employed to analyse gene expression initially are at best not very high through-put and so limiting in the quantity of data that can be extracted. Therefore, microarray experiments were used as a follow-up experiment, as described in Chapter 4.

The data obtained by the powerful microarray technique opens many different avenues and provides data on changes in expression of numerous genes involved in adipogenesis. The changes that were expected e.g. PPARγ, CD36 etc did indeed behave as in the SybrMan experiments in Chapter 3. The exact expression levels may be some what different as neither technique is wholly quantitative. Interestingly, Nurr1 over-expression did appear to have an affect on C/EBPα, not seen by SybrMan. However, this could be dependent on primer design and position. This needs to be followed up for future work. At this early stage it cannot be categorically stated whether Nurr1 over-expression directly or indirectly results in the gene expression changes evident from the microarray data. A number of the genes with altered expression do indeed have NGFIB response elements in their promoters. It could also be postulated that Nurr1 changes in PPARγ expression mediate downstream effects. It is possible that the early reduction in PPARγ expression is responsible for the decrease in expression of many of the adipogenic genes. The gene changes of most interest are those at the earlier time point, 72hrs, as the changes at this point are

the ones that shape the adipogenic pattern. The microarray results also present interesting data on a number of cytokines (adipokines) which were then followed up using ELISA's. The results obtained at the mRNA level accurately predicted those obtained when monitoring protein release using the ELISA technique.

So this data taken in conjunction with the data presented in the previous chapter strengthens the role for Nurr1 in adipogenesis both at the gene expression and secretory function level.

To date all the data concentrate on the over-expression of Nurr1 in 3T3-L1 cells and even though this provides a wealth of functional data, it would also be useful to know what would happen if the transient induction of Nurr1 were not to occur. What is unknown at this stage is whether or not the transient induction of Nurr1 is required for adipogenesis, by either switching off the genes which maintain the cells in the preadipocyte state, or for inducing very early adipogenesis events. The Nurr1 homozygote knock-out is embryonic lethal and in the timescale of the PhD it has not been possible to obtain the heterozygote mice and is definitely worth considering for future work. The alternative option was to employ the current exciting and novel siRNA technology. Although timescales, did not allow full exploitation of this technique, it did show that both Nurr1 and nur77 mRNA expression can successfully be diminished to near insignificant levels (Chapter 5). It is known that Nurr1 can compensate for nur77 expression as is evident in the nur77 knock-out mice, although the opposite was not reported for Nurr1, perhaps due to the nature of the lethality – no known role for nur77 in dopamine synthesis. So it was investigated whether or not knock-down of both genes would be required to perform the subsequent experiments. It would appear that the results obtained in these very preliminary experiments mirror that obtained in animal models. Further work is planned to evaluate the knock-down of these genes in differentiating 3T3-L1 cells.

In conclusion, through the use of many molecular biology and cellular techniques a role for Nurr1 has emerged in adipose tissue and adipogenesis. Here in the very early novel results are presented suggesting that this nuclear receptor may play an opposing role to PPARγ, and hence have a putative role in adipogenesis, lipid accumulation and adipocyte secretory function, with implications for insulin resistance at the adipocyte level. Adipogenesis and the program of events which bring about the differentiation of pre-adipocytes to adipocytes have been extensively studied over the last number of years. More recently interest in the events which inhibit or alter the

adipogenic programme has grown. However the genes studied to date have produced varied results depending on the cell types and conditions used. This could be due to the fact the programme of events are controlled at the transcriptional level and only changes in transcriptional events will bring about true changes.

The data presented in this thesis provides an excellent outlay for future work. The data obtained by the microarray study also provides a vast amount of information that can be utilised and scrutinised further to fully elucidate the mechanism of action of Nurr1 in other adipose tissue functions.

Of course, further work is required to tease out the exact role of Nurr1 in adipogenesis and this will rely on using over-expression and siRNA techniques as laid out below, perhaps with some adaptations.

One approach which could be employed is to reduce the variability produced by the adenovirus over-expression. As a method it is an excellent means to introduce your gene of interest into cultured cells, however there is intrinsic variability associated with it. A relatively new method using, more established techniques would be the use of the Amaxa nucleofector – a method based on electroporation (and cell specific solutions) to introduce DNA to cells. DNA is delivered directly to the cell nucleus and expression can be detected very shortly after nucleofection. It is also possible to generate stable integrants of the gene of interest, thereby eliminating the need to continually transfect cells, reducing experiment to experiment variation.

The siRNA work would also need further validation and follow-up on the effect of knock-down on adipogenesis. From the perspective of siRNA expression, the above technique could also be employed to increase the transfection efficiency. Similar experiments to those employed in the over-expression studies could then be used to fully elucidate the effect of Nurr1 on adipogenesis.

Some very preliminary work has been performed (not reported here) on the induction of Nurr1 by other stimuli and it is possible to induce its expression through IL-1β, forskolin and PGE2. However, sustained expression has never been achieved. There are reports in the literature of activators of Nurr1, so it would be interesting to confirm the activity of these "activators" and examine their effects during differentiation in 3T3-L1 cells, primary mouse cells or even human primary cells. Also, from a pharmacological stand-point, investigations into Nurr1/RXR specific agonists would be hugely exciting and perhaps the best way forward to modulate Nurr1 regulated pathways.

Another avenue which would be tremendously advantageous in the dissection of Nurr1 in adipose tissue function would be through the use of animal models. There are a number of approaches which could be used.

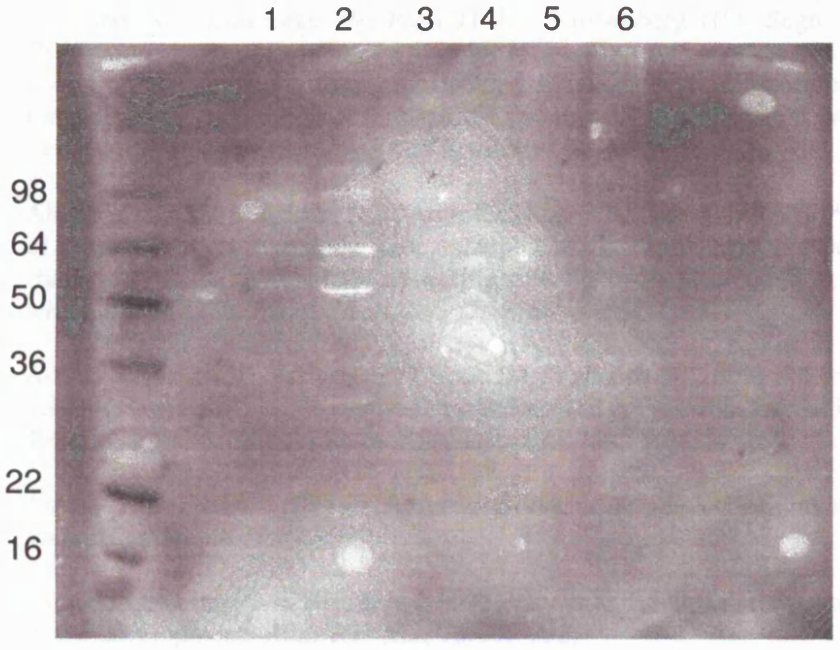
i) Obtain heterozygote Nurr1 mice (Thomas Perlmann, Karolinska Institute, Stockholm) and extract adipose tissue for analysis. Tissue could be fractionated into adipocytes and stromal vascular fractions which could be subsequently cultured for differentiation experiments.

ii) Tissue transplant studies: Over-expression of Nurr1 using adenovirus in 3T3-L1 cells and then transplant these cells into mice and analyse the effects.

iii) Generation of adipose tissue specific knock-outs (possibly conditional knock-outs) and again analyse the effects. These types of experiments would depend on the outcome from the siRNA work.

The pathways referred to in Chapter 4, NO pathway, steroidogenesis and cytokine production are hugely exciting and perhaps some of the more interesting and novel data to come from the microarray experiment. Investigations into the pathways, several of which are over-lapping, in obesity associated pathologies would provide today's society with greater insight into both the development of metabolic disease and provide targeted drug therapies.

Supplementary Data



Supplementary Figure showing Western Blot using Nurr1 specific antibody. Lane 1&2 Nurr1 Gal4 LBD, Lane 3&4 NOR-1 Gal4 LBD, Lane 5&6 nur77 Gal4 LBD. Lysates from transient transfections. Expected band at approximately 30kD is faintly evident in lanes 1 and 2 but some evidence in 5 and 6. Many other products also detected.

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