The potential roles of interactions between STAT3, Hsp90, and Hop in the maintenance of self-renewal in mouse embryonic stem cells

A thesis submitted in fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

of

Rhodes University

Mokgadi Michael Setati

February 2008

ABSTRACT

Self-renewal of mouse embryonic stem (mES) cells is dependent upon the presence of leukemia inhibitory factor (LIF). LIF induces tyrosine phosphorylation and nuclear translocation of STAT3 (signal transducer and activator of transcription 3) which is thought to promote self-renewal by inducing key target genes. The molecular chaperone heat shock protein 90 (Hsp90) is involved in signal transduction pathways and regulates STAT3 activity in different cell types. However, the role of Hsp90 in regulating STAT3 activity in mES cells has not previously been investigated. The aim of this study was to investigate if Hsp90 interacts with STAT3 in mES cells and to determine if this interaction is important for the maintenance of self-renewal. It was found that when mES cells were cultured for 24.0 hours in the absence of LIF, the expression levels of total STAT3, tyrosine-phosphorylated STAT3 (pYSTAT3), and the pluripotency marker, Nanog, were down regulated. However, the expression level of Hsp90 was found to be slightly up-regulated over the same period. Significantly, it was found that the amount of STAT3 in differentiating mES cells available for binding to Hsp90 was decreased upon down-regulation of STAT3 by LIF withdrawal. Therefore, STAT3-Hsp90 interactions in mES cells were dependent on the presence of LIF, which suggested that the reduction in STAT3-Hsp90 interaction may have resulted from the low levels of STAT3. Despite a dramatic reduction in the expression levels of pYSTAT3 upon 24.0 hours of culture of mES cells in the presence of the STAT3 tyrosine phosphorylation inhibitor, cucurbitanin I, there was no obvious reduction in the levels of total STAT3, Oct-3/4 or Nanog. These results suggested that the levels of unphosphorylated STAT3 rather than pYSTAT3, maybe more important in the maintenance of mES cells self-renewal.

ACKNOWLEDGMENTS

I would like to thank:

- ✓ My supervisor, Professor Blatch, G.L. for his unflinching support throughout this project and for his unparalleled dedication to his students.
- ✓ Dr. Murray P.A. for her continued support and motivation and time helping me with my project while in Liverpool.
- ✓ Dr. Longshaw V.M. for inspiring me throughout my project.
- ✓ Prof. Edgar D. for supporting me throughout my project while in Liverpool.
- ✓ Prof. Toft D.O. (Department of Biochemistry and Molecular Biology, Mayo Clinic, USA) for his generous supply of mouse monoclonal anti-human Hsp90 antibodies.
- ✓ All members of the following research groups: The Chaperone research group (Rhodes University) and the University of Liverpool Stem cell consortium (University of Liverpool) for providing a good environment for my research studies.
- ✓ To Lesibe Rapolai for being patient and supportive throughout my studies.
- ✓ My mother, Nthapong, my father, Nthobonye, my brothers, Kailane and Mahlodi; my sisters, Noko, Selaelo, Mmakwena and Molatelo and my nephews and nieces for all their support.
- ✓ Doctors: C.A Togo, E. Prinsloo, and A. Shonhai for their time they spent in proof-reading my thesis.
- ✓ NRF (South Africa) and The Royal Society (UK) for funding my project.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER THREE

RESULTS

CHAPTER FOUR

APPENDIX

LIST OF FIGURES

CHAPTER ONE

CHAPTER THREE

LIST OF TABLE

LIST OF ABBREVIATIONS

ATP	Adenosine 5´-triphosphate
ATPase	Adenosine 5´-triphosphatase
BMP	Bone morphogenic protein
BAG-1	Bcl-2-associated athanogene-1
Cdx-2	Caudal homeobox protein 2
Coup-tf1	Chicken ovalbumin upstream promoter transcription factor 1
DNA	Deoxyribose Nucleic Acid
EDTA	ethylenediamine tetra-acetic acid
EEVD	glutamate-glutamate-valine-aspartate
EGFR	Epidermal growth factor receptor
EGFP	enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ESCs	Embryonic stem cells
FCS	Foetal calf serum
GCSF	Granulocyte-colony stimulating factor
GCNF	Germ Cell Nuclear factor
gp130	Glycoprotein 130
HDAC	histone deacetylase
HEPES	N-(2 hydroxyethyl) piperrazine-N-` (2-ethanesulphonic) acid
hESCs	Human embryonic stem cells
Hop	Hsp70/Hsp90 organizing protein
HS cells	Haematopoetic stem cells
Hsps	Heat shock proteins
Hsp90	Heat shock protein 90
Hsp70	Heat shock protein 70
Hsp40	Heat shock protein 40
HSCs	Haematopoietic stem cells
4HT	4-Hydroxytamoxifen
GTP	Guanosine 5´triphosphate

ICM	Inner cell mass
IL-6	Interleukin 6
JAK	Janus kinase
KCl	Potassium chloride
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MEFs	Mouse fibroblasts
MITF	Microphthalmia transcription factor
mES cells	Mouse embryonic stem cells
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen orthophosphate
NLS	Nuclear localizing sequence
NSCs	Neural stem cells
PBS	Phosphate buffered saline
PIAS1	Protein inhibitor of activated STAT1
PIAS3	Protein inhibitor of activated STAT3
PIASxα	Protein inhibitor of activated xα
PIASxβ	Protein inhibitor of activated xβ
PIASγ	Protein inhibitor of activated γ
PIAS3	Protein inhibitor of activated STAT3
pYSTAT3	Tyrosine-phosphorylated STAT3
SCs	Stem cells
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src homology 2
STATs	Signal Transducer and activator of transcriptions
STAT3	Signal Transducer and activator of transcription 3
STAT3ER	Signal Transducer and activator of transcription 3 Estrogen receptor
SUMO	Small ubiquitin-like modifier
TBS	Tris-buffered saline
TBST	TBS-tween
TPR	Tetratrico peptide repeat

LIST OF SYMBOLS

α	Alpha
β	Beta
^{o}C	Degree Celsius
M	Molar
mM	Millimolar
nM	Nanomolar
µg	Micrograms
µl	Microlitres
L	Litres
g	Grams
mg	Milligrams
kDa	Kilo Daltons
min	Minutes
mol	Mole
ml	Millilitre(s)
%	Percent or g/100 ml
U	Units
V	Volts
xg	Relative centrifugal force to gravity

LIST OF AMINO ACIDS AND NUCLEIC ACIDS

Listed below are the international Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature of 20 amino acids.

AMINO ACIDS

NAME	THREE LETTER CODE	SINGLE LETTER CODE
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

NUCLEIC ACIDS

PURINES	Adenine	Guanine		
PYRIMIDINES	Cytosine	Guanine	Uracil	Thiamine

CHAPTER ONE

INTRODUCTION

1. GENERAL INTRODUCTION

1.1 STEM CELLS

Stem cells (SCs) are undifferentiated cells that reside in the embryo, fetus and adult. They have under certain conditions, the ability to self-replicate for long periods without undergoing differentiation (Kirschstein and Skirboll, 2001). The best characterized types of SCs include those derived from the pre-implantation embryo; namely, embryonic stem (ES) cells, neural stem (NS) cells and haematopoietic stem (HS) cells (Kirschstein and Skirboll, 2001).

1.2 EMBRYONIC STEM CELLS

Development of the mouse embryo begins with fertilization of the oocyte by sperm resulting in the formation of a zygote having the genetic material of both parents (Kirschstein and Skirboll, 2001). Further developmental process of the zygote into an embryo occurs at three different stages, the blastulation, gastrulation, and organogenesis stage (Kirschstein and Skirboll, 2001). Blastulation occurs during the pre-implantation stage of embryonic development, and results from cleavage of the fertilized egg to generate a spherical layer of approximately 128 cells surrounding a fluid-filled cavity called the blastocoel. At this stage, the embryo is known as the blastocyst (Kirschstein and Skirboll, 2001). The blastocyst consists of two primary cell types: the inner cell mass (ICM) which contains the cells of the embryo, and the trophoblast (Kirschstein and Skirboll, 2001). The trophoblast forms the outer layer of the embryo (Figure 1.1, lower panel F) and is required for implantation into the uterus (Gonzales *et al*., 1996; Spagnoli and Hemmati-Brivanlou, 2006).

ES cells are cells derived from the ICM of the blastocyst at approximately day 4 (Kirschstein and Skirboll, 2001; Chambers *et al*., 2003). They resemble the *in vivo* population of cells known as the epiblast. Unlike NS cells and HS cells, ES cells are pluripotent, which means that an individual cell has the potential to differentiate into all cell types derived from the three embryonic germ layers, the ectoderm, mesoderm and endoderm (Niwa, 2001; Burdon *et al*., 2002; Kinoshita *et al*., 2007). The most

important properties of ES cells is that they can be cultured for prolonged periods without differentiating while retaining their pluripotency potential (Smith, 2001).

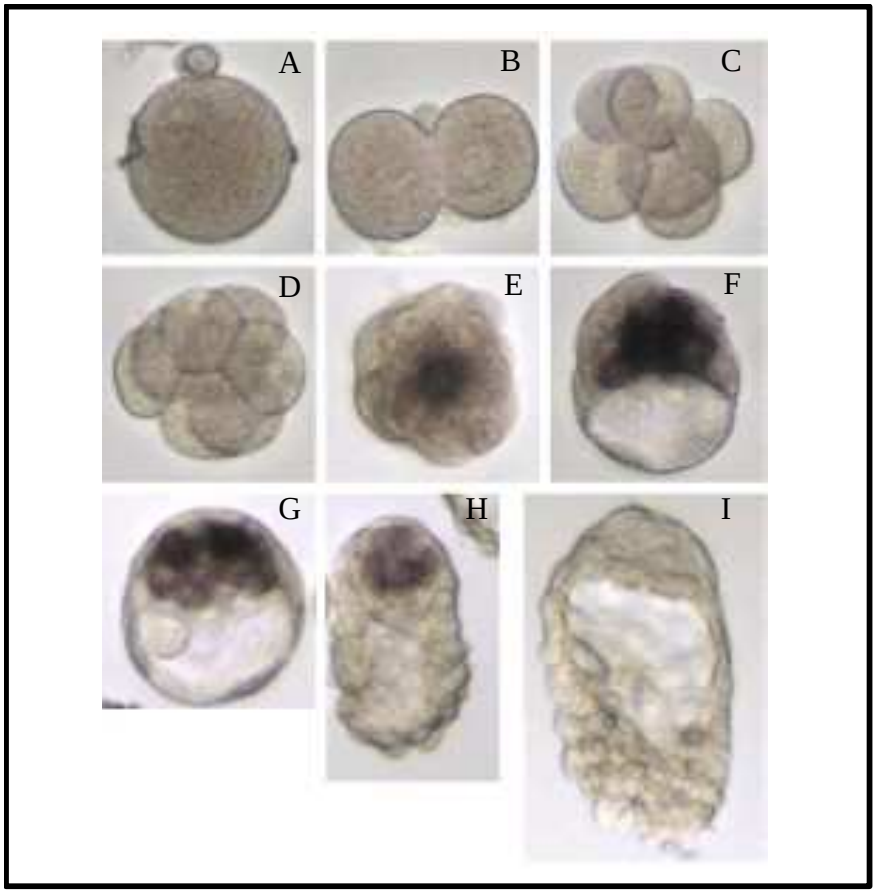

Figure 1.1 Phase contrast micrographs on the development of the embryo to the pre-implantation blastocyst in mice. Note the change in cell number, (top panel: A (embryo of single cell), B (embryo of two cells), C (embryo of 6 cells); Middle panel: embryo of 8 cells (D), late morula (E) and early blastocyst (F), Bottom panel: embryos at expanded (G), hatched (H) and implanting stage (I) (adapted from Chambers *et al*., 2003).

1.2.1 Mouse embryonic stem cell pluripotency

Cellular pluripotency is defined as the ability of a cell to differentiate into various types of cells belonging to the three embryonic germ layers: ectoderm, mesoderm, and endoderm (Niwa, 2001). Mouse ES cell lines were first established in the early 1980s. Their isolation from the pre-implantation blastocyst involved careful isolation and careful cultivation on mouse embryonic fibroblasts (MEFs) to prevent differentiation (Wobus and Boheler, 2005). However, the cytokine, leukaemia inhibitory factor (LIF) was subsequently used to maintain mES cells in the absence of MEFs. To date, the cultivation of mES cells require either the presence of MEFs or LIF to retain their pluripotency (Evans and Kaufman, 1981). The ability of mES cells to spontaneously differentiate when allowed to aggregate in the absence LIF has been reported (Murray

and Edgar, 2001; Spagnoli and Hemmati-Brivalou, 2006). This included formation of embryoid bodies from which early embryonic cell lineages are derived (Murray and Edgar, 2001; Spagnoli, and Hemmati-Brivanlou, 2006).

In light of the limited culture conditions of mES cells, described above, it is important to first understand the mechanisms by which they regulate the balance between differentiation and self-renewal potential during extended periods of culture in order to manipulate them reliably (Chambers, 2004). Several transcription factors have been identified to be critical both for the formation of the ICM during mouse pre-implantation development and self-renewal. They include signal transducer and activator of transcription 3 (STAT3), Octamer 3/4 (Oct-3/4) and homeodomain protein (Nanog) (Figure 1.2; Chambers *et al*., 2003; Wobus and Boheler, 2005; Kinoshita *et al*., 2007). Withdrawal of LIF from the culture medium triggers down-regulation of these factors and at the same time causes over expression of Oct-3/4 repressors such as caudal homeobox protein 2 (Cdx-2), Chicken ovalbumin upstream promoter transcription factor 1(Coup-tfl), and Germ Cell Nuclear factor (GCNF) (Kinoshita *et al*., 2007).

The level of Oct-3/4 expression has been shown to dictate how mES cells should differentiate and whether they should continue to proliferate (Kirschstein and Skirboll, 2001). Artificial increases in the levels of Oct-3/4 expression results in endodermal and mesodermal differentiation (Niwa, 2001; Kirschstein and Skirboll, 2001). However, inhibition of Oct-3/4 expression in mES cells cultured in the presence of LIF resulted in trophoctoderm or neuronal differentiation (Niwa, 2001; Kisrchstein and Skirboll, 2001; Chambers *et al*., 2003; Chen *et al*., 2007). The expression of GABP (GA-repeat binding protein) α is associated with undifferentiated mES cells (Kinoshita *et al*., 2007). In the study to determine the role of GABPα in mES cells, Kinoshita *et al* (2007) showed that GABPα over-expression maintained the expression levels of Oct-3/4 in mES cells cultured for 8.0 days even in the absence of LIF, however, differentiation-associated genes were also expressed. This data suggests that LIF maintains self-renewal of mES cell and that its presence down-regulates the expression of differentiation-associated markers. Therefore, maintenance of self-renewal and pluripotency in ES cells is influenced by LIF and the balance

between differentiation and self-renewal transcription factors (Kisrchstein and Skirboll, 2001; Wobus and Boheler, 2005).

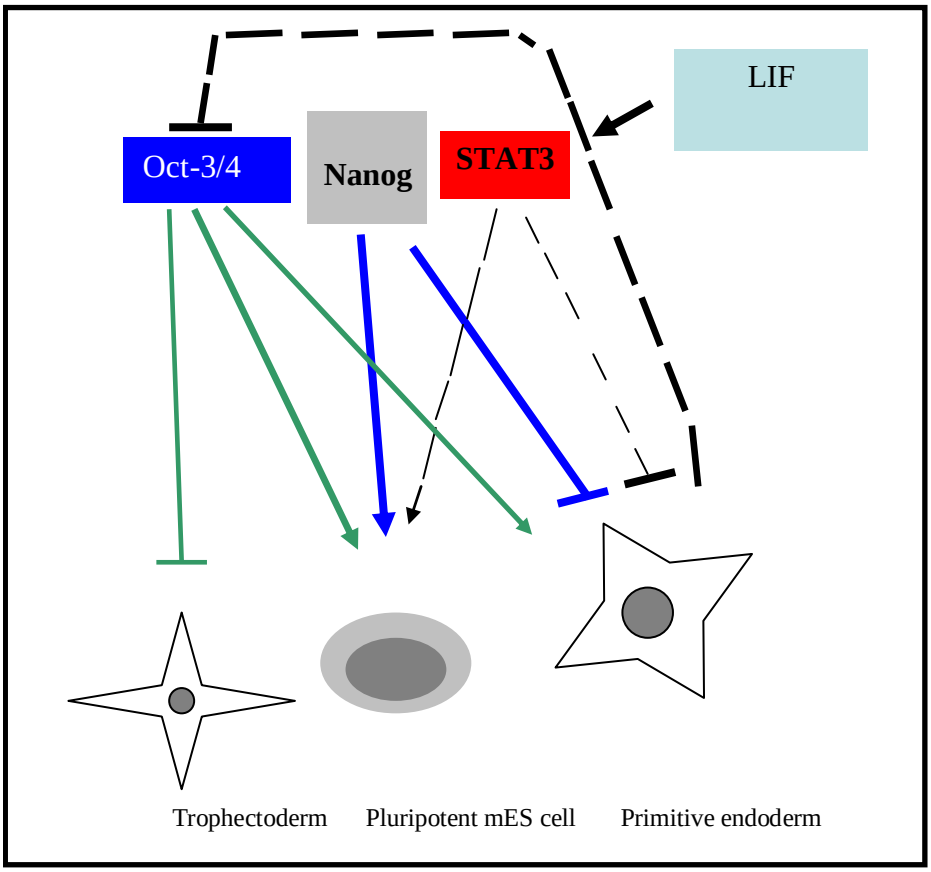

Figure 1.2 Diagrammatic representation of the involvement of the transcription factor network between Nanog, Oct-3/4 and STAT3 in the maintenance of self-renewal in mouse embryonic stem cells. Inhibitory pathways are indicated by hammer head. Stimulatory pathways are indicated by arrows (adapted from Chambers *et al*., 2003).

1.2.2 STAT3 structure and function

The latent transcription factor, STAT3 is among seven of the STAT family of signal transduction proteins predominantly found in the cytoplasm in their monomeric forms (Shi *et al*., 1996; Turkson and Jove, 2000; Zhang *et al*., 2000). This family of transcription factors is comprised of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Blaskovich *et al*., 2003; Sato *et al*., 2005). These proteins play a dual role of transducing biological information from cell surface receptors to the cytoplasm and as transcription factors by binding to target genes that regulate gene expression (Blaskovich *et al*., 2003; Sun *et al*., 2005). Furthermore, the STAT family also participates in the regulation of genes that are in involved in the regulation of

essential cell processes that include acute phase response, inflammation, cell growth, and differentiation (Zhang *et al*., 2000; Blaskovich *et al*., 2003; Sun *et al*., 2005).

Of the STAT family members, STAT3 is mostly expressed in the kidney, liver, and spleen. After expression, STAT3 is spliced to give two isoforms called STAT3α and STAT3β (Zhiyuan and Kone, 2004). The difference between STAT3β and STAT3α is that STATβ lacks the 55 C-terminal amino acid residues which are present in STAT3α. Furthermore, STAT3β is constitutively expressed and has seven additional amino acids residues at its C-terminus (Zhiyuan and Kone, 2004). However, DNA binding and transcriptional activities of STAT3α and STAT3β can be activated by the same set of cytokines and growth factors, and both can either form homodimers or heterodimers with STAT1 (Schaefer *et al*., 1997). Activation of either isoforms including constitutive activation of STAT3β, correlated with phosphorylation of tyrosine 705. The activated STAT3β had greater DNA binding specificity and its stability in transfetected COS-7 cells was more than that of STAT3α (Schaefer *et al*., 1997). However, relative to DNA binding activity, STAT3α was shown to be transcriptionally more active than STAT3β in transfected cells (Schaefer *et al*., 1997).

The STAT proteins are 750 to 850 amino acids residues long. The N-terminal domain of STAT proteins has approximately 125 amino acids and is comprised of four antiparallel alpha (α) helices (α1, α2, α3 and α4) joined together by short loops (Figure 1.3, Becker *et al*., 1998). This domain is responsible for mediating interactions between STAT proteins and the cytoplasmic domain of the LIF receptor through the phosphotyrosine residues of STAT proteins as well as in the activation of dimerization between STAT proteins (Becker *et al*., 1998; Song *et al*., 2004; Sato *et al*., 2005; Ma and Cao, 2006). The N-terminal domain is followed by a coiled-coil domain; a DNA binding domain; a linker domain, and a Src homology 2 (SH2) domain (Becker *et al*., 1998). The SH2 domain in linked to C-terminal trans-activation domain (Figure 1.4) (Becker *et al*., 1998; Zhang *et al*., 2000; Sato *et al*., 2005; Ma and Cao, 2006). Dimerization of STAT3 leads to nuclear translocation, DNA binding and expression of target genes (Zhiyuan and Kone, 2004). The coiled-coil domain contains approximately 135 to 315 amino acids and is essential for cytokine and growth factor-stimulated recruitment of STAT3 to the receptor (Zhiyuan and Kone, 2004). Furthermore, this domain has been shown to be responsible for

dimmer formation, nuclear translocation, and DNA binding. The DNA binding domains contains approximately 320-480 amino acids and recognizes members of the GAS family of enhancer and seems to regulate nuclear export (Zhiyuan and Kone, 2004).

The linker domain of STAT3 proteins is approximately 480 to 575 amino acids long (Zhiyuan and Kone, 2004). This domain has been shown to be important in the binding of DNA. Furthermore, it has been implicated in the regulation of nuclear export in resting cells (Zhiyuan and Kone, 2004). The SH2-domain (amino acids 575 to 680) shares with other SH2 domain a central three-stranded β-pleated sheet (Becker *et al*., 1998; Figure 1.3: strands B, C, and D).

This domain is flanked by helix αA and strand βA and βG, is the most highly conserved motif and play a role in the docking of STAT proteins to the tyrosine phosphorylated subunits of the gp130 receptor (Zhiyuan and Kone, 2004). The motif of the SH2-domain is important for promoting dimerization and may also associate with the activating JAK (Zhiyuan and Kone, 2004). The C-terminal domain of STAT proteins contains an autonomously functioning transcriptional activation domain. This domain also functions in protein-protein interaction and is absent from spliced isoforms of STAT1, STAT3, and STAT4 (Zhiyuan and Kone, 2004).

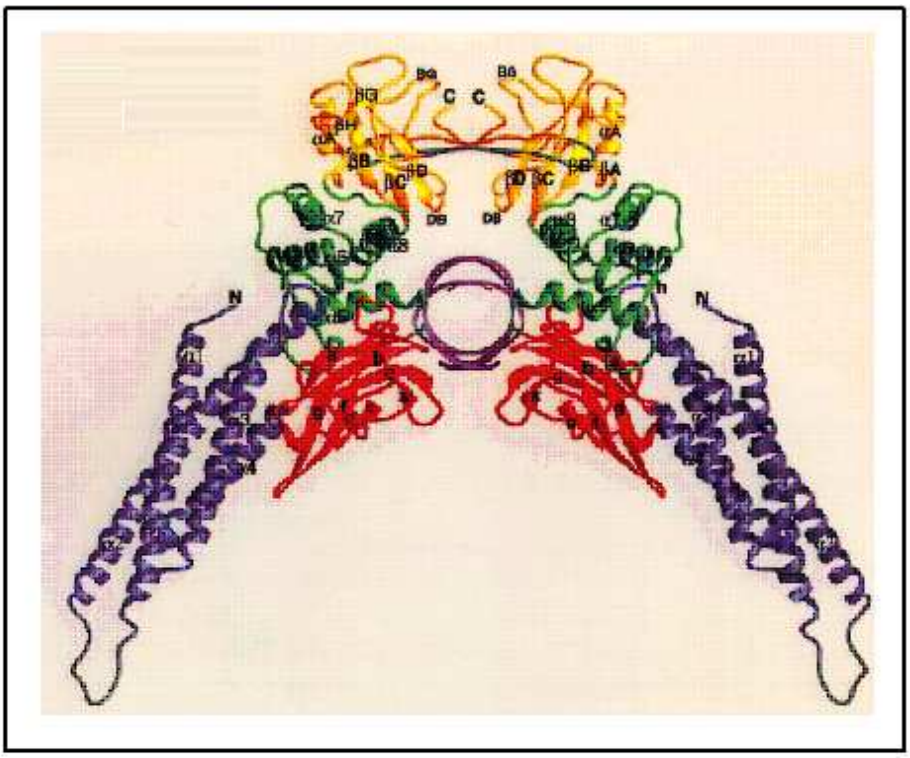

Figure 1.3 Ribbon diagram of the STAT3β homodimer-DNA complex. The N-terminal 4-helix bundle is shown in blue, the β-barrel domain is shown in red, the connector domain is shown green, and the SH2 domain and the phosphotyrosine-containing region is shown in yellow. Disordered regions between helix α1 and α2 and the residues 689 to 701 have been modeled in grey (adapted from Becker *et al*., 1998).

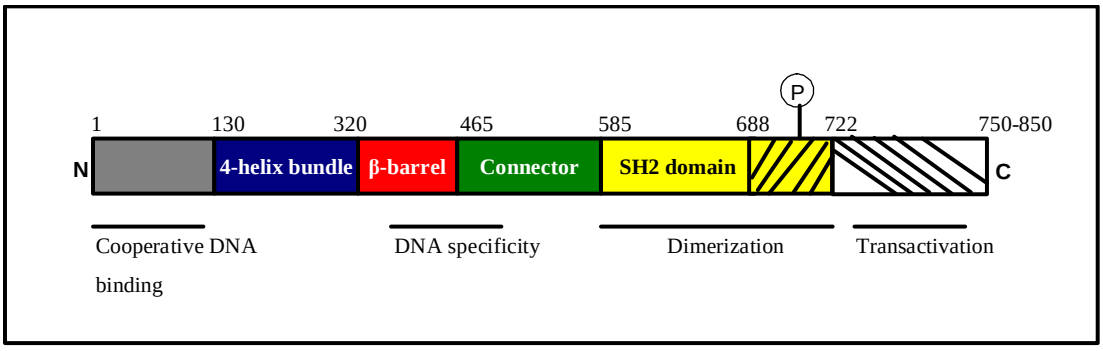

Figure 1.4 Schematic representation of the structural features of STAT3 protein. The domain structure and the domain boundaries of STAT3β are shown: the 130 N-terminal amino acid residues mediate co-operativity in binding to multiple DNA sites; the coiled-coil domain (amino acid residues 130-320) is essential for cytokine and growth factor-stimulated recruitment of STAT3 to the receptor as well as dimer formation, nuclear translocation, and DNA binding; the DNA binding domain (amino acid residues 320-465) confer DNA binding specificity but are not sufficient for DNA binding; the connector/linker domain lie within residues 465-585; residues 585-688 contains the Src-homology-2 (SH2) domains and mediates dimerization; the phosphorylated tyrosine is located around residue 705; The transactivation domain (amino acid residues 722-750) is responsible for transcriptional activation (adapted from Becker *et al*., 1998).

Although the nuclear translocation of STAT proteins is a key control point towards cell growth and proliferation, no classical nuclear localizing sequence (NLS) was found was found in STAT proteins, and the mechanisms of their nuclear translocation were unclear for a long time (Ma *et al*., 2003). However, studies on STAT1 have shown that an Arginine/Lysine-rich element in the DNA binding domain was important in the interferon-induced nuclear translocation of STAT1 and STAT2 in MCF-7 cells. Furthermore, leucine 407 which is located in the DNA binding domain of STAT1 has also been shown to be required for nuclear translocation (Ma *et al*., 2003). The results have also demonstrated that the NLS of STAT1 and STAT5b was located in the DNA binding in which Lys-410/417 and Leu-407 were defined to be critical residues for STAT1 nuclear translocation (Ma *et al*., 2003). Furthermore, five double or triple mutants (Arg-335/Lys-340, Lys-348/Arg-350/Lys-354, Lys-363/Lys-365/Lys-370, Arg-379/Arg-382/Lys-383) in the full length STAT3 were studied. Furthermore, single mutant with mutation in Leu-411 corresponding to Leu-407 in STAT1 was also studied. (Ma *et al*., 2003). The results of this study showed that mutation in Arg-414/Arg-417 that corresponded to Leu-407/Leu-413 in STAT1 resulted in a loss of nuclear translocation induced by epidermal growth factor (EGF). These data suggest that two elements, Arg-214/215 in the coiled-coil domain could be the potential NLS for nuclear translocation whereas Arg-414/417 in the DNA binding domain could be required for STAT3 nuclear translocation in response to EGF in MCF-7 cells (Ma *et al*., 2003).

The DNA in the STAT3β-DNA adopts a B-form DNA-like conformation and is slightly less wounded with 10.7 base pairs per turn (Becker *et al*., 1998). As shown in figure 1. 5, four loops per monomer are in contact with the sugar-phosphate backbone of both DNA strands and recognizes bases in the major grooves. Three of these (loops ab, cx, and ef) protrude from the β-barrel domain whereas the fourth loop (loop gα5) links the β-barrel and connector domains (Becker *et al*., 1998). Loops cx and ef each contribute only one or two DNA-binding residues, respectively whereas loops ab and gα5 provide multiple contacts to the DNA (Becker *et al*., 1998).

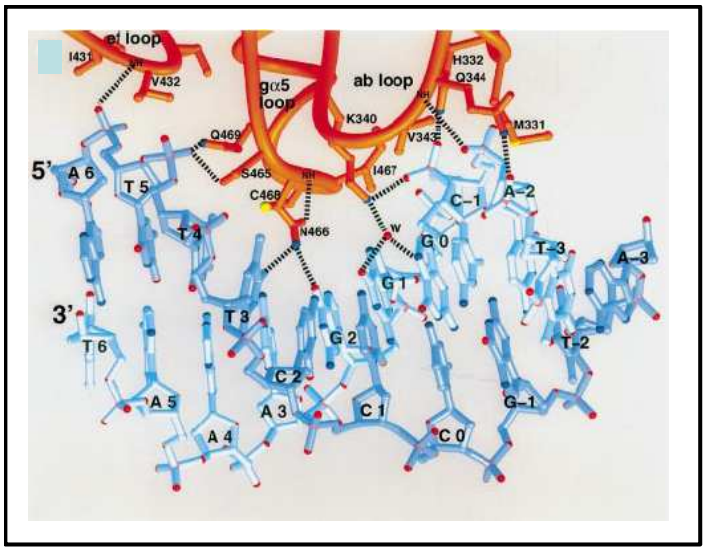

Figure 1.5 DNA recognition by the STAT protein. Ribbon diagram of the interactions of one monomer with DNA. Arginine 382 of loop cx is omitted for clarity. Polar interactions are indicated with dashed lines (adapted from Becker *et al*., 1998).

As shown in figure 1.6, polar residues in helix α8 (Lys-573, Lys-574) and of the SH2 domain (Gln-643) are facing towards the DNA. However their proximity is not enough to make physical contact with the appropriate DNA residues (Becker *et al*., 1998). The interaction between the DNA and STAT3 protein is shown to be possible between the amide and the side chain of Glutamine (Gln-344) and the phosphate groups. Further interaction is shown to be between the sugar residues and the amino acids Met-331 and Val-343 (Becker *et al*., 1998).

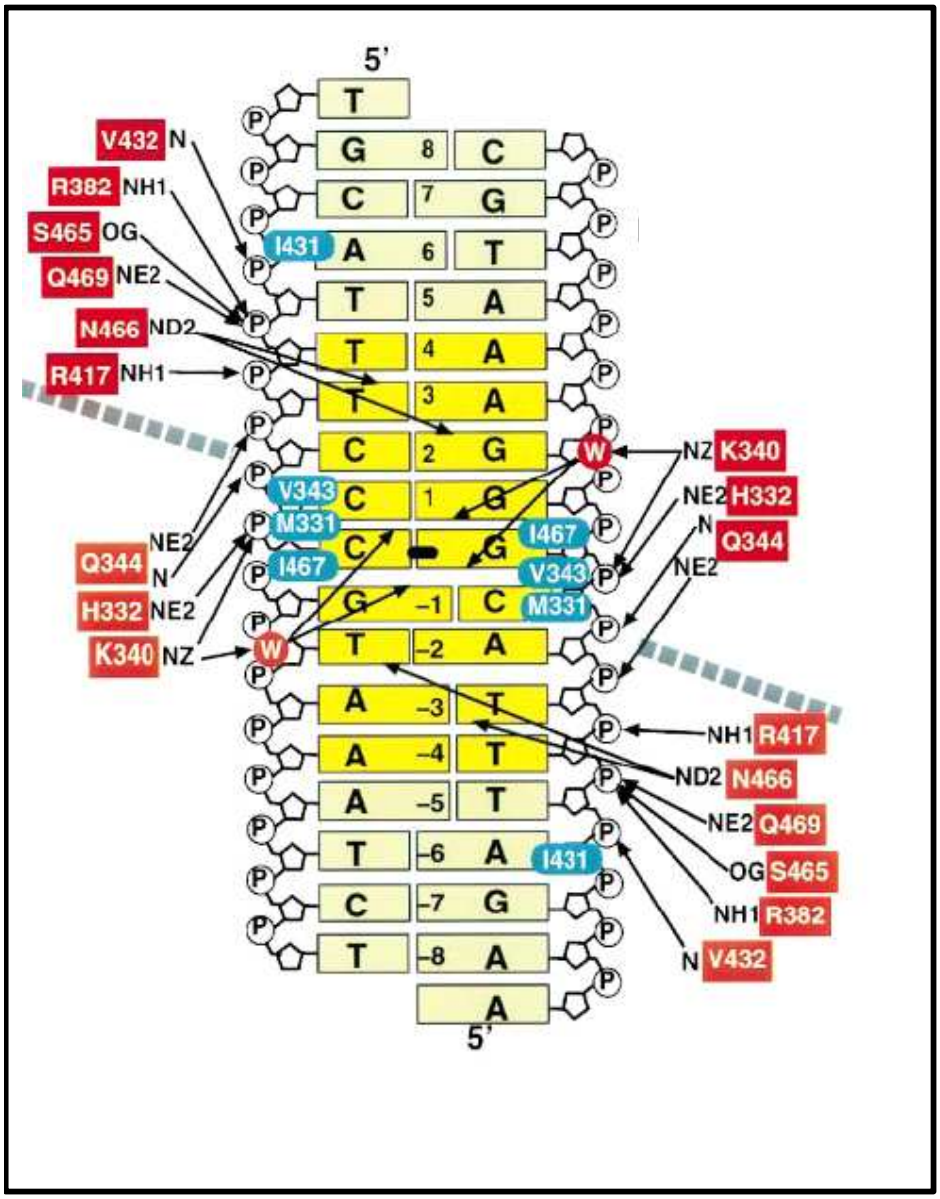

Figure 1.6 DNA recognition by STAT protein. Interactions between polar residues (shown in red), the hydrophobic residues (shown in turquoise) of the protein and DNA. The pseudo-dyad coinciding with the crystallographic dyad is shown in black. The central 9 base pair corresponding to the consensus DNA sequence is shown in yellow (adapted from Becker *et al*., 1998).

The involvement of STAT3 in oncogenesis has been well studied: in many cases, it has been found that high levels of transcriptionally active STAT3, which results from constitutive tyrosine phosphorylation, is present in many human cancer cells (Turkson and Jove, 2000; Blaskovich *et al*., 2003). Furthermore, the use of molecular and pharmacological tools in disease-related models have shown that STAT3 plays a role in oncogenesis through constitutive tyrosine phosphorylation, and provides proof that

STAT3 is a target for cancer drugs (Turkson and Jove, 2000; Sato *et al*., 2003; Sato *et al*., 2005; Sun *et al*., 2005).

1.2.3 Inhibition of activated STAT proteins by PIAS

In unstimulated cells, a STAT protein is predominantly found in the cytoplasm whereas microphthalmia transcription factor (MITF) and inhibitor of activated signal transducer and activator of transcription 3 (PIAS3) were colocalised in the nucleus (Levy *et al*., 2001). Upon activation by tyrosine phosphorylation in response to ligand stimulation, STATs form dimers through the SH2-phosphotyrosyl interactions (Levy *et al*., 2001; Long *et al*., 2004). These dimers then translocate into the nucleus to activate transcription. Protein inhibitor of activated signal transducer and activator of transcription (PIAS) protein is a family comprised of the following members, inhibitor of activated signal transducer and activator of transcription 1 (PIAS1); PIAS3; inhibitor of activated signal transducer and activator of transcription xα (PIASxα); inhibitor of activated signal transducer and activator of transcription xβ (PIASxβ) and inhibitor of activated signal transducer and activator of transcription γ (PIASγ) (Duval *et al*., 2003; Sonnenblick *et al*., 2004).

Structurally, PIAS proteins contain several conserved domains: the N-terminal SAP (Saf-A/B, acinus and Pias) box with the LXXLL signature, which is required for the trans-repression of STAT1 activity by PIASγ; the MIZ-Zn finger/RING domain which is essential for SUMO (small ubiquitin-like modifier) ligase activity; and the C-terminal domain which is required for nuclear retention and binding of PIAS3 to the nuclear co-activator TIF2 (Duval *et al*., 2003, Long *et al*., 2004; Sonnenblick *et al*., 2004; Levy *et al* ., 2006).

These proteins were identified due to their ability to bind specific proteins such as, potassium ion (K^+) channel and ribose nucleic acid (RNA) helicase II (Duval *et al*., 2003). PIAS3 was originally identified as a specific inhibitor of STAT3 and its mode of inhibition was through the binding of activated STAT3 which resulted in the inhibition of DNA-binding as well as STAT3-mediated gene expression (Long *et al*., 2004; Yamamoto *et al*., 2003). Two homologues of PIAS, PIASxα and PIASxβ were shown to interact with the androgen receptor (AR) and the homeodomain protein Msx2 respectively (Long *et al*., 2004). PIASγ antagonize Smad-mediated

transcriptional responses by interacting with Small mothers against decapentaplegics (Smads) and histone deacetylase (HDAC) (Long *et al*., 2004).

MITF is a basic helix-loop-helix leucine zipper (bHLH-Zip) DNA-binding protein (Levy *et al*., 2006). Physical abnormalities resulting from mutations on the MITF gene have been reported. They include among others, deafness, bone loss, small eyes, and poorly pigments eyes and skin (Levy *et al*., 2006; Sonnenblick *et al*., 2004). MITF has also been shown to have key regulatory roles in both mast cells and in melanocytes. In the study to determine the role of MITF in gene regulation, PIAS3 was shown to be a physiological regulator of MITF-induced transcriptional activity and that STAT3 does not interfere in the interactions between PIAS3 and MITF (Levy *et al*., 2001).

1.2.4 LIF-STAT signaling pathway

The mechanisms for self-renewal and pluripotency of ES cells have recently been reported to involve both extrinsic (LIF, and the bone morphogenetic protein-BMP) and intrinsic (Oct 3/4 and Nanog) factors (Mitsui *et al*., 2003; Chambers, 2004; Humphrey *et al*., 2004). Further studies also showed that the activity of Nanog was independent of STAT3 (Chambers *et al*., 2003; Humphrey *et al*., 2004; Puente *et al*., 2006).

Mouse ES cells can be maintained for a prolonged period when cultured either on feeder cells, or in the presence of cytokines of the interleukin (IL)-6 family, which includes LIF, IL-6, IL-11, ciliary neutrophic factor, oncostatin M and cardiotropin-1 (Matsuda *et al*., 1999; Burdon *et al*., 2002). The signal generated by these cytokines is mediated through a trans-membrane cell surface receptor complex composed of the low affinity LIF receptor (LIFR) and gp130 (Figure 1.7). However, the LIFR/gp130 receptor complex does not have intrinsic protein kinase domains, but are in association with the Janus kinase (JAK) protein family of non-receptor cytoplasmic tyrosine kinases (Matsuda *et al*., 1999; Humphrey *et al*., 2004). Among this group of kinases are JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) (Shi *et al*., 1996; Rajasingh and Bright, 2006).

Previous studies have shown that tyrosine phosphorylation of STAT3 due to cytokine or growth factor binding to the receptor was sufficient for its nuclear translocation where they bind DNA and regulate transcription of their target genes that are essential for self-renewal and pluripotency in mES cells. However low levels of the ligand-independent constitutive nuclear expression of STAT1 and STAT3 were also reported in COS-1 and NIH3T3 cells (Ma *et al*., 2003). Furthermore, a mutant STAT3 containing just the N-terminal portion of STAT3 was constitutively localized in the nucleus a breast cancer cell line, MCF-7 (Ma *et al*., 2003).

Binding of LIF with the LIFR/gp130 heterodimer results in the rapid activation of JAK and subsequent tyrosine phosphorylation of the gp130 on its tyrosine residues. These phosphorylated tyrosine residues of gp130 serve as a docking site for SH2 domain-containing signaling molecules such as STAT3, and for protein tyrosine phosphatase that contains two SHP2 domain (SHP2) (Matsuda *et al*., 1999; Zhang *et al*., 2000). Furthermore, the binding of STAT3 to the gp130 receptor leads to its phosphorylation at a single tyrosine residue at the carboxyl-terminus by JAK. The events following tyrosine phosphorylation of STAT3 include homodimer formation and nuclear translocation (Niwa *et al*., 1998; Matsuda *et al*., 1999; Zhang *et al*., 2000; Humphrey *et al*., 2004).

To demonstrate that STAT3 activation was sufficient and necessary in suppressing mES cell differentiation, Burdon *et al* (2002) used mES cells expressing STAT3ER, a fusion protein consisting of STAT3 and the ligand-binding domain of estrogen receptor, to show that mES pluripotency could be maintained by activation of STAT3ER by a synthetic estrogen receptor ligand, estradiol, even in the absence of LIF (Figure 1.7).

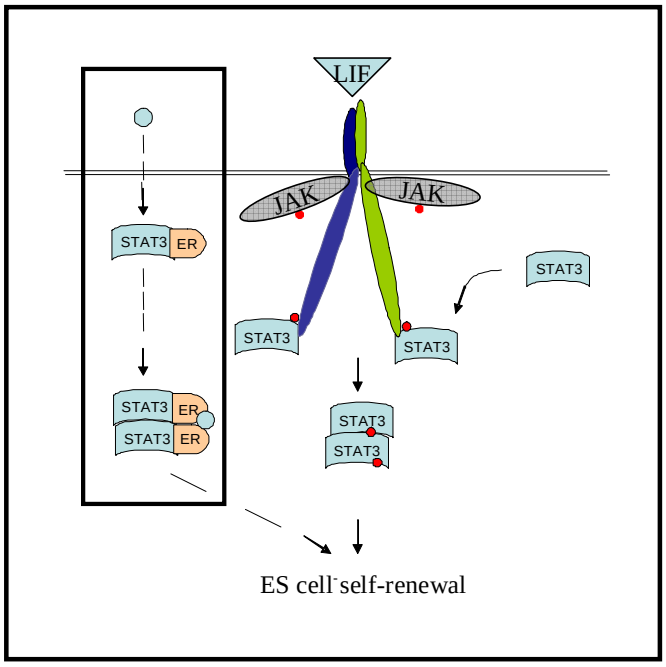

Figure 1.7 LIF-dependent activation of STAT3 blocks mES cells differentiation and promotes self-renewal. LIF stabilizes the association of LIFR and gp130 cytokine receptor. The resultant activation of JAK kinases (JAK) causes the recruitment of STAT3 through its coiled-coil domain to the gp130 receptor. Once on the receptor, it is then tyrosine phosphorylated on the SH2 domain. Once phosphorylated, the STAT3 monomers then form dimers through their SH2 domain, and then translocate into the nucleus where they control transcription of genes that promote self-renewal. Activation of STAT3ER, (a fusion protein consisting of STAT3 and estrogen receptor) by estradiol also promoted self-renewal in mES cells (adapted from Burdon *et al*., 2002).

1.3 THE PROMISE OF STEM CELL RESEARCH

Since their initial derivation in the 1980s, mES cells have been used extensively to generate genetically engineered mice. This is due to the fact that mES cells have the capacity to colonize the germ line with ease, resulting in the formation of chimeric animals with functional gametes (Kirschstein and Skirboll, 2001). This allowed mES cells to be used as vehicles in the introduction of genetic modifications for the production of genetically engineered mice (Kirschstein and Skirboll, 2001).

Stem cell research holds great promise for regenerative medicine and tissue engineering and provides exciting new avenues for treating cardiovascular diseases (Yin *et al*., 2006). ES cells are of particular interest because of their pluripotency and their unlimited capacity for self-renewal. Human ES cells could potentially be used as

a source of differentiated cells from the human body (Haynes and Pouton, 2007). These ES cell- derived tissues/cells could be used as model systems for the identification of drug targets and toxicity testing, as well as for the screening of various therapeutics (Haynes and Pouton, 2007).

Human ES (hES) cells may also be used for the generation of various types of cells and tissues for replacement therapy for human degenerative disease such as Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and stroke (Kirschstein and Skirboll, 2001; Tai and Svendsen, 2004; Haynes and Pouton, 2007). However, major barriers towards the development of ES cell based therapies arise from the inability to culture hES cells from animal-free components. These exposures present a risk of retroviral transfer or infection with other pathogens that could be transmitted to patients. The inability to maintain hES cells in the absence of feeder cell lines is mainly due to the fact that unlike mES cells, hES spontaneously differentiate when cultured in a medium supplemented with LIF (Ginis *et al*., 2004). A greater understanding of the molecular mechanisms underlying LIF-mediated self-renewal of mES cells may lead to the design of culture conditions that are capable of maintaining the self-renewal of hES cells in the absence of feeder cell lines.

1.4 MOLECULAR CHAPERONES

Molecular chaperones are integral components of the cellular machinery that assist many signaling molecules to maintain their activation-competent state (Sőti *et al*., 2005). Molecular chaperones function by capturing unfolded polypeptides through their exposed hydrophobic residues, stabilizing and preventing misfolded polypeptides from accumulating under physiological conditions and in stressed cells (Chiosis, *et al*., 2004; Odunga *et al*., 2004; Sőti *et al*., 2005; Huen and Chan, 2005).

Prolonged stress results in a compromised immune response, defective development and pathologies such as stroke, myocardial reperfusion damage, ischemia, cancer, amyloidosis as well as other neurodegenerative disease (Nardi *et al*., 2006; Gooljarsingh, *et al*., 2006). Molecular chaperones form large complexes and have a large number of co-chaperones that regulate their activities that include their ability to bind substrates (Sőti *et al*., 2005). Furthermore, they also assist in the regulation and co-ordination of cellular networks that include those of signaling and the transcriptional pathway, the cytoskeletal, membrane and the organelle network

(Figure 1.8). Molecular chaperones are also involved in stress, disease and aging (Figure 1.8, Sőti *et al*., 2005).

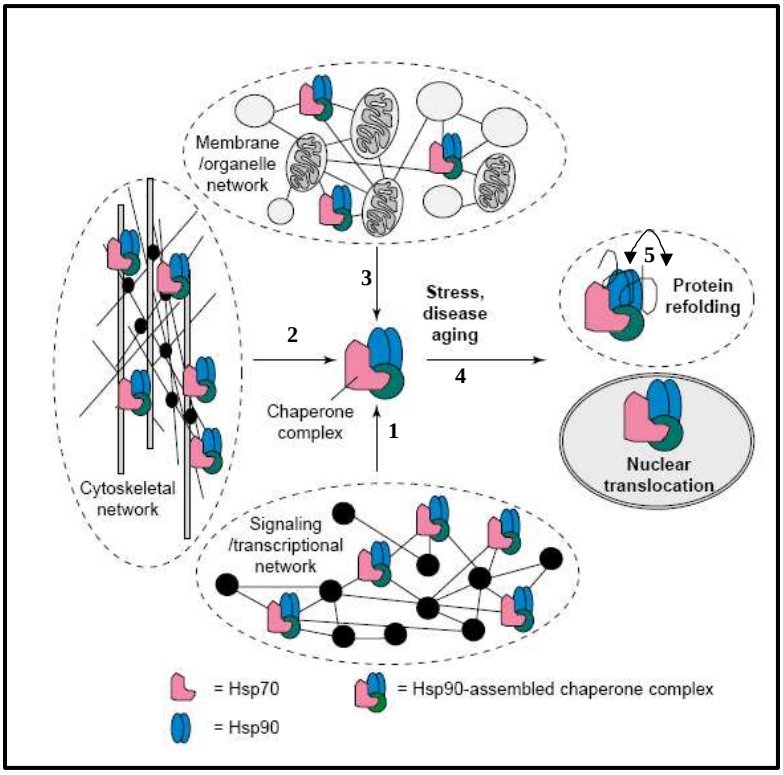

Figure 1.8 A schematic representation showing network working of various pathways by chaperone complex. These networks are represented as follows, 1: signaling/transcriptional network; 2: cytoskeletal network; 3: membrane/organelle net work; 4: Chaperone complex are involved in stress, disease and aging; Chaperone complex also play a role in stress, disease and, aging were they aid in protein refolding (5) and in nuclear translocation (adapted from Sőti *et al*., 2005).

As shown in table 1.1, the major classes of chaperones are heat shock proteins; namely, the Heat shock protein 40 (Hsp40); Heat shock protein 60 (Hsp60); Heat shock protein 70 (Hsp70); Heat shock protein 90 (Hsp90); Heat shock protein 100 (Hsp100); and the small heat shock proteins (Fink, 1999; Mehta *et al*., 2005).

Molecular chaperones involved in the folding of newly synthesized proteins primarily recognize their substrate proteins via their exposed hydrophobic residues (Fink, 1999). Although the general biochemical properties of certain molecular chaperones are well studied (Pratt, 1998; Jackson *et al*., 2004; Brown *et al*., 2007), less is known about how these chaperones interact with substrate proteins to form stable complexes. The best studied chaperone complex is the steroid aporeceptors that contain Hsp90

and Hsp70, the Hsp70/Hsp90 organizing protein (Hop), Hip and the immunophillins, FK506 binding protein-54/52 (FKBP54/52) (Freeman *et al*., 1996; Wegele *et al*., 2006). Recent investigations have also shown the involvement of protein co-factors as part of the chaperone complex (Fink, 1999).

Table1.1 Key members of the Heat shock protein family (adapted from Mehta *et al*., 2005).

*Hsp MEMBER		LOCATION	DESCRIPTION
Low molecular weight Hsps			
	Ubiquitin	Cytoplasm/nucleus	Facilitates targeting and removal of denatured proteins
	Hsp10	Mitochondria	Co-factor for Hsp60
	Hsp27	Cytoplasm/Nucleus	
	Aβ-crystalin	Cytoplasm	Cytoskeletal stabilization Intracellular actin dynamics
Hsp40			
	Hsp40	Cytoplasm/Nucleus	Regulate Hsp70 activity, Binds non-native protein
	ERjs	Endoplasmic Reticulum(ER)	Regulate Hsp70, BiP and involved in protein translocation
Hsp60			
	Hsp60	Mitochondria	Molecular chaperone
Hsp70			
	Hsp72	Cytoplasm/nucleus	Highly stress inducible, protects against ischemia
	Hsp73	Cytoplasm/nucleus	Constitutively expressed molecular chaperone
	Hsp75	Mitochondria	Induced by stress including hypoxia
	BiP	ER lumen	Protein import and folding within the ER
Hsp90	Hsp90	Cytoplasm/migrate to nucleus	Part of steroid receptor complex and involved in maturation of signaling molecules in general
Hsp110	Hsp110	Nucleus/cytoplasm	Thermal tolerance
	Hsp105	Cytoplasm	Protein refolding

*Abbreviations in use: Hsp: Heat shock protein; Hsp10: Heat shock protein 10; Hsp27: Heat shock protein 27; Hsp40: Heat shock protein 40; Hsp60: Heat shock protein 60; Hsp90; Heat shock protein 90; Hsp70: Heat shock protein 70; ER: endoplasmic reticulum.

1.4.1 Heat shock protein 90 (Hsp90), structure and function

Heat shock protein 90 (Hsp90) defines a family of molecular chaperones that are highly conserved from prokaryotes to eukaryotes (Minami *et al*., 1994; Brown *et al*., 2007). Mammalian Hsp90 has two isoforms, Hsp90α and Hsp90β, which are mainly present as α-α and β-β homodimers and are encoded by separate genes (Minami *et al*.,

1994; Fink, 1999; Bernstein *et al*., 2001). As shown in figure 1.9, Hsp90 is composed of three domains. The N-terminal domain contains the ATP- and geldanamycin-binding site. Hydrolysis of ATP is important for *in vivo* the functioning of Hsp90 (Jackson *et al*., 2004; Prodromou and Pearl, 2006). Human Hsp90 exhibit low ATPase activity, however this activity was increased upon binding glucocorticoid receptor (GR) (McLaughlin *et al*., 2002). The middle domain, the major site for the binding of client proteins, is connected to the N-terminal domain through a highly charged linker domain. The C-terminal domain contains the dimerisation interface and a conserved pentapeptide sequence motif, MEEVD responsible for binding of tetratricopeptides (TPR) containing proteins (Jackson *et al*., 2004; Brown *et al*., 2007).

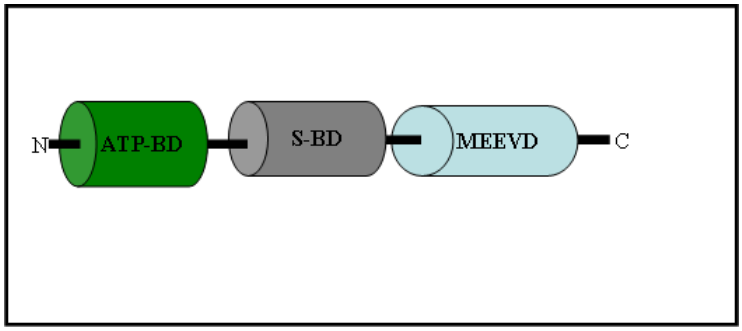

Figure 1.9 Schematic representation of the structural features of Hsp90. The N-terminal consists of an ATP bindng domain (ATP-BD) linked to a substrate-binding domain (S-BD) and at the C-terminal, contains conserved amino acids sequences, MEEVD that serve as the binding site for tetra tricopeptide repeat TPR-containing co-chaperones (adapted from Young *et al*., 2001).

Like other stress proteins such as Hsp70, Hsp90 is an abundant evolutionary conserved molecular chaperone found among all eukaryotic cells and constitute approximately 2.0 percent of the cytosolic proteins (Minami *et al*., 1994). Hsp90 is primarily cytosolic (Table 1.1); however, a small amount of Hsp90 rapidly accumulates in the nucleus in response to stress. Hsp90 primarily functions as a multiprotein chaperone complex with co-chaperones that include Hsp70, Hsp40, and Hop, (Jackson *et al*., 2004; Gooljarsingh, *et al*., 2006). Figure 1.10 shows some of the co-chaperones that interact with Hsp90 and the processes in which they are involved, (Jackson *et al*., 2004).

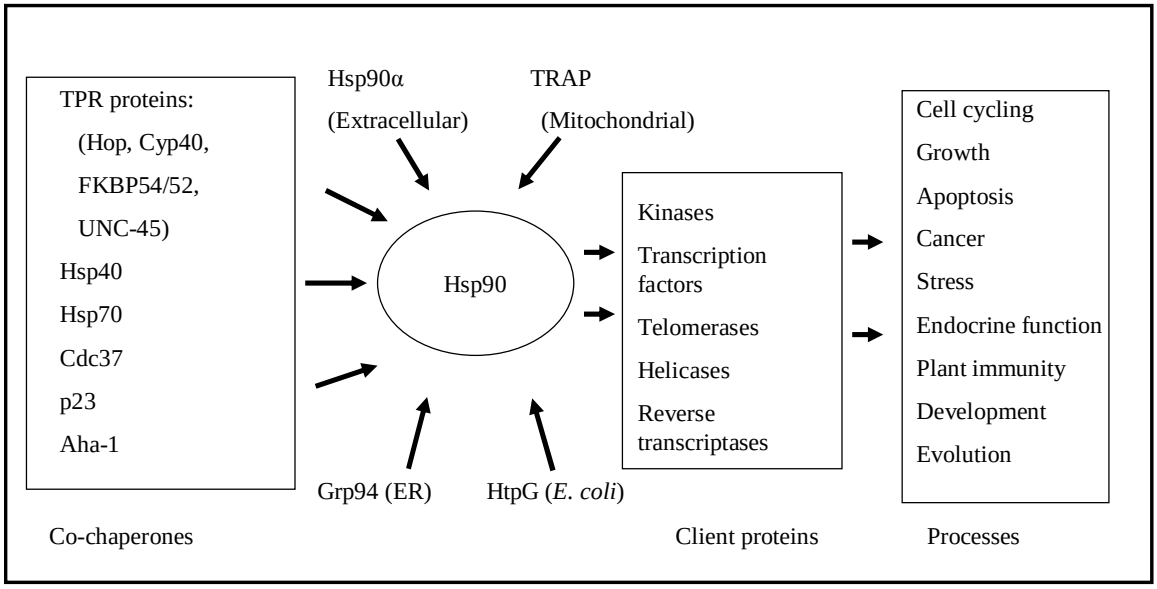

Figure 1.10 Diagrammatic representation of co-chaperones and clients proteins that complexes with Hsp90. Cytosolic Hsp90 and its homologs Grp94, TRAP and HtpG interact with numerous co-chaperones that form the cellular assembly machine. Hsp90 acts on a range of client proteins thereby controlling many cellular processes (adapted from Jackson *et al*., 2004).

Although essential in cell viability, the Hsp90-chaperone complex has been extensively implicated in many cellular processes, such as cell cycling, apoptosis, cancer, stress response, endocrine function, plant immunity, development and evolution (Fink, 1999; Jackson *et al*., 2004; Younes and Georgakis, 2005; Brown *et al*., 2007). It can be seen, therefore, that Hsp90 functions both as a typical heat shock protein, as reflected by its increased expression in cells under stress, and a ubiquitously expressed molecular chaperone. The chaperone functions of Hsp90 include preventing newly synthesized peptides from forming aggregates, protein maturation and the activation of a wide variety of proteins. Furthermore, Hsp90, in partnership with other chaperones such as Hsp70 and Bag1, play a critical role in the regulation of cellular networks (Sőti *et al*., 2005).

Using luciferase as a substrate, Wegele *et al* (2006) demonstrated that the Hsp90 molecular chaperone complex was efficient in preventing luciferase from aggregating (Figure 1.11). However, in order for this process to be successful, other Hsp40, Hsp70 and co-chaperone, Hop need to be involved.

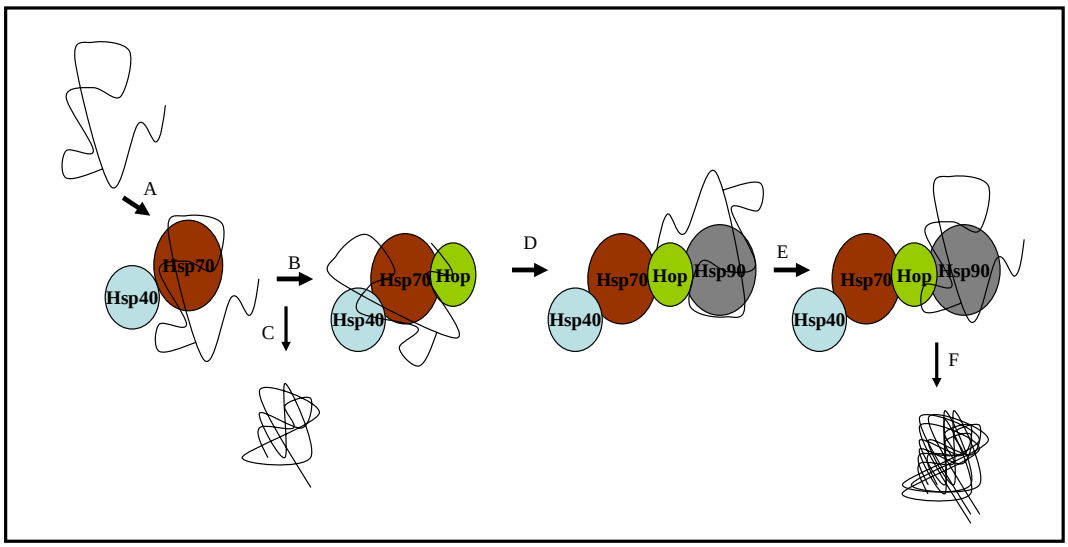

Figure 1.11 Model for the chaperone pathway of the Hsp70/Hsp90 complex; partitioning between folding and aggregation. Following release from the ribosome, Unfolded luciferace is captured by the Hsp40/Hsp70 chaperone complex (A); however, in the absence of the Hsp40/Hsp70 chaperone complex, the unfolded luciferase tends to aggregate (C); the captured luciferase is followed by the binding of Hop to the C-terminus of Hsp70 (B); the binding of Hop to Hsp70 efficiently allows the binding of Hsp90 to Hop, a step which is critical in the transfer of luciferase from Hsp70/Hsp40 chaperone complex to Hsp90 (D); The captured luciferase is transferred to Hsp90 (E); release of the folded luciferase (F) (adapted from Wegele *et al*., 2006).

1.4.2 Heat Shock protein 70/Heat shock protein 90 organizing protein (Hop)

Co-chaperones are an integral part of the chaperone folding mechanisms and are involved in regulation of chaperone activity. Furthermore, they also present client proteins to chaperones and enable indirect "communication" between different chaperone systems (Travers and Fares, 2007).

Heat shock protein 70/Heat shock protein 90 organizing protein (Hop) is a 60-kDa protein that under certain conditions, forms part of the Hsp90 chaperone machinery. Structurally, Hop is defined by the presence of three 34 amino acids, helix-turn-helix tetratricopeptides repeat (TPR) motifs (Young *et al*., 2001). These motifs are clustered into domains each consisting of three TPRs, named TPR1, TPR2A and TPR2B with the NLS (responsible for nuclear translocation) overlapping the TPR2A domain

(Figure 1.12) (Johnson *et al*., 1997; Hernandez *et al*., 2002; Odunuga *et al*., 2004; Wegele *et al*., 2006). TPRs are protein-protein interaction modules present in a number of proteins that are functionally unrelated (Blatch and Lässle, 1999). These motifs were shown to play a critical role in the functioning of chaperones, trafficking of protein and in cell cycle (Blatch and Lässle, 1999).

Among the three domains, only TPR1 and TPR2A have been well studied (Odunuga *et al*., 2004). These domains have been shown to specifically interact with Hsp70 and Hsp90 through their conserved carboxyl-terminal EEVD sequence with the N-terminal TPR1 domain binding to the C-terminal of Hsp70, and the central TPR2 domain binding to the TPR receptor site on the C terminal of Hsp90 (Johnson *et al*., 1997; Odunuga *et al*., 2004; Longshaw *et al*., 2004; Carrigan *et al*., 2006; Americo *et al*., 2007).

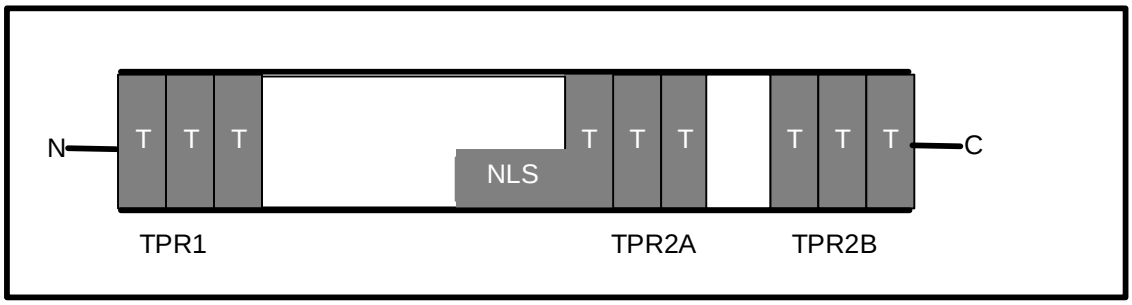

Figure 1.12 A schematic representation of the Hop protein. It is a 60 kDa protein consisting of three TPR domains (TPR1; TPR2A and TPR2B) and a nuclear localization signal (NLS). The N and C termini of Hop are indicated (adapted from Odunuga *et al*., 2004).

Hop was shown to serve as a linker (co-chaperone) between Hsp90 and Hsp70 in reticulocyte and NIH3T3 lysate, since an association between the two molecular chaperones was impossible without it (Johnson *et al*., 1997). Yeast Hop was shown to modulate the chaperoning activity of Hsp70 and Hsp90 upon interaction. However, this modulation was compromised when the respective TPR1 and TPR2 domains were deleted (Song and Masison, 2005). Though deletion of both TPR1 and TPR2 had no adverse effect on either Hsp70 or Hsp90-clent protein activity, the pathway to the formation of a folded protein was impaired (Song and Masison, 2005). An X-ray crystallographic structure on TPR2a and MEEVD has been studied It showed the presence of basic side chains on the TPR2a domain to be essential in the formation of the binding pocket (carboxylate clump) (Scheufler *et al*., 2000). Since Hop is able to interact with Hsp70 and Hsp90 at the same time, this interaction essential in the

targeting of Hsp90 to Hsp70-bound proteins (Chen and Smith, 1998). Further studies also showed the involvement of Hop in the maturation of client proteins as well as in the dissociation of Hsps from the chaperone complex (Odunuga *et al*., 2004; Carrigan *et al*., 2005). To study the relevance of carboxylate clump, point mutation along the basic side chains was performed had an effect of the binding of Hsp90. consequently, mutations along the MEEVD sequence failed to effect Hop binding (Chen *et al*., 1998).

The nuclear localization signal of Hop play an essential role in the shuttling of proteins between the cytoplasm and the nucleus. NLS is a polypeptide sequences that are essential in the trafficking of proteins destined for nuclear transport (reviewed in Allen *et al*., 2000). This polypeptide sequence is comprised of minor lysine arm (Lys-222-Lys-223), a spacer region consisting of 13 amino acids and the major lysine arm (Lys-237-239) (Daniel *et al*., 2007). Studies on subcellular localization of Hop in mammalian cells showed that was predominantly in the nucleus suggesting that, under normal physiological conditions, Hop was predominantly cytoplasmic (Lässle *et al*., 1997; Longshaw, *et al*., 2004). However, nuclear localization of Hop was observed when cells were subjected to heat stress (Daniel *et al*., 2007). In a study to demonstrate that the NLS was essential for nuclear localization, Longshaw *et al* (2004) used the NLS fused to Enhanced Green Fluorescent Protein (EGFP) and showed that the NLS was responsible for nuclear localization of EGFP. Furthermore, treatment of mouse fibroblast cells with nuclear export inhibitor, Leptomycin-B resulted in the accumulation of Hop in the nucleus (Longshaw *et al*., 2004).

The NLS of Hop plays an important role in the trafficking of Hsp90-bound proteins in the nucleus (Odunuga *et al*., 2004). However, Bild *et al* (2002) have shown that nuclear localization of STAT3 was initiated through endocytic vesicles in transit from the cell membrane to the perinuclear region in response to growth factor. Since a grater proportion of Hop was observed when nuclear export was inhibited or during the condition of G1/S arrest, it is likely therefore that the movement of Hop between the cytoplasm and the nucleus is regulated by the cell cycle (Longshaw, 2002; Longshaw *et al*., 2004). The role of Hop in the maintenance of mES cells self-renewal has not been shown before. Since LIF play an important role in the mES cells self-

renewal, it is likely therefore that the expression levels of Hop in mES cells is regulated by the presence of LIF.

1.4.3 Heat shock proteins, STAT3 and mES cell pluripotency

Mouse ES cells can be maintained for a number of passages without undergoing differentiation when cultured in the presence of LIF or a feeder layer of fibroblasts (Williams *et al*., 1998). These are characterized by the presence of a large clear nucleus and little cytoplasm (Tielens *et al*., 2006). LIF is a glycoprotein of 179-amino acids with a predicted molecular weight ranging from 32-62 kDa. This compound known to induce differentiation of M1 Myeloid leukemia cells and well as inhibitory effects on mES cell differentiation (Gearing *et al*., 1989; Gearing *et al*., 1991; Metcalf, 1990). The effect of LIF is initiated through its interaction with the LIFR/gp130 trans-menbrane receptor and this leads to tyrosine phosphorylation of STAT3 (Burdon *et al*., 2002).

Mouse ES cells can be assessed for pluripotency by monitoring the pluripotency markers. These markers include alkaline phosphatase activity (ALP), Oct-3/4 and Stage Specific Embryonic Antigen-1 (SSEA-1) (Berrill *et al*., 2004; Tielens *et al*., 2006). Mouse Oct-3/4 is a transcription factor with 352 amino acids (Tielens *et al*., 2006). It belongs to Class V family of POU transcription factor and has the potential to bind octamer motif sequence ATGCAAAT (Pesce *et al*., 1997). The expression levels of oct-3/4 in early embryogenesis and thereafter in ES cells suggest its significance in the formation, self-renewal and maintenance of pluripotent ES cells (Pesce *et al*., 1997; Niwa, 2001).

The expression of Nanog was shown to be essential in the maintenance of mES cell self-renewal because its targeted disruption resulted in endodermal differentiation (Mitsui *et al*., 2003). Furthermore, Nanog expression was shown to be able to maintain self-renewal of mES cell independent of LIF-STAT activation. These findings suggest that LIF-STAT signaling is independent of Nanog functioning (Mitsui *et al*., 2003).

The molecular chaperone, Hsp90, is a molecular chaperone which under different physiological conditions, associates mainly with proteins involved in transcriptional

regulation and signal transduction pathways (Sato *et al*., 2003). The role of Hsp90 in the maintenance of self-renewal in mES cells has not been studied before, however its involvement in the IL-6-mediated signaling pathway has been studied (Sato *et al*., 2003). Using human embryonic kidney carcinoma cell line-293T, Sato *et al* (2003) showed that Hsp90 physically interacts with STAT3 through its N-terminal region and that this interaction was necessary for stabilizing STAT3. Furthermore, in a study using MDA-MB-468 human breast carcinoma cells, Song *et al* (2004) showed that though STAT3 was in complex with Hsp27, the levels of Hsp27 expression was regulated by STAT3 levels and that STAT3 could be involved in activation of Hsp27 through serine phosphorylation (Song *et al*., 2004).

The ability of ES cells to regulate the pathways between differentiation and self-renewal is complex. Partly regulation of these pathways involves the restriction and activation of new protein expression rather than the addition of newly expressed genes (Battersby *et al*., 2007). Hsp27 is among one of the small heat shock family of proteins. Hsp27 is also known as 24, 28, or 29 kDa protein (Song *et al*., 2004). This has been shown to play a critical role in tumor development as well as in resistance to chemotherapy (Song *et al*., 2004). However, Hsp27 along with Hsp90, have been shown to be part of the molecular chaperones involved in the assembly and protection of STAT3, dimerization as well as in the translocation to the nucleus (Song *et al*., 2004). Previous study has shown that the levels of Hsp27 were down-regulated when mES cells were induced to differentiate by using chemically defined media (Battersby *et al*., 2007). However, when embryonic carcinoma cells were induced to differentiate in response to retinoic acid, the expression levels of Hsp27 was up-regulated (Stahl *et al*., 1992). These observed differences were attributed to the use of different cell lines (Battersby *et al*., 2007).

In a study to compare proteomic analysis between mES cells and vascular smooth muscle cells, Yin *et al* (2006) have demonstrated by Western analysis that heat shock proteins were more abundant in mES cells than in vascular smooth muscles cells. These heat shock proteins include Hsp27, Hsp60, and Hsp90. However, the levels of Hsp20, a smaller heat shock protein that regulates actin polymerization was more abundant in smooth muscles cells than in mES cells (Yin *et al*., 2006).

The interaction between STAT3 and Hsp90 has been shown before (Sato *et al*., 2003). This association was reported to be essential in the translocation of STAT3 from the membrane rafts to the nucleus after IL-6 stimulation (Sőti *et al*., 2005). However, the interactions between STAT3 and Hsp90 in the maintenance of self-renewal in mES cells has not been studied before, therefore, it is likely that the maintenance of self-renewal in mES cells could be regulated by the ability of the Hsp90-chaperone complex to interact with STAT3, regulate and stabilize STAT3 in a conformation amenable for activation and/or nuclear translocation.

1.5 PROBLEM STATEMENT: Information on chaperoning activity of Hsp90 and the role of STAT3 in mES cells is novel and limited. The study of their interactions in mES cells will provide a better understanding on the roles they play in the regulation of self-renewal and pluripotency

1.6 RESEARCH HYPOTHESIS: The function and the role of STAT3 in the maintenance of self-renewal and pluripotency in mES cells is promoted by the chaperone activity of Hsp90 and its co-chaperones.

1.7 BROAD QUESTION: Do STAT3 and Hsp90 interact in mES cells; if so, is the interaction affected by withdrawal of LIF?

1.8 THE AIMS OF THIS STUDY WERE TO INVESTIGATE:

- The effect of LIF withdrawal on the expression levels of STAT3, pYSTAT3, Hsp90 and Hop.
- Whether Hsp90 interacts specifically with STAT3 in the absence and in the presence of LIF.
- The effect of STAT3 tyrosine phosphorylation inhibitor (cucurbitacin I) on the expression levels of STAT3, pYSTAT3, Hsp90 and Hop.

CHAPER TWO

MATERIALS AND METHODS

2.1 MATERIALS

All major chemicals, culture media, molecular biology reagents and equipment are listed in the Appendix section with the grade and vendor details.

2.2 ROUTINE MAINTENANCE OF MOUSE ES CELLS

Mouse ES cells (E-14 cell line) were kindly donated by Dr. Murray, P.A from the University of Liverpool, England, United Kingdom. The line was originally derived in 1985 by Hooper *et al* (1987).

Mouse ES cells were maintained on 0.1% (w/v) gelatine-coated 6 cm tissue culture dishes in mES cells culture medium, which comprised the following: Advanced® DMEM supplemented with 2.0 % (v/v) foetal calf serum (FCS), 1.0 % (v/v) 200.0 mM L-Glutamine, 0.01% (v/v) 50.0 mM 2-mercaptoethanol, and 1000 U/ml LIF. mES cells cultured were incubated in a humidified atmosphere at 37.0 °C with 10.0 % (v/v) CO_2 in air.

Cells were typically split 1:3 every 3 to 4 days. All solutions were preheated to 37°C before use. Six centimetre (6.0 cm) dishes were coated with 3 ml 0.1% (w/v) gelatin for 10 min at room temperature. Cells were trypsinized by first aspirating the medium from the dish, adding 3 ml 1x Trypsin/EDTA in calcium and magnesium-free phosphate buffered saline (PBS), and incubating the dish at for 3-5 min at room temperature. The trypsinized cells were then transferred to a sterile 15.0 ml conical tube containing an equal volume of DMEM containing 10% (v/v) FCS to stop the trypsin reaction.

The cells were pelleted by centrifugation for 2.5 min at 100 g (800 rpm) in a desk top centrifuge. The supernatant was discarded and the pellet was resuspended in 3.0 ml of mES cells culture medium. 1.0 ml of cell suspension was transferred into each of three 6.0 cm gelatin-coated culture dishes containing 2.5 ml of mES cells culture medium. The medium was changed every third day.

2.3 IMMUNOFLUORESCENCE STAINING AND CONFOCAL MICROSCOPY

Mouse ES cells were cultured in mES cell culture medium for 24.0 hours in the presence or absence of LIF using 6.0 cm Nunc culture dishes. When confluency was reached, the cell were harvested by trypsination and then collected by centrifugation (see section 2.2). The pellet was resuspended in 4.5 ml of culture medium. About 0.5 ml of the cell suspension was transferred to each of the 6.0 3.5 cm Nunc culture dishes which have been gelatinized for 10.0 minutes. Two milliliters of culture medium was added to each dishes and incubation was continued until confluency was reached. After incubation, the medium was aspirated and the cells were fixed at room temperature with 4.0 % (w/v) paraformaldehyde for 10.0 min, washed three times in PBS and stores at 4.0 °C. In order to inhibit non-specific binding of the primary and secondary antibodies, blocking solution (10.0 % (v/v) normal goat serum and 0.1 % (v/v) Triton X-100 in PBS) was added to each culture dish and incubated at room temperature for 40.0 minutes.

The blocking solution was then aspirated and, the primary antibody solution was applied, which comprised the following: 1.0 % (v/v) normal goat serum and 0.1 % (v/v) Triton X-100 in PBS and the appropriate primary antibodies. Primary antibody concentrations were as follows: rabbit polyclonal anti-mouse STAT3 antibody (1:100); mouse monoclonal anti-human Oct-3/4 antibody (1:500); rabbit polyclonal anti-human Nanog antibody (1:500) (see appendix A8). Samples were incubated overnight at 4°C in a humidified chamber.

After overnight incubation, the cells were washed three times with PBS to remove unbound primary antibodies and the secondary antibody solution was applied, which comprised the following: 1.0 % (v/v) normal goat serum and 0.1 % (v/v) Triton X-100 in PBS and the appropriate secondary antibodies. Secondary antibody concentrations were as follows: chicken anti-rabbit IgG-Alexa 488 (1:500) for detection of STAT3 and Nanog); goat anti-mouse IgG_{2b}-Alexa 594 (1:1000) for detection of Oct-3/4 (see appendix A9). Samples were incubated for 2.0 hours in the dark at room temperature in a humidified chamber.

After incubation, the cells were washed twice in PBS and were counterstained with DAPI (0.05 ng/ml in PBS) for 5.0 min in the dark at room temperature. Cells were then washed twice in PBS and mounted in fluorescent mounting medium.

Samples were analysed using a Leica TCS-SP2 confocal fluorescent microscope with 40X oil objective. Images were recorded digitally using Leica software. The excitation wavelength for Alexa ^{Fluor} 488 was 488nm, and the emission was captured at 500-550 nm. The excitation wavelength for Alexa ^{Fluor} 595 was 595 nm and the emission was captured at 600 – 650 nm. Image quality was digitally optimized and merges were generated using Adobe Photoshop® software, version 6.0. In all cases, care was taken to ensure that the same parameter changes were applied to all images.

2.4 WESTERN BLOT DETECTION OF Hsp90, Hop, STAT3, AND pYSTAT3

2.4.1 Protein extractions from mES cells cultured with and without LIF

Mouse ES cells were cultured in mES cells culture medium for 24.0 hours in the presence or absence of LIF.

The cells were then washed three times in ice-cold PBS and harvested by trypsination (method discussed in section 2.2). The cell suspension was transferred into 15.0 ml conical tube and centrifuged at 100 g (800 rpm) for 2.0 min. After centrifugation, the supernatant was removed and the pelleted cells were resuspended in 100.0 µl of ice-cold lysis buffer (0.037 M Hepes, 0.05 M sucrose, 0.1 M KF, 0.6 % (w/v) sodium cholate, and 1.0 %, v/v protease inhibitor cocktail), and incubated on ice for 10.0 min on a rocking platform, followed by centrifugation at 12000 g for 15.0 minutes at 4 $^{\circ}$C. Protein concentration was determined using the Bradford Assay (Bradford, 1976). Briefly, samples were diluted ten times (1.0 µl of the sample and 9.0 µl of 1x PBS) using 1x PBS. One part (1.0 µl) of the diluted samples was mixed with 1.0 ml of the Bradford reagent. The mixture was mixed and the absorbance readings were taken at 595 nm. The blank sample contained 1.0 µl of 1x PBS and 1.0 ml of the Bradford reagent.

The absorbance values were added on the following formula. Bovine serum albumin (BSA) was used as a standard:

$$\textbf{Sample concentration} = \frac{\textbf{Absorbance value + 0.0027}}{\textbf{0.5514}}$$

The resultant concentration value was multiplied by ten to give the final protein concentration in µg/µl.

The lysate were either used or stored at -20.0°C until further use.

2.4.2 Sodium dodecyl–polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis

Twenty two µl of 2.0 µg/µl protein lysate, 11.4 µl of SDS-PAGEloading buffer and 4.5 µl of SDS-PAGE sample treatment buffer were transferred to a centrifuge tube and then boiled for 6.0 minutes (both SDS-PAGE loading buffer and SDS-PAGE sample treatment buffer were commercially available (see appendix A5). Alternatively, SDS-PAGE sample treatment buffer and SDS-PAGE loading buffer were prepared according to a standard recipe (see appendix A2).

After cooling to room temperature, 10 µl were loaded into each well of a pre-cast 4-12 % SDS-PAGE gel (or prepared 4-12 % SDS-PAGE gel (see appendix A3).

The tank was filled with NuPAGE® MOPS SDS Running Buffer (see appendix A5). Alternatively, SDS-running buffer was prepared according to standard recipe (see appendix A4).

The loaded samples were resolved at 150 V using a Consort E132 power pack. The gel was removed and resolved proteins were transferred onto nitrocellulose

membrane. The transfer was carried out for 1.5 h at 30.0 V using Consort E 321 power pack.

2.4.3 Blocking of the membrane and incubation with the primary antibody

The membrane was stained with Ponceau Stain, photographed and then blocked for one hour at room temperature using 5.0 % (w/v) of non fat powder milk, (Marvel, UK) in Tris-buffered saline-Tween (TBST), pH 7.6.

After blocking, the membrane was incubated in 5.0 % (w/v) non fat powder milk (Marvel, UK) in TBST, pH7.6 containing one of the following primary antibodies: mouse monoclonal anti-human pYSTAT3 antibody (1:100); mouse monoclonal anti-human STAT3 antibody (1:100); mouse monoclonal anti-chicken Hop antibody (1:500); mouse monoclonal anti-human Hsp90β antibody (1:500); rabbit polyclonal anti-mouse β-actin antibody (1:500) (see appendix A8). Incubation was performed overnight at $4^{\circ}C$ on a rocking platform.

2.4.4 Detection of STAT3, pYSTAT3, Hop, Hsp90 and β-actin

After overnight incubation, the membranes were washed three times in TBST, pH7.6 and then re-incubated in 5.0 % Marvel in TBST, pH7.6 containing the appropriate secondary antibody: goat anti-mouse IgG_1-HRP (1:2000) for the detection of STAT3, pYSTAT3, Hop and Hsp90; goat anti-rabbit IgG-HRP (1:2000) for the detection of β-actin (see appendix A8). Incubation was performed for 1.0 hour at room temperature on a rocking platform. After incubation, the membranes were washed as above.

After the final wash, the membranes were immersed in Chemiluminiscent buffer (Sigma) and chemiluminiscent reaction buffer (Sigma), mixed in the ratio of 1:1 for 5.0 min. After immersion for 5.0 min, the membranes were sealed in Cling Film for development by autoradiography.

2.5 IMMUNOPRECIPITATION

2.5.1 Immunoprecipitation of Hsp90, STAT3 and Hop from mES cells lysate cultured in the presence and in the absence of LIF

Mouse ES cells were cultured in mES cell culture medium for 24.0 hrs in the presence or absence of LIF (see section 2.3). Cells were then harvested and lysed as above. Samples of the protein lysate (300 µg) were incubated for two hours with one of the following antibodies: mouse monoclonal anti-human Hsp90 β (1.5 µg; a kind gift from Prof. Toft, D.O, Department of Biochemistry and Molecular Biology, Mayo Clinic, USA) ; mouse monoclonal anti-human STAT3 antibody (2.0 µg); and mouse monoclonal anti-chicken Hop (2.0 µg). After incubation, the complexes were each transferred to microfuge tubes containing 12.0 µl of Protein G Plus/Protein-A agarose suspension and incubation was continued overnight on ice on a rocking platform.

After overnight incubation, the complexes were centrifuged for 1.0 min at 2100 g and the supernatants were removed. The pelleted complexes were washed four times by centrifugation at 525 g for 30.0 sec at 4°C with ice-cold PBS. After the last wash, 40.0 µl of SDS-PAGE sample treatment buffer was added and then boiled for 10.0 minutes. After boiling, the samples were cooled to room temperature and 10.0 µl was resolved by SDS-PAGE and Western blot analysis was conducted for the detection of Hsp90, STAT3 and Hop.

The negative controls were conducted with mES cells lysate incubated with 12.0 µl of Protein G Plus/Protein-A agarose suspension (no antibody) and the positive controls were conducted with free mES cell lysate.

Western blot detections of pYSTAT3, STAT3, Hsp90 and Hop were carried out as in section 2.4.

2.6 STAT3 TYROSINE PHOSPHORYLATION INHIBITION STUDY

2.6.1 Inhibitor preparation and inhibition

Five milligrams (5.0 mg) of the inhibitor, Cucurbitacin I (Merck, Cat no. 238590) from the plant *Cucumis sativus L* was dissolved in 2.0 ml of absolute ethanol to give a final stock concentration of 1.0 mM. This was then stored at -20°C until further use. For inhibition studies, the stock was diluted to 0.2 mM using mESC culture medium.

Mouse ES cells were cultured for 24.0 h in LIF-containing mES cells culture medium (see section 2.3) supplemented with the following concentrations of inhibitor: 0, 50 and 250 nM**.**

Western blot detections of pYSTAT3, STAT3, Hsp90 and Hop were carried out as in section 2.4.

CHAPTER THREE

RESULTS

3.1 THE EFFECTS OF LIF WITHDRAWAL ON THE EXPRESSION LEVELS OF STAT3, pYSTAT3, Hsp90 AND Hop IN MOUSE EMBRYONIC STEM CELLS

The expression levels of pYSTAT3, total STAT3, Hsp90 and Hop in mES cells were investigated 24.0 hours following LIF withdrawal. Although earlier studies have shown that LIF induces the activation of the JAK-STAT3 pathway in mES cells, leading to increased levels of pYSTAT3 (Rajasingh and Bright, 2006), the effect of LIF on levels of total STAT3, Hsp90 and Hop has been little-studied. As expected, the levels of pYSTAT3 were reduced following LIF withdrawal (Figure 3.1 A). However, it was found that the levels of total STAT3 were also reduced (Figure 3.1 B), indicating that the reduction in pYSTAT3 levels is due, at least in part, to reduced expression of total STAT3. In contrast, the expression levels of Hsp90 were slighty increased following LIF withdrawal (Figure 3.2 A), whereas Hop levels were unchanged (Figure 3.2 B), suggesting that the expression of Hop is not regulated by the LIF-STAT signaling pathway.

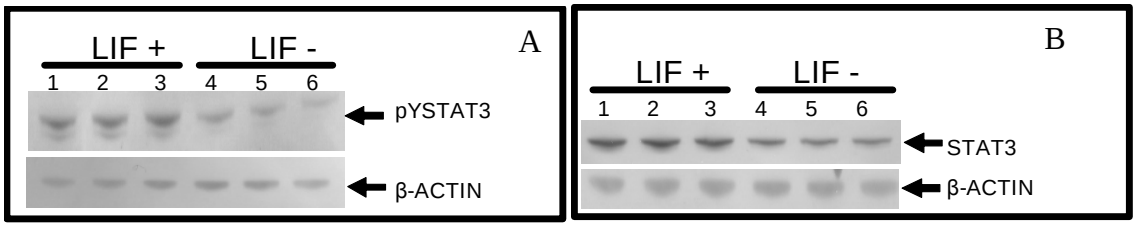

Figure 3.1 Western analysis of pYSTAT3 and total STAT3 levels in mES cells. Levels of pYSTAT3 (A) and total STAT3 (B) were determined 24.0 h following LIF withdrawal. β-actin was used as the loading control. Lane 1-3 represent samples from the same experiment in triplicates.

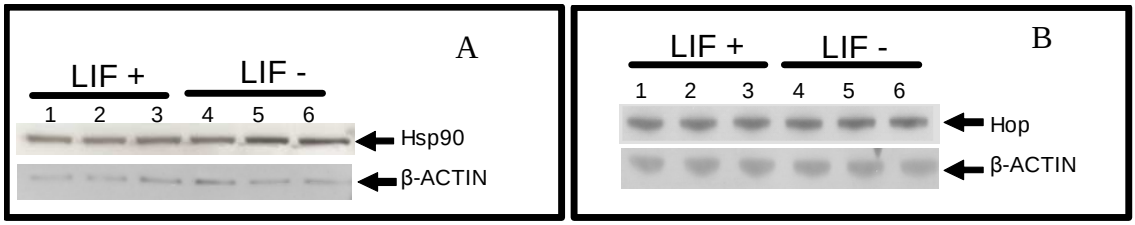

Figure 3.2 Western analysis of Hsp90 and Hop levels in mES cells. Levels of Hsp90 (A) and Hop (B) were determined 24.0 h following LIF withdrawal. β-actin was used as the loading control. Lane 1-3 represent samples from the same experiment in triplicates.

3.2 IMMUNOFLUORESCENCE ANALYSIS OF THE EFFECT OF LIF WITHDRAWAL ON STAT3, Oct-3/4 AND Nanog EXPRESSION IN mES CELLS

3.2.1 The effect of LIF withdrawal on the expression of STAT3 in mouse ES cells

It is well established that the LIF-STAT signaling pathway play a crucial role in the maintenance of self-renewal in mouse ES cells (Kinoshita *et al*., 2007). Immunostaining was performed to investigate the expression of STAT3. In the study to determine the effect of LIF withdrawal on the expression levels of STAT3, mES cells were cultured for 24.0 hours in the presence and in the absence of LIF. Mouse ES cells were stained for the nuclear DNA using DAPI, (Figure 3.3, middle panel, blue) and for STAT3 (Figure 3.3, right, top and bottom panel, green). The top and bottom panels on the left are the bright field images.

Contrary to the Western analysis, it was found that 24.0 hours following LIF withdrawal, the levels of total STAT3 were reduced (Figure 3.3, compare top and bottom panels, right).

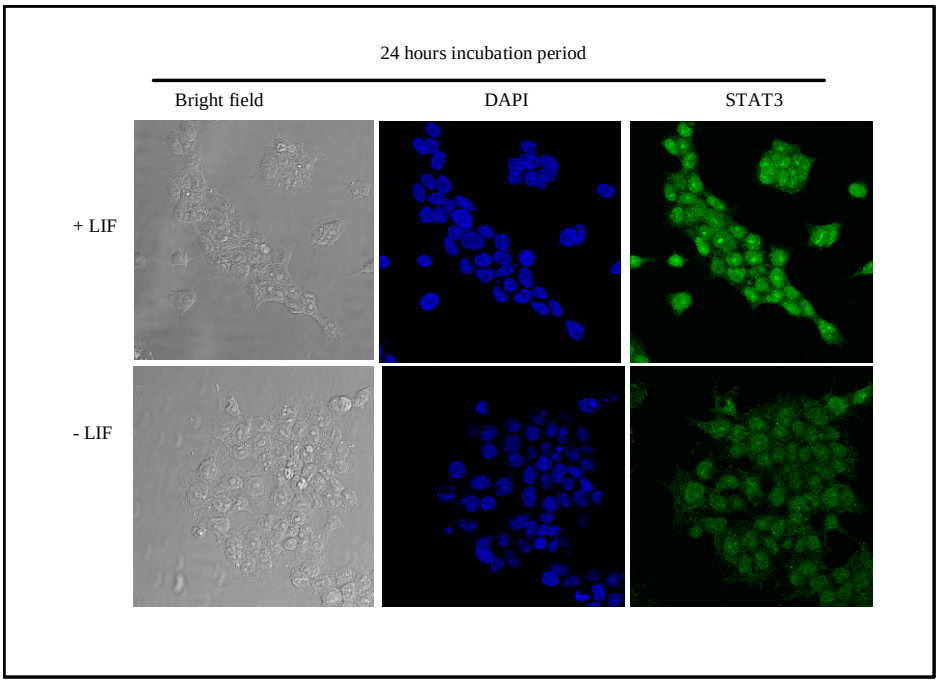

Figure 3.3 Immunofluorescence analysis of STAT3-expressing mES cells following 24.0 hours of LIF withdrawal. Mouse ES cells (E-14 cell line) were cultured for 24.0 hours in the presence of LIF (+LIF) and in the absence of LIF (+LIF). Cells were fixed and then stained for STAT3 (green; right hand panel) and for the nucleus (DAPI staining; blue; middle panel). Images were taken from the same magnifications using confocal fluorescence microscopy on a Leica TCS-SP2 confocal microscope.

Mouse ES cells were again cultured for four days in the presence (Figure 3.4, top panel) and in the absence of LIF (Figure 3.4, bottom panel). Mouse ES cells were then fixed and stained for STAT3 (Figure 3.4, top and bottom panels, right). In this, we have shown that STAT3 over-expression in mES cells depended on the presence of LIF (Figure 3.4, top panel, right). However, when mES cells were allowed to grow for four days in the absence of LIF, the expression levels of STAT3 were dramatically reduced (Figure 3.4, bottom panel, right). The top and bottom panel on the left are the bright field images.

These data seems to suggest that LIF promotes STAT3 over-expression in mES cells and that this STAT3 over-expression could be is essential in the maintenance of self-renewal.

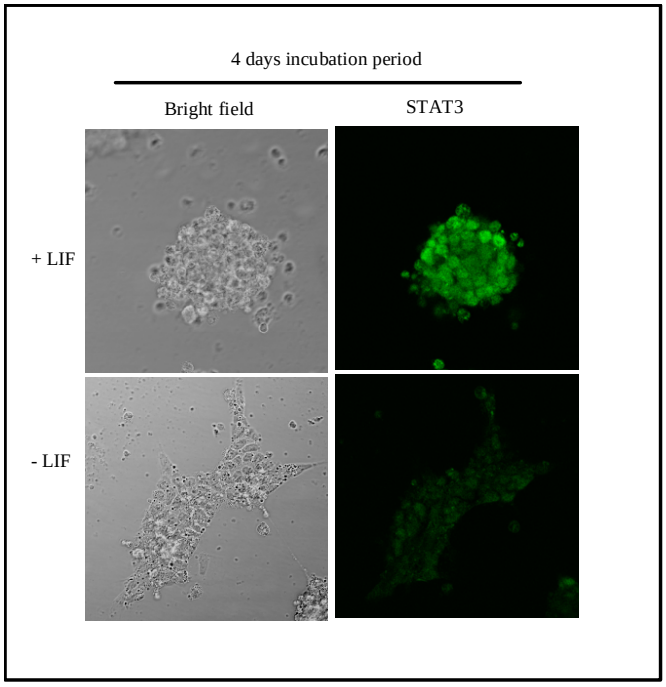

Figure 3.4 Immunofluorescence analysis of STAT3-expressing mES cells following 4.0 days of LIF withdrawal. Mouse ES cells (E-14 cell line) were culture for 4.0 days in the presence of LIF (+LIF) and in the absence of LIF (-LIF). Cells were fixed and then stained for STAT3 (green; right hand panel) and for the nucleus (DAPI staining; blue; middle panel). Images were taken from the same magnification using confocal fluorescence microscopy on a Leica TCS-SP2 confocal microscope.

3.2.2 The effect of LIF withdrawal on the levels of Nanog and Oct-3/4 expression in mES cells

Mouse ES cells *in vitro* can replicate indefinitely to produce 1 to 10 billion cells without differentiating. These cells express the pluripotency marker, Oct-3/4 which is required in maintenance of the pluripotent, undifferentiated state of ES cells (Kirschstein and Skirboll 2001). In mES cells, inhibition of Oct-3/4 expression results in differentiation toward trophectoderm lineage (Niwa, 2001). In the study to determine the effect of LIF withdrawal of on the expression level of Nanog and Oct-3/4 in mouse ES cells, mouse ES cells were cultured for 24.0 hours in the presence of LIF (Figure 3.5, top panels) and in the absence of LIF (Figure 3.5, bottom panel) and then stained for Nanog (Figure 3.5, top panel, green, right and bottom panel, green right) and for the nuclear DNA (Figure 3.5, top panel, blue, middle and bottom panel, blue middle). The top and bottom panel on the left show the bright field images.

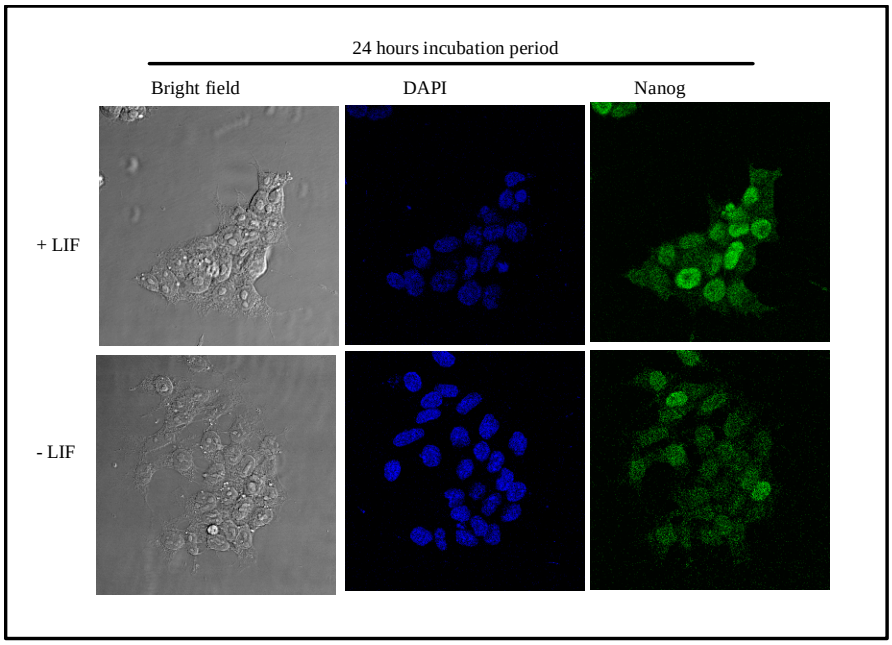

Figure 3.5 Immunofluorescence analysis of the effect of LIF withdrawal on Nanog-expressing mES cells. Mouse ES cells (E-14 cell line) were cultured for 24.0 hours days in the presence of LIF (+LIF) and in the absence of LIF (-LIF) fixed and then stained for mES cells pluripotency marker, Nanog (green). Images were taken from the same magnifications using confocal fluorescence microscopy on a Leica TCS-SP2 confocal microscope.

Mouse ES cells were again cultured for four days in the presence of LIF (Figure 3.6, top panel) and in the absence of LIF (Figure 3.6, bottom panel). After incubation, cells were fixed and stained for Nanog (Figure 3.6, top and bottom panel, green) and for Oct-3/4 (Figure 3.6, top and bottom panel, red). The bright field images on the top

and bottom panel are indicated: the first and third panels from the left to the right. It is well known colonies of pluripotent ES cells are round (see Figure 3.4, top left panel and Figure 3.6 top left panel), however, it should be noted here that some colonies spread out and lost their characteristic round shape despite being cultured in the presence of LIF but yet retained the pluripotecy (see Figure 3.3, 3.5 and 3.7 top left panel). These observations could be due to the degradation of LIF in the culture medium.

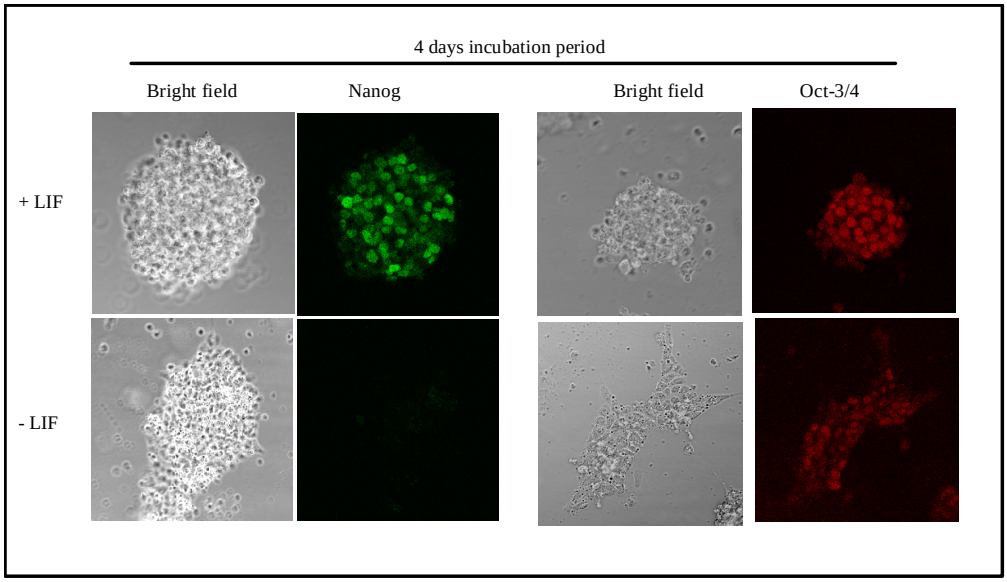

Figure 3.6 Immunofluorescence analysis of the effect of LIF withdrawal on Nanog and Oct-3/4-expressing mES cells. Mouse ES cells (E-14 cell line) were cultured for four days in the presence of LIF (+LIF) and in the absence of LIF (+LIF) fixed and then stained for mES cells pluripotency markers, Nanog (green) and Oct-3/4 (red). Images were taken from the same magnification using confocal fluorescence microscopy on a Leica TCS-SP2 confocal microscope.

In this study, we have shown that the when mES cells are cultured in the absence of LIF, the expression levels of Nanog-expressing mES cells were slightly reduced (Figure 3.5, compare top and bottom panel, green, right). Furthermore, the expression levels of Nanog-expressing mES cells were barely detectable when mES cells were cultured for four days in the absence of LIF (Figure 3.6, compare top and bottom panel, green).

There was no obvious reduction in the expression levels of Oct-3/4 in mES cells cultured for 24.0 hour in the absence of LIF (Figure 3.7; compare top and bottom panel, red, right). Furthermore, when mES cells were cultured for four days in the

absence of LIF, the levels of Oct-3/4 down-regulation was far less as compared to that of Nanog (Figure 3.6, compare top and bottom panel, red, right).

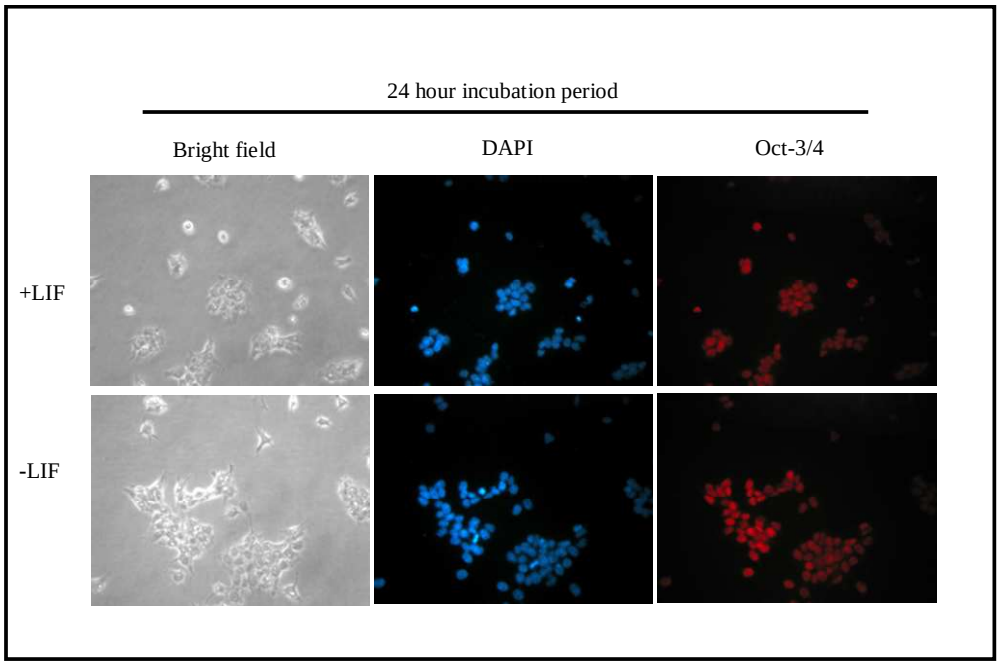

Figure 3.7 Immunofluorescence analysis of the effect of LIF withdrawal on the expression levels of Oct-3/4 in mouse ES cells. Mouse ES cell line E-14 was cultured for 24.0 hours in the presence of LIF (+LIF) and in the absence of LIF (-LIF). The cells were fixed, stained and then analysed using fluorescence microscope for the expression of Oct-3/4. Images were taken from the same magnifications.

The results from this study seems to suggest that the absence of LIF promotes down-regulation of mES cell pluripotency marker Nanog and that the expression levels of Oct-3/4 in mES cells cultured for 24.0 hour to four days in the absence of LIF is not down-regulated, similar findings were reported by Kinoshita (Kinoshita *et al*., 2007).

3.3 STAT3 AND Hsp90 OCCUR IN A COMMON COMPLEX IN mES CELLS

The role of molecular chaperones mainly involves prevention of target protein from aggregation as well as ensuring correct folding and assembly of the target protein (Pearl and Prodromou, 2001). Previous studies have shown that Hsp90 interact with many proteins that are involved n the regulation of transcription and in signal transduction pathway (Schulte, *et al*., 1995). These proteins include among others, steroid hormone receptors, protein kinases, and transcription factors (Pratt *et al*., 2004). Sato *et al* (2003) have demonstrated that Hsp90 and STAT3 interact and that this interaction is important in the function of IL-6 which was mediated through

STAT3 in human embryonic kidney carcinoma cell line, 293T (Sato *et al*., 2003). However, the interaction between Hsp90 and STAT3 in mES cells has not been shown before.

To show that Hsp90 and STAT occur in a common complex, mES cells, mES cell lysate were obtained from mES cells cultured for 24.0 hours in the presence of LIF, immunoprecipitated with Protein G/ plus A agarose suspension and mouse monoclonal anti-human Hsp90β, and resolved by SDS-PAGE gel.

To show that the immunoprecipitation had worked, the resolved proteins were first analyzed by Western blotting for the presence of Hsp90. Hsp90 was detected in the test sample (Figure 3.8, lane 3), and the detection was at the same position as the detection of Hsp90 in the positive control (Figure 3.8, lane1). There was no detection of Hsp90 on the negative control (Figure 3.8, lane 2). The results from this study indicated that the immunoprecipitation of Hsp90 had worked.

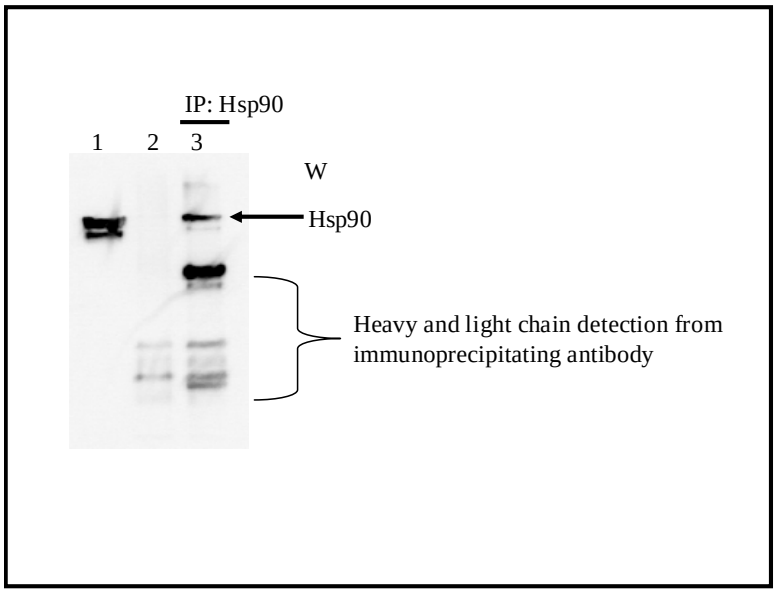

Figure 3.8 Mouse monoclonal anti-human Hsp90β can immunoprecipitate Hsp90. Mouse ES cells were cultured for 24.0 hours in the presence of LIF. Cell lysate were obtained, immunoprecipitated, loaded into each well, resolved by SDS-PAGE and then analysed by western blotting. Lane 1: (positive control) free mES cell lysate; lane 2: (negative control) mES cells lysate subjected to immunoprecipitation without antibody; Lane 3: mES cell lysate subjected to immunoprecipitated with mouse monoclonal anti-human Hsp90β antibody. The membranes were subjected to Western analysis (W) for Hsp90.

To determine if STAT3 was complex to Hsp90, a second Western analysis for the detection of Hsp90 was conducted on the Hsp90 immunoprecipitate. As shown in figure 3.9 (lane 3), STAT3 was detected in the test sample and the detection was at the same position as the detection of STAT3 in the positive control (lane1). There was no detection in the negative control (lane 2). The results from this study indicate that in mES cells, Hsp90 and STAT3 occur in a common complex *in vivo.* These results are consistent with previous work by Sato *et al* (2003) which showed that STAT3 and Hsp90 physically interacted in human embryonic kidney carcinoma cell line, 293T.

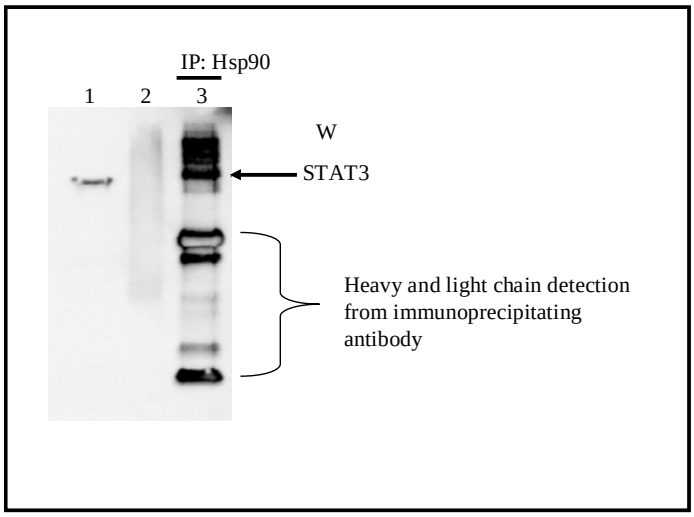

Figure 3.9 Hsp90 and STAT3 occur in a common complex in mES cells. Mouse ES cells were cultured for 24.0 hours in the presence of LIF. Mouse ES cell lysate were obtained, immunoprecipitated, loaded into each well, resolved by SDS-PAGE and then analysed by Western blotting. Lane 1: (positive control) free mES cell lysate; lane 2: (negative control) mES cells lysate subjected to immunoprecipitation without antibody; Lane 3: mES cell lysate subjected to immunoprecipitation with mouse monoclonal anti-human Hsp90β antibody. The membranes were subjected to Western analysis (W) for STAT3.

3.4 SELF-RENEWAL IN mES CELLS POTENTIALLY DEPENDS ON THE INTERACTIONS BETWEEN STAT3, Hsp90, AND Hop

Previous study on human embryonic kidney carcinoma cell line, 293T has shown that Hsp90 and STAT3 was in common complex (Sato *et al*., 2003). Furthermore, the results also showed that the interaction was more upon interleukin (IL)-6 stimulation. In the study to determine the effect of LIF (an IL-6 family of cytokine) withdrawal on the interactions between Hsp90, STAT3 and Hop, mES cells were cultured for 24.0 hours in the presence of LIF and in the absence of LIF

Mouse monoclonal anti-human Hsp90β antibodies were used for immunoprecipitation of lysate obtained from mES cells cultured in the presence (Figure 3.10, lane 3-4) and in the absence of LIF (figure 3.10, lane 5-6). Mouse ES cell lysate were used as positive control, (Figure 3.10, lane 1). For the negative control, mES cell lysate obtained from mES cells cultured in the presence of LIF were immunoprecipitated with protein G/ Plus protein A agarose suspension and then subjected to Western blotting (Figure 3.10, lane 2).

The membranes were subjected to Western analysis of STAT3 (Figure 3.10, top panel) and Hop (Figure 3.10, bottom panel). The amount of STAT3 immunoprecipitated with anti-mouse Hsp90 antibodies from mES cell lyates obtained from mES cells cultured in the presence of LIF was greater than the amount of STAT3 immunoprecipitated with anti-mouse Hsp90 antibodies from mES cell lyates obtained from mES cell cultured in the absence of LIF (Figure 3.10, compare lane 3-4 and lane 5-6, top panel). The amount of Hop that formed complexes with Hsp90-STAT3 was slightly reduced upon 24.0 hours of LIF withdrawal (compare Figure 3.10, lanes 3-4 and lanes 5-6. bottom panel). The results from this study suggested that LIF promoted the interaction between STAT3 and Hsp90 and that this interaction was essential in the maintenance of mES cell self-renewal.

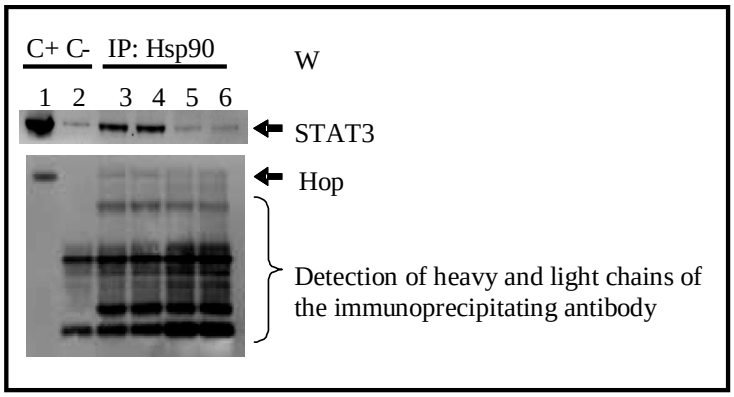

Figure 3.10 The effect of LIF withdrawal on the interactions between STAT3, Hsp90, and Hop in mES cells. Briefly, mES cells were cultured for 24.0 hours with and without LIF. Cell lysate were obtained, immunoprecipitated, loaded into each well, resolved by SDS-PAGE and then analysed by western blotting. Top panel: Lane 1: (Positive control) Free mES cell lysate; lane 2: (Negative control) mES cells lysate subjected to immunoprecipitation without antibody; Lane 3-4: cell lysate obtained from mES cells cultured in the presence of LIF and then immunoprecipitated with mouse monoclonal anti-Hsp90; Lane 5-6: cell lysate obtained from mES cells cultured in the absence of LIF and then immunoprecipitated with mouse monoclonal anti-Hsp90 antibody. The membranes were subjected to Western analysis (W) for STAT3, (top panel) and Hop, (bottom panel).

The immunoprecipitation experiment was repeated. Mouse ES cells were cultured in the presence of LIF (Figure 3.11, lanes 3, 5, and 7) and in the absence of LIF (Figure 3.11, lanes 4, 6 and 8). Mouse ES cell lysate from mES cells cultured in the presence of LIF were used as positive control (Figure 3.11, lane 1). Mouse ES cells obtained from mES cell cultured in the presence of LIF and then subjected to immunoprecipitation using Protein G/Plus A agarose suspension were used as negative control (Figure 3.11, lane 2).

Equal amount of mES cell lysate were subjected immunoprecipitation using mouse monoclonal anti-human Hsp90 antibodies (Figure 3.11, lane 3-4); mouse monoclonal anti-human STAT3 antibodies (Figure 3.11, lane 5-6) and mouse monoclonal anti-chicken Hop antibodies (Figure 3.11, lane 7-8).

In this study, it was shown that the amount of Hsp90 that immunoprecipitated with mouse anti-human Hsp90 monoclonal antibody from lysate obtained from mES cells cultured in the presence of LIF was higher than the amount of Hsp90 immunoprecipitated from mES cell lysate obtained from mES cell cultured in the absence of LIF (Figure 3.11, compare lane 3 and 4, top panel). In contrast to these results, when using anti-mouse monoclonal anti-human STAT3 antibodies, the

amount of Hsp90 immunoprecipitated from mES cell lysate obtained from mES cell cultured in the presence of LIF was lower than the amount of Hsp90 immunoprecipitated from mES cell lysate obtained from mES cells cultured in the absence of LIF (Figure 3.11, compare lane 5 and 6, top panel). Using mouse monoclonal anti-chicken Hop antibodies, the amount of Hsp90 immunoprecipitated from mES cell lysate obtained from mES cells cultured in the presence of LIF was slightly more than the amount of Hsp90 immunoprecipitated from mES cell lysate obtained from mES cells cultured in the absence of LIF (Figure 3.11, compare lane 7 and 8, top panel).

Since the intensities of the heavy and light chains are not the same, these suggested that loading was not equivalent. Therefore, conclusion on the effect of LIF withdrawal on the interaction between, STAT3, Hsp90, and Hop would be invalid. However, what can be drawn from this study is that Hsp90 was in common complex with STAT and Hop

The amount of Hop that formed complexes with Hsp90 in mES cell lysate obtained from mES cells cultured in the presence of LIF and immunoprecipitated with mouse anti-human Hsp90 monoclonal antibodies was more than the amount of Hop that formed complexes with Hsp90 in mES cell lysate from mES cells cultured in the absence of LIF. However, the levels of Hop protein detection in these immunoprecipitations was relatively low and the protein bands were only faintly visible (Figure 3.11, compare lane 3 and 4, bottom panel).

The amount of Hop immunoprecipitated with either mouse monoclonal anti-human STAT3 or mouse monoclonal anti-chicken Hop from mES cell lysate obtained from cells cultured either in the presence or in the absence of LIF was the same (Figure 3.11, compare lane 5 and 6 or lane 7 and 8, bottom panel). These results further stress that Hop is not regulated by the LIF-STAT signaling pathway.

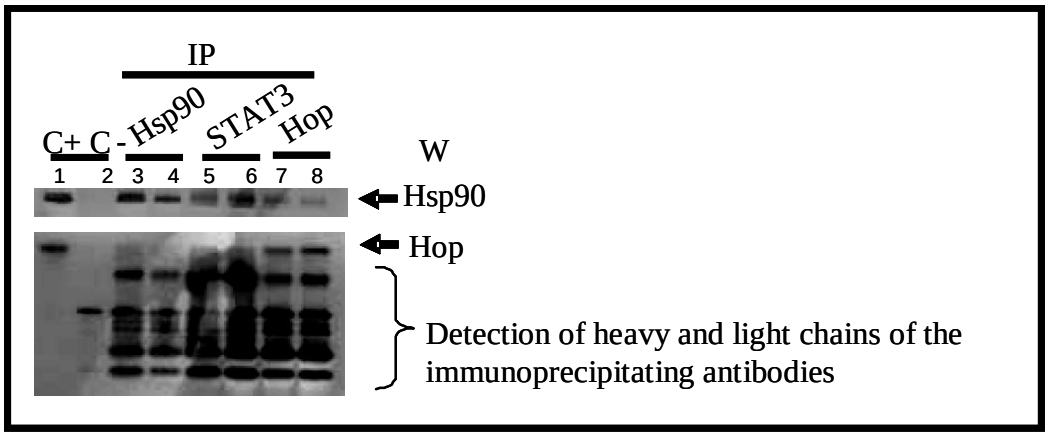

Figure 3.11 Western blot analysis of the effect of LIF withdrawal on the interactions between Hsp90, STAT3 and Hop in mES cells. Briefly, mES cells (Line E-14) were cultured for 24.0 hours in the presence of LIF and in the absence of LIF. Cell lysate were obtained, immunoprecipitated with antibodies against Hsp90, STAT3 and Hop, resolved by SDS-PAGE and then analysed by Western blotting. Top panel: lane 1: (Positive control) free mES cell lysate; lane 2: (negative control): mES cell lysate subjected to immunoprecipitation without immunoprecipitating antibody; lane 3-8: immunoprecipitating antibodies (IP) are indicated for immunoprecipitation on the lysate from mES cells cultured in the presence of LIF (lanes 3, 5, and 7) and in the absence of LIF (lanes 4, 6 and 8). The membranes were subjected to Western analysis (W) of Hsp90, top panel and Hop, bottom panel.

3.5 THE EFFECT OF STAT3 TYROSINE PHOSPHORYLATION INHIBITOR (CUCURBITACIN I) ON LEVELS OF pYSTAT3, STAT3, Hsp90 AND Hop

Cucurbitacin I is a member of the cucurbitacin family of compound that are isolated from various plant families such as Cucurbitaceae and Cruciferae. This family of compounds include: cucurbitacin A, cucurbitacin B, cuccurbitacin E, cucurbitacin I, and cucurbitacin Q (Sun *et al*., 2005).

Previous immunohistochemical studies on human tumor cells (cell line A549), have shown that cucurbitacin I inhibited the activation of STAT3 and JAK2 (Sun *et al*., 2005). Furthermore, using Western blot analysis, Blaskovich *et al* (2003) have shown that cucurbitacin was effective against STAT3 activation. However, inhibition of STAT3 activation did not affect the expression levels of STAT3 (Blaskovich *et al*., 2003).

The effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on mES cell self-renewal was determined. Briefly, the effect of the following concentrations of cucurbitacin I: 0; 25 and 250 nM on the expression levels of STAT3, pYSTAT3,

Hsp90 and Hop were studied. Mouse ES cells were cultured for 24.0 hours in the presence of LIF and in the presence of cucurbitacin I. Lysate were obtained and resolved on SDS-PAGE gel and analysed by Western blotting. Equal amount of proteins were loaded and to confirm this, β-actin was used as a loading control. In the study to determine the effect of cucurbitacin I on the expression levels of pYSTAT3 and STAT3, β-actin levels were found to be equivalent (Figure 3.12 A, bottom panel, lanes 1-9; Figure 3.12 B, bottom panel, lanes 1-9). Similarly, β-actin levels were equivalent for the experiment on Hsp90 and Hop (Figure 3.13 A and B, lanes 1-9, bottom panel).

Therefore, this indicated that loading was equivalent in all the lanes allowing accurate analysis of pYSTAT3, STAT3, Hsp90 and Hop. For pYSTAT3, there was no obvious reduction in the expression levels of pYSTAT3 after 24.0 hours of incubation in presence of 50 nM of cucurbitacin I (compare Figure 3.12 A, lanes 1-3, top panel and lanes 4-6, top panel); however, reduction in the expression levels of pYSTAT3 was observed when mES cells were cultured in the presence of 250 nM of cucurbitacin I (compare Figure 3.12 A, lanes 1-3, top panel and lanes 7-9, top panel).

There was no obvious reduction in the expression levels of STAT3, (Figure 3.12 B, top panel), despite a dramatic reduction in the expression levels of pYSTAT3. These results correlate with previous studies on NIH3T3 mouse fibroblast cells (Blaskovich *et al.*, 2003).

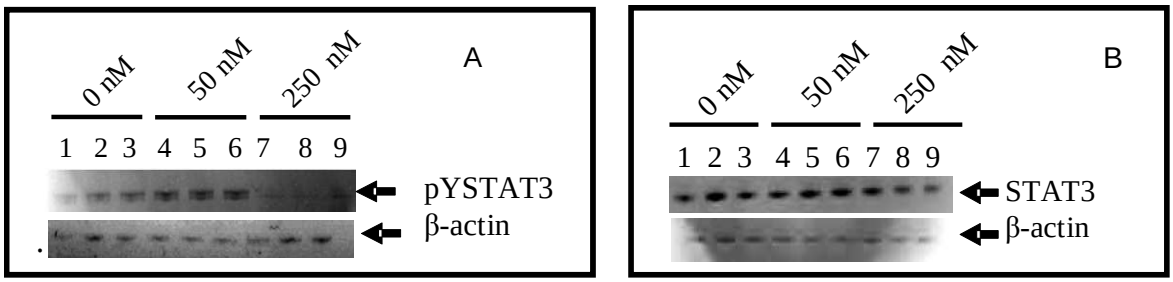

Figure 3.12 Western blot analysis of the effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the expression and tyrosine phosphorylation of STAT3 in mES cells. Mouse ES cells (E-14 cell line) were cultured for 24.0 hours in the presence of LIF and STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I (0 nM, lane 1-3; 50 nM, lanes 4-6 and 250 nM lanes 7-9). Equal amounts of mES cell lysate were obtained, loaded into each well, resolved by SDS-PAGE and then analysed by Western blotting. Membranes were detected for A: top panel: pYSTAT3 and for β-actin, bottom panel; B: STAT3, top panel and β-actin, bottom panel. Lane 1-3 represent samples from the same experiment in triplicates.

There was no reduction in the expression levels of Hsp90 (Figure 3.13 A, top panel) or of Hop (Figure 3.13 B, top panel), when mES cells were cultured for 24.0 hours in the presence of either 50 nM or 250 nM of cucurbitacin I.

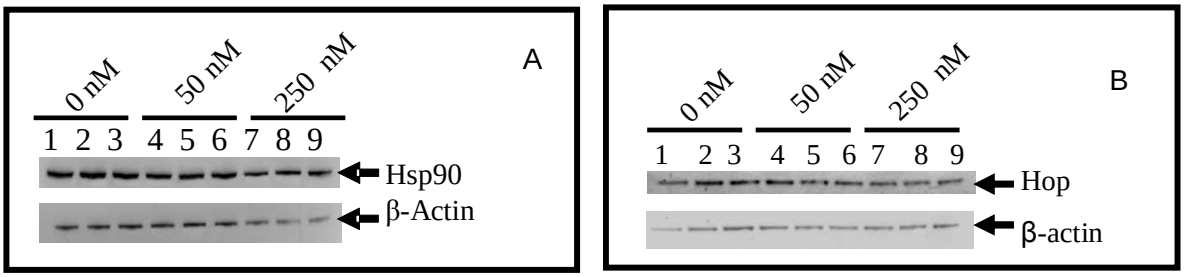

Figure 3.13 Western blot analysis of the effect of STAT3 tyrosine phosphorylation inhibitor, Cucurbitacin I on the expression levels of Hsp90 and Hop in mES cell Line E-14. Mouse ES cells were cultured for 24 hours in the presence of LIF and STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I (0 nM, lane 1-3; 50 nM, lanes 4-6 and 250 nM lanes 7-9). Equal amounts of mES cell lysate were obtained, loaded into each well, resolved by SDS-PAGE and then analysed by Western blotting. Membranes were detected for A: top panel: Hsp90 and for β-actin, bottom panel; B: Hop, top panel and β-actin, bottom panel. Lane 1-3 represent samples from the same experiment in triplicates.

3.6 THE EFFECT OF STAT3 TYROSINE PHOSPHORYLATION INHIBITOR (CUCURBITACIN I) ON MOUSE ES CELL DIFFERENTION

3.6.1 The effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the expression levels of STAT3 in mES cells

The effect of cucurbitacin I on the expression levels of STAT3-expressing mES cells was determined. Mouse ES cells were cultured for 24.0 hours in the absence of STAT3 tyrosine phosphorylation inhibitor cucurbitacin I (0 nM) (Figure 3.14, top panel) and in the presence of STAT3 tyrosine phosphorylation inhibitor cucurbitacin I (250 nM) (Figure 3.14, bottom panel). After incubation, the cells were fixed and stained for STAT3 (Figure 3.14, top and bottom panel, green, right) and for nuclear DNA (Figure 3.14, top and bottom panel, blue, middle). The bright field images are shown on the left of both the top and the bottom panel.

There was no obvious reduction in the levels of STAT3, (Figure 3.14, compare top panel right, green (0 nM) and bottom panel, right, green (250 nM). The results from this study suggest that when mES cells are cultured in the presence of LIF and cucurbitacin I (250 nM) for 24.0 hours, the expression levels if STAT3 is not down-regulated.

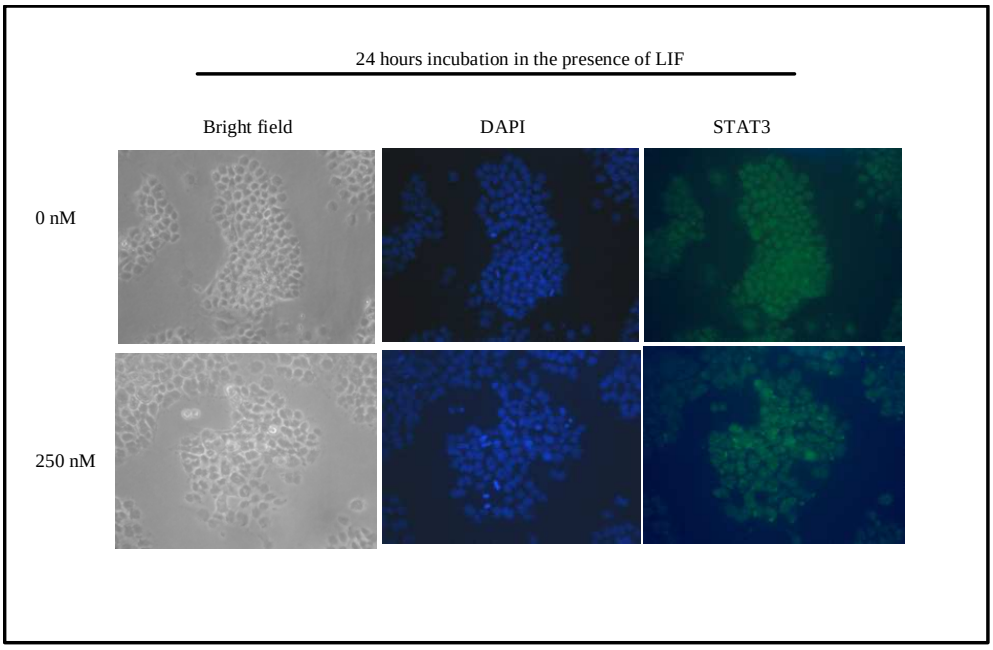

Figure 3.14 Immunofluorescence analysis of the effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the levels of STAT3 expression in mES cells. Mouse ES cell line E-14 was cultured for 24 hours in the presence of LIF and containing 0 nM and 250 nM of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I. The cells were fixed, stained, and then analysed using fluorescence microscope for the expression of STAT3. Images were taken from the same magnifications.

3.6.2 The effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the levels of Oct-3/4 and Nanog

In the study to determine the effect of STAT3 tyrosine phosphorylation inhibitor, mouse ES cells were cultured for 24.0 hours in the presence of LIF and without STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I (Figure 3.15, top panel) and with STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I (Figure 3.15, bottom panel). After incubation, mES cells were fixed and stained for DNA and Oct-3/4 or Nanog.

There was no obvious reduction in the expression levels of either Oct-3/4 (Figure 3.15, compare top panel (0 nM), red, right and bottom panel (250 nM), red, right) or Nanog (Figure 3.16, compare top panel (0 nM), green, right and bottom panel (250 nM), green, right). The bright field images are shown on the left of both the top and the bottom panel.

The results from this study suggested that inhibition of tyrosine phosphorylation of STAT3 by cucurbitacin I in mES cells cultured in the presence of LIF for 24.0 hours was insufficient to induce down-regulation of mES cells pluripotency markers (Oct-3/4 and Nanog). Thus, inhibition of STAT3 phosphorylation may not be sufficient to induce differentiation. However, this has not been fully tested in this study.

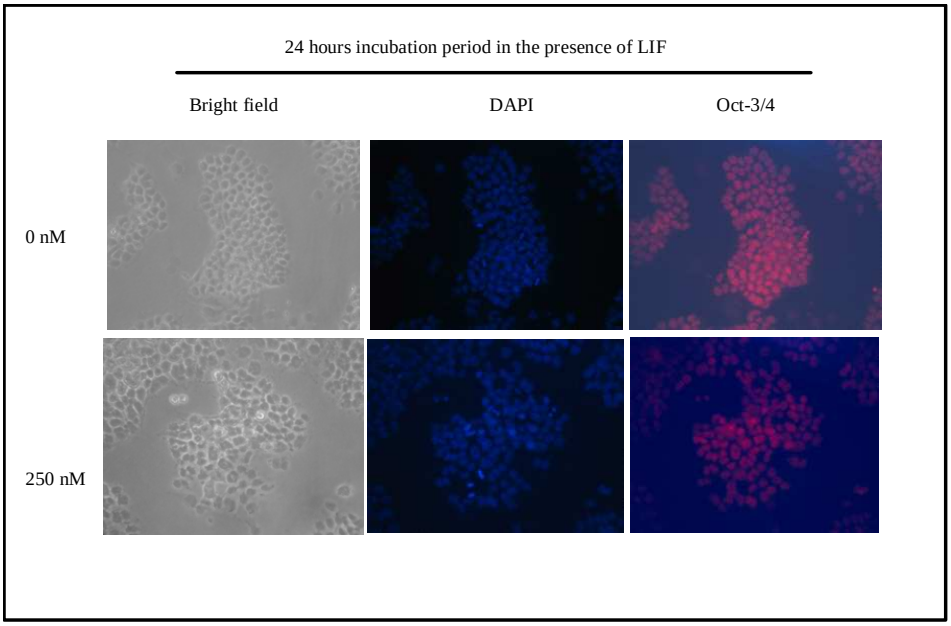

Figure 3.15 Immunofluorescence analysis of the effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the levels of Oct-3/4 expression in mES cells. Mouse ES cell line E-14 were cultured for 24 hours in the presence of LIF and containing 0 nM and 250 nM of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I. The cells were fixed, stained, and then analysed using fluorescence microscope for the expression of Oct-3/4. Images were taken from the same magnifications.

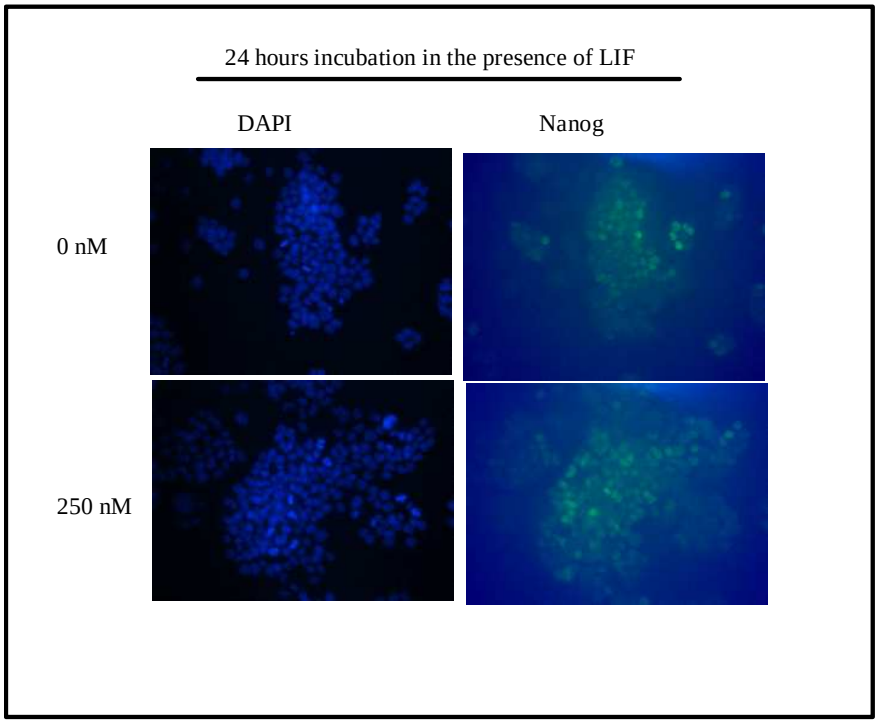

Figure 3.16 Immunofluorescence analysis of the effect of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I on the levels of Nanog expression in mES cells. Mouse ES cell line E-14 were cultured for 24 hours in the presence of LIF and containing 0 nM and 250 nM of STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I. The cells were fixed, stained, and then analysed using fluorescence microscope for the expression of Nanog. Images were taken from the same magnifications.

CHAPTER FOUR

DISCUSSIONS AND CONCLUSIONS

Heat shock proteins play an important role in the maintenance of cell viability under physiological conditions by protecting proteins from degradation and misfolding. Hsp90 is a molecular chaperone involved in the activation and maturation of a wide variety of client proteins such as steroid hormone receptors, transcription factors and kinases (Fang *et al*., 1996; Prescott and Coetzee, 2006). The chaperoning activity of Hsp90 depends on its ability to hydrolyse ATP. The chaperoning activity Hsp90 also depends on its ability to form complexes with Hsp70 and Hop. The maintenance of mES cells in an undifferentiated state mainly depends on the presence of LIF and an adhesion surface. The strong and the weaker adhesion surfaces promote differentiation of mES cells (Konno *et al*., 2006) whereas surfaces with an adhesion surface midway the strong and the weak surface promote self-renewal. In this study, it was shown using Western analysis that when mES cells were cultured for 24.0 hours in the absence of LIF, the expression levels of Hsp90 were slightly up-regulated to a level above those of mES cells cultured in the presence of LIF. These observations could primarily be due to stress response signaling resulting from the withdrawal of LIF, cytokine required for the maintenance of self-renewal of mES cells.

The JAK/signal transducers and activators of transcription (STAT3) pathways are utilized by a wide range of cytokines to regulate gene expression. These cytokines included among others, IL-6, LIF etc. The presence of these cytokines primarily induces recruitment and tyrosine phosphorylation of STAT3, a step which leads to STAT3 dimerisation through interactions between with the SH2 domain and phosphorylated tyrosine. It is widely believed that STAT3 proteins in dimerised form are essential in the maintenance of self-renewal in mES cell.

It is clear that LIF and STAT3 have been shown to be essential for mES cell self-renewal (Raz *et al*., 1999). However, although it has been shown that LIF leads to dimerisation and transcriptional activation of STAT3 in various cell types (e.g. hepatocytes), it is not yet clear if trancriptionally active STAT3 is essential for maintenance of mES cell self-renewal. Given that unphosphorylated STAT3 has multiple functions, it is possible that the effect of STAT3 on mES cell is not mediated

through direct activation of STAT3 target genes. In order to determine the role of LIF in the maintenance of self-renewal in mES cells, the levels of total STAT3 were investigated following short-term culture in the absence of LIF. Although numerous studies have shown that the levels of pYSTAT3 increase following LIF stimulation, the effect of LIF on total STAT3 levels has not previously been investigated. In this study, it was shown that when mES cells were cultured in the absence of LIF for 24.0 hours, levels of total STAT3 were dramatically reduced. Furthermore, it was shown that the levels of tyrosine phosphorylation of STAT3 from mES cells cultured in the presence of LIF was more than the levels of tyrosine phosphorylation of STAT3 obtained from mES cells cultured in the absence of LIF.

Previous studies have shown that tyrosine phosphorylation of STAT3 followed by its translocation in the nucleus was essential in the activation of genes that promotes self-renewal and pluripotency in mES cells. In the study to determine the effect of LIF withdrawal on the intensity of STAT3 expression in mES cells, we have shown that when mES cells were cultured in the absence of LIF for 24.0 hours, the intensity of STAT3 staining in mES cells was lower than the intensity of STAT3 staining from mES cells cultured in the presence of LIF, an observation that was in line with Western blot detection of STAT3. Furthermore, when mES cells were cultured for 4.0 days in the absence of LIF, the intensity of STAT3 staining dramatically decreased. These findings suggest that LIF is not only required in the tyrosine phosphorylation of STAT3 but is essential for the over-expression of STAT3. When mES were stained for the pluripotency markers, Oct-3/4 and Nanog, the intensities of Oct-3/4 were the same as those of mES cells cultured for 24.0 hours in the absence of LIF. These finding were also recently reported by another research group (Kinoshita *et al*., 2007). However, there was a noticeable change in the intensities of Nanog staining. These findings suggested that Nanog expression was downregulated prior to Oct-3/4,

When mES cells were cultured for 4.0 days in the absence of LIF, the intensity of Oct-3/4 staining in mES cells was reduced. In contrast to Oct-3/4 staining of mES cells cultured for 24.0 hours in the absence of LIF, Nanog staining of mES cells was reduced to a level lower than those of Oct-3/4 staining. Furthermore, when mES cells were cultured for 4.0 days in the absence of LIF, the intensity of Nanog staining of

mES cells was barely detectable. The results from this showed that the timing of Nanog downregulation was correlated with loss of STAT3 expression.

JSI-124 is a plant natural product which has previously been identified as cucurbitacin I. cucurbitacin I is a member of the cucurbitacin family of compounds that are isolated from various plant families such as the Cucurbitaceae and Cruciferae. These plants have for centuries been used as folk medicines in countries such as China and India. However, until recently, little was known about their biological activities. Some cucurbitacins have been shown to have anti-inflammatory and analgesic as well as cytotoxic effects. Furthermore, cucurbitacins have also been shown to inhibit DNA, RNA, and protein synthesis in HeLa cells, endothelial cells, and T lymphocytes. Furthermore, some cucurbitacins have been shown to suppress skin carcinogenesis, inhibit cell adhesion and disrupt the actin and vimentin cytoskeleton in prostate carcinoma cells.

Although reports on the biological activities of cucurbitacin suggest anti-proliferative activity and possible anti-tumor activity, their ability to inhibit self-renewal and pluripotency in mES cells has not been tested before. In this study to determine the effect of STAT3 tyrosine phosphorylation inhibitor on the pluripotecy and self-renewal, mES cells were cultured for 24.0 hours in the presence of LIF and STAT3 tyrosine phosphorylation inhibitor, cucurbitacin I and then Western blot performed to detect STAT3, pYSTAT3 and Hsp90 levels. These results showed that expression levels of pYSTAT3 were dramatically reduced when mES cells were cultured for 24.0 hours in the presence of 250 nM of cucurbitacin I. However, the expression levels of STAT3 were not affected. These results correlate with the work done previously on NIH3T3 mouse fibroblast cells (Blaskovich *et al.*, 2003). The expression level of Hsp90 remained unchanged after 24.0 hours of culture under the same culture conditions.

Using immunofluorenscence staining, mES cells were cultured for 24.0 hours in the presence of LIF and cucurbitacin and then stained for Oct-3/4, STAT3 and Nanog. It was shown that the intensities of STAT3 staining remain the same as the intensity of STAT3 from mES cells cultured in the absence of cucurbitacin I, an observation that was in line with the Western blot detection of STAT3 from mES cells cultured under

the same culture conditions. Furthermore, there was no obvious reduction in the intensity of Oct-3/4 and Nanog staining when mES cells were cultured for 24.0 hours in the presence of cucurbitacin I.

The data seem to suggest that though Nanog and STAT3 levels are down regulated within 24.0 hours of LIF withdrawal, but the levels are unchanged following 24.0 hours of culture in the presence of STAT3 tyrosine phosphorylation inhibitor implying that the effect of LIF on Nanog expression or ES cell differentiation is not mediated by pYSTAT3, but might be dependent on unphosphorylated STAT3.

Hsp70/Hsp90 organizing protein (Hop) is a co-chaperone whose expression has been shown to be required in linking Hsp70 and Hsp90 together through their EEVD domains. This linking has been shown to be responsible for the transfer of Hsp70-bound substrate to Hsp90. However, recent studies have shown Hop to be more than an Hsp70/Hsp90 co-chaperone (Daniel *et al*., 2007). To determine the effect of LIF on the expression levels of Hop, it has been shown here that when mES cells were allowed to grow for 24.0 hours in the absence of LIF, the expression levels of Hop remained unchanged. These results showed that the expression levels of Hop are not regulated by LIF. In the study to determine the effect of cucurbitacin I on the expression levels of Hop, it was shown despite a dramatic reduction in the expression levels of pYSTAT3 when mES cells were cultured for 24.0 hours in the presence of 250 nM of cucurbitacin I, that the expression levels of Hop remained unchanged. Interestingly, the levels of Hop that co-precipitated with Hsp90 from mES cells lysate cultured in the absence of LIF were slightly lower than the levels of Hop that co-precipitated with Hsp90 from mES cells lysate culture in the presence of LIF. Therefore, the role of Hop in the maintenance of mES cell self-renewal remains unclear. Future work on the role of Hop in the maintenance of self-renewal in mES cells would include knock-down studies of Hop from mES cells cultured in the presence and absence of LIF and immunohistochemical and Western blot analysis of Nanog, Oct-3/4 and STAT3 as well as immunoprecipitation of Hsp90/STAT3 to evaluate a change in the levels of STAT3 that co-precipitate with either Hsp90 or STAT3.

Hsp90 is implicated in the maintenance of conformation, stability and function of key proteins that are involved in signal transduction pathways. Previous studies on human embryonic kidney carcinoma 293T cells have shown that Hsp90 complexes with STAT3 (Sato *et al*., 2003). However, the interaction between Hsp90 and STAT3 and the role of this interaction in mES cells has not been shown before. In this study, it was shown for the first time that Hsp90 and STAT3 co-precipitate from mES cells lysate. Therefore, these results indicated that in mES cells, Hsp90 and STAT3 occur in a common complex *in vivo*.

When LIF was withdrawn from the culture medium for 24.0 hours, a decrease in the levels of STAT3 co-precipitating with Hsp90 was observed. Since the levels of Hsp90 increased upon LIF withdrawal, taken together, these data suggested that under conditions of self-renewal the amount of STAT3 complexed with Hsp90 was greater than that under conditions that promoted differentiation. Therefore, these data suggested that Hsp90 and STAT3 interaction plays an important role in the maintenance of self-renewal in mES cells. Since the levels of pYSTAT3 decreased dramatically upon LIF withdrawal and that total STAT3 levels in mES cells that co-precipitated with Hsp90 decreased upon LIF withdrawal, it may be that the major sub-population of STAT3 co-precipitating with Hsp90 was pYSTAT3 (Sato *et al*., 2003). Unfortunately, the levels of pYSTAT3 were below detection using Western analysis. Therefore, immunoprecipitation studies were carried out by Western analysis of the whole STAT3 population. Future work may therefore include bulk purification or fractionation of phosphoprotein populations from mES cells lysate in the presence and absence of LIF.

REFERENCES

Americo, T.A., Chiarini, L.B., and Liden, R. (2007) Signaling induced by Hop/STI-1 depends on endocytosis, *Biochem. Biophys. Res. Commun.* **358**: 620-625.

Battersby, A., Jones, RD., Lilley, K., McFarlane, R.J., Brag, H., Allen, N.D., and Wakeman, J.A. (2007) Comparative proteomic analysis reveals differential expression of Hsp27 following the directed differentiation of mouse embryonic stem cells, *Biochimica Biophysica Acta* **1773**: 147-156

Becker, S., Groner, B., and Müller, C.W. (1998) Three-Dimensional structure of the STAT3β homodimer bound to DNA, *Nature* **394**: 145-151.

Berrill, A., Tan, H.L., Wuang, S.C., Fong, W.J., Choo, A.B.H., and Oh, S.K.W. (2004) assessment of stem cell markers during long-term culture of mouse embryonic stem cells, *Cytotechnology* **44**: 77-91.

Bernstein, S.L., Russell, P., Wong, P., Fishelevich, R., and Smith, L.E.H. (2001) Heat Shock protein 90 in retinal ganglion cells: Association with axonal transported proteins, *Visual Neuroscience* **18**: 429-436.

Bild, A.H., Tuckson, J., and Jove, R. (2002) Cytoplasmic transport of STAT3 by receptor-mediated endocytosis. *Eur. Mol. Biol.* **13**: 3255-3262.

Blaskovich, M.A., Sun, J., Cantor, A., Turkson, J., Jove, R., and Sebti, S.A. (2003) Discovery of JSI-124 (Cucurbitacin I), a selective Janus Kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor ctivity against human and murine cancer cells in mice, *Cancer Res.* **63**: 1270-279.

Blatch, G.L. and Lässle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions, *Bioassays* **21**: 932-939.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem* **72**: 248-254.

Brown, M.A., Zhu, L., Schmidt, C., and Tucker, P.W. (2007) Hsp90-From signal transduction to cell differentiation, *Biochem. Biophys. Res. Commun.* **363**: 241-246.

Burdon, T., Smith, A., and Savatier, P. (2002) Signaling, cell cycle and pluripotency in embryonic stem cells, *Trends Cell Biol.* **12**: 432-438.

Carrigan, P.E., Sikkink, L.A., Smith, D.F., and Ramirez-Alvarado, M. (2006) Domain: domain interactions within Hop, the Hsp70/Hsp90 organizing protein, are required for protein stability and structure, *Protein science* **15**: 522-532.

Carrigan, P.E., Riggs, D.L., Chinkers, M. and Smith, D.F. (2005) Functional comparison of human and drosophila Hop reveals novel role in steroid receptor maturation, *J. Biol. Chem.* **280**: 8906-8911.

Chambers, I., Colby D., Robertson, M., Nichols, J., Lee, S., Tweedie, S, and Smith, A. (2003) Functional expression cloning of Nanog, a pluripotent sustaining factor in embryonic stem cells, *Cell* **113**: 643-655.

Chambers, I. (2004) The molecular basis of pluripotency in mouse embryonic stem cells, *Cloning Stem Cell* **6**: 386-391.

Chen, S. and Smith, D.F. (1998) Hop as an adaptor in the heat shock protein 70 (Hsp70) and Hsp90 chaperone machinery, *J. Biol. Chem.* 273: 35194-35200.

Chen, S.Y., Sullivan, W.P., Toft, D.O. and Smith D.F. (1998) Differential interactions of p23 and TPR-containing proteins Hop, Cyp40, FKBP52 and FKBP52 with Hsp90 mutants, *Cell Stress chap* 3, 118-123.

Chen, S., Choo, A.B.H., Nai-Dy, W., Heng-Phon, T., and Ho, S.K.W. (2007) Knockdown of *Oct-4* or *Sox-2* attenuates neurogenesis of mouse embryonic stem cells, *Stem Cell Dev.* **16**: 413-420.

Chiosis, G., Vilenchik, M., Kim, J., and Solit, D. (2004) Hsp90: the vulnerable chaperone, *DDT* **9**: 881-888.

Daniel, S., Bradley, G., Longshaw, V.M., Sőti, C., Csermely, P. and Blatch, G.L (2007) Nuclear translocation of the phosphoprotein Hop (Hsp70/Hsp90 organizing protein) occurs under heat shock, and its proposed nuclear localization signal is involved in Hsp90 biding, Rhodes University, Grahamstown.

Duval, D., Duval, G., Kedinger, C., Poch, O, and Boeuf, H. (2003) The ´PINIT` motif, of a newly identified conserved domain of the PIAS protein family, is essential for nuclear retention of PIAS3L, *FEBS* **554**: 111-118.

Evans, M.J., and Kaugman, .M.H. (1998) Establishment in culture of the pluripotential cells from mouse embryos, *Nature* **292**: 154-156.

Fang, Y., Fliss, A.E., Robbins, D.M, and Caplan, A.J. (1996) Hsp90 regulates androgen receptor hormone binding affinity *in vivo Amer. Soc. Biochem. Mol. Biol.* **271**: 28697-28702.

Fink, A.L (1999) Chaperone-mediated protein folding, *Physiol, Rev* **79**: 425-449.

Freeman, B, C., Toft, D.O., and Morimoto, R.I. (1996) Molecular chaperone machines: chaperone activities of the cyclophilin Cyp-40 and the steroid aporeceptor-associated protein p 23, *Science* **274**: 1718-1720.

Gearing, D.P., Thut, C.J., VandenBos, T., Gimpel, S.D., Delaney, P.M, King, J., Price, V., Cosman, D. and Beckmann, M.P. (1991) Leukemia inhibitory factor receptor is structurally related to IL-6 signal transducer, pg130, *J. EMBO* **10**: 2839-2848.

Gearing, D.P., Nicola, N.A., Metcalf, D., Foote, S., Willson, T.A., Gough, N.M. and Williams, R.L. (1989) Production of Leukaemia inhibitory factor in Escherichia coli by a novel procedure and its use in maintaining embryonic stem cells in culture, *Biotech.* **7**: 1157-1161.

Ginis, I., Luo, Y., Miura, T., Thies, S., Brandenberger, R., Gerrecht-Nir, S., Amit, M., Hoke, A., Carpenter, M.K., Itskovitz-Ellldor, J., and Rao, M.S. (2004). Differences between human and mouse embryonic stem cells, *Dev. Biol.* **269**: 360-380.

Gonzales, D.S., Jones, J.M., Pinyopummintr, T., Carnevale, E.M., Ginther, O.J., Shapiro, S.S., and Bavister, B.D. (1996) Trophectoderm projections: a potential means for locomotion, attachment and implantation of bovine, equine and human blastocysts, *Human Reproduction* **11**: 2739-2745.

Gooljarsingh, L.T., Fernades, C., Yan, K., Zhang, H., Grooms, M., Johanson, K., Sinnamon, R.H., Kirkpatrick, R.B., Kerrigan, J., Lewis, T., Arnone, M., King, A.J., Lai, Z., Copeland, R.A., and Tummino, P.J. (2006) A biochemical rational for the anticancer effects of Hsp90 inhibitors: slow, tight binding inhibition by geldanamicin and its analogues, *PNAS* **103**: 7625-7630.

Hernandez, M.P., Sullivan, W.P., and Toft, D.O. (2002) The assembly and intermolecular properties of the Hsp970-Hop-Hsp90 molecular chaperone complex, *J. Biol. Chem.* **227**: 38294-38304.

Haynes, J.M., and Pouton, C.W. (2007) Embryonic stem ells as sources of models for drug discovery, *Nature Reviews* **6**: 605-616.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultural cells, *Nature* **326**: 292-295.

Huen, N.Y.M., and Chan, H.Y.E. (2005) Dynamic regulation of molecular chaperone gene expression in polyglutamine disease, *Biochem. Biophys. Res. Commun.* **334**: 1074-1084.

Humphrey R.K., Beattie, G.M., Lopez, A.D., Bucay, N., King, C.C., Firpo, M.T., Rose-John, S., and Hayek, A. (2004) Maintenance of pluripotency in human embryonic stem cells is STAT3 independent, *Stem Cells* **22**: 522-530.

Jackson, S.E., Queitsch, C., and Toft, D. (2004) Hsp90: from structure to phenotype, *Nature Str. Mol. Biol.* **11**: 152-1155.

Johnson, B.D. Schumacher, R.J., Ross, E.D., and Torf, D.O. (1997) Hop modulates Hsp70/Hsp90 interactions in protein folding *J. Biol. Chem.* **273**: 3679-3686.

Kinoshita, K., Ura, H., Akagi, T., Usuda, M., Koide, H., and Yokoda, T. (2007) GABPα regulates Oct-3/4 expression in mouse embryonic stem cells. *Biochem. Biophys. Res. Commun.* **353**: 686-691.

Kirschstein R, and Skirboll, L.R. (2001) Stem cells: Scientific progress and future directions, *Terese Winslow* 1-106.

Konno, T., Kawazoe, N., Chen, G. and Ito, Y. (2006) Culture of mouse embryonic stem cells on photoimmobilized polymers, *J. Bioscience Biotech*. **102**: 304-310.

Lässle, M., Blatch, G.L., Kndra, V., Takatori, T. and Zetter, B.R. (1997) Stress-inducible, murine protein mSTI1. Characterization of binding domain for Heat shock proteins and *in vitro* phosphorylation by different kinases, *J. Biol. Chem.* **272**: 1876-1884.

Levy, C., Nechushtan, H., and Razin, E. (2001) A new role for the STAT3 inhibitor, PIAS3: A repressor of microphthalmia transcription factor, *J. Biol. Chem.* 1-30.

Levy, C., Lee, Y., Nechushtan, H., Schueker-Fuman, O., Sonnenblick, A., Hacohen, S. and Razin, E. (2006) Identifying a common molecular mechanism for inhibition of MITF and STAT3 by PIAS3, *Blood* **107**: 2839-2845.

Long, J., Wang, G., Matsuura, I., He, D., and Liu, F. (2004) Activator of Smad transcriptional activity by protein inhibitor of activated STAT3 (PIAS3), *PNAS* **101**: 99-104.

Longshaw, V.M. (2002) The phosphorylation and nuclear localization of the co-chaperone murine stress-inducible protein 1, Rhodes University, Grahamstown.

Longshaw, V.M., Chapple, J.P., Balda, M.S., Cheetham, M.E., and Blatch, G.L. (2004) Nuclear translocation of the Hsp70/Hsp90 organising protein mSTI1 is regulated by cell cycle kinases, *J. Cell Sci.* **117**: 701-710.

Ma, J. and Cao, X. (2006) Regulation of STAT3 nuclear import α5 and α7 via two different functional sequence elements, *Cell. Signaling* **18**: 1117-1126.

Ma, J., Zhang, T., Novotny-Diermayr, V., Tan, A.L.C., and Cao, X. (2003) A novel sequence in the coiled-coil domain of STAT3 essential for its nuclear translocation *J.Biol. Chem.* **278**: 29252-29260.

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokoda, T. (1999) STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells, *EMBO. J.* **18**: 4261-4269.

McLauglin, S.H., Smith H.W. and Jackson, S.E. (2002) Stimulation of the weak ATPase activity of human Hsp90 by a client protein, *J. Mol. Biol.* **314**: 787-798.

Mehta, T.A., Greenman, J., Ettelaie, C., Venkatasubramaniam, A., Chetter, I.C., and McCollum, P.T. (2005) Heat shock proteins in vascular disease-A review, *Eur. J. Vasc. Endovasc. Surg.* **29**: 395-402.

Metcalf, D. (1990) The induction and inhibition of differentiation in normal and leukaemic cells, *Phil. Trans. R. Soc. Lond. B.* **327**: 99-109

Minami, Y., Kimura, Y., Kawasaki, H., Susuki, K., and Ichiro, Y. (1994) The carboxyl- terminal region of mammalian Hsp90 is required for its dimerization and function *in vivo*. *Mol. Cell Biol.* **14**: 1459-1464.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) The homeoprotein Nanog is required for the maintenance of pluripotency in mouse epiblast and ES cells, *Cell* **113**: 631-642.

Murray, P.A., and Edgar, D. (2001) The regulation of embryonic stem cell differentiation by leukemia inhibitory factor (LIF), *Differentiation* **68**: 227-234.

Nardi, G, Végh, E.M, Prohárszka, Z., and Csermely, P. (2006) Chaperone-related immune dysfunction: an emergent property of distorted chaperone network, *Trends immune.* **27**: 74-79.

Niwa, H., Burdon, T., Chambers, I. and Smith, A. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3, *Genes Dev.* **12**: 2048-2060.

Niwa, H. (2001) Molecular mechanisms to maintain stem cell renewal of ES cells, *Cell Structure and function* **26**: 137-148.

Odunuga, O.O, Longshaw, V.M, Blatch, G.L. (2004) Hop: more than an Hsp70/Hsp90 adaptor protein, *Bioassayas* **26**: 1058-1068.

Pesce, M., Wang, X., Wolgemuth, D.J. and Schőler, H. (1997) Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation, *Mechanisms of Differentiation* **71**: 89-98.

Prescott, J., and Coetzee, G.A., (2006) Molecular chaperones throughout the life cycle of the androgen receptor, *Cancer Letter* **231**: 12-19.

Pratt, W.B. (1998) The Hsp90-based chaperone system: Involvement in signal transduction from a variety of hormone and growth factor receptor, *Proc. Soc. Exp. Biol. Med.* **4**: 420-434.

Pratt, W.B., Galigniana, M.D., Harrell, J. and DeFranco, D.B. (2004) Role of Hsp90 and Hsp90-binding immunophillins in signaling protein movement, *Cell signaling* **16**: 857-872.

Prodromou, C. and Pearl, L.H. (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery, *Ann. Rev. Biochem.* **75**: 271-294.

Pearl, L.H., and Prodromou, C. (2001) Structure, function and mechanism of the Hsp90 molecular chaperone, *Adv. Protein Chem.* **59**: 157-186.

Puente, L.G., Borris, D.J., Carrière, J.F., Kelly, J.F., and Megeney, L.A. (2006) Identification of candidate regulators of embryonic stem cell differentiation by comparative phosphoprotein affinity profiling, *Mol. Cellular Pro.* **5**: 57-67.

Rajasingh, J., and Bright, J.J. (2006) 15-Deoxy-$\Delta^{12,\ 14}$-Prostaglanding J_2 regulates leukemia inhibitory factor signaling through Jak-STAT pathway in mouse embryonic stem cells, *Exp. Cell. Res.* **312**: 2538-2546.

Raz, R., Lee, C.K., Cannizzaro, L.A., D`Eustachio, P. and Levy D.E. (1999) Essential role of STAT3 for embryonic stem cell pluripotency, *Proc. Natl. Acad. Sci.* **96**: 2846-2851.

Sato, N., Tsuruma, R., Imoto, S., Sekine, Y., Muramoto, R., Sugiyama, K., and Matsuda, T. (2005) Nuclear retention of STAT3 through the coiled-coil domain regulates its activity, *Biochem. Biophys. Res. Commun.* **336**: 617-624.

Sato, N, Yamamoto, T, Sekine, Y, Yumioka, T, Junicho, A, Fuse, H, and Matsuda, T. (2003) Involvement of heat-shock protein 90 in the interleikin-6-mediated signaling pathway through STAT3, *Biochem. Biophys. Res. Commun.* **300**: 847-852.

Schaefer, T.S., Sanders, L.K., Park, O.K., and Nathans, D. (1997) Functional differences between STAT3α and STAT3β, *Mol. Cell. Biol.* **17**: 5307-5316.

Scheufker, C., Briner, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U. and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine, *Cell* **101**:190-210.

Schulte, T.W., Blagoklonny, M.V., Ingui, C., and Neckers, L. (1995) Disruption of Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association, *J. Biol. Chem.* **270**: 24585-24588.

Shi, W., Inoue, M., Minami, M., Takeda, K., Matsumoto, M., Matsuda, Y., Kishimoto, T., and Akira, S. (1996) The genomic structure and chromosomal localization of the mouse STAT3 gene, *Int. Immun.* **8**: 1205-1211.

Smith, A.G. (2001) Embryo-derived stem cells of mice and men, *Anna Rev. Cell. Dev. Biol.* **17**: 435-462.

Song, Y., and Masison, D.C. (2005) Independent regulation of Hsp70 and Hsp90 chaperones by Hsp70/Hsp90-organizing protein Sti1 (Hop), *J. Biol. Chem.* **280**: 34178-34185

Song, H., Ethier, S.P., Dziubinski, M.L., and Lin, J. (2004) STAT3 modulates heat shock 27kDa protein expression in breast epithelial cells, *Biochem. Biophys. Res. Commun.* **314**: 143-150.

Sonnenblick, A., Levy, C., and Razin, E. (2004) Interplay between MITF, PIAS3, and STAT3 in mast cells and melanocytes, *Mol. Cell. Biol.* **24**: 10584-10592**.**

Sőti, C, Pal, C., Papp, B., and Csermely P. (2005) Molecular chaperones as regulatory elements of cellular networks, *Curr. Opin. cell Biol.* **17**: 210-215.

Spagnoli, F.M. and Hemmati-Brivanlou, A. (2006) Guiding embryonic stem cells towards differentiation: lessons from molecular embryology, *Cur. Opin. Gen. Dev.* **16**: 469-475.

Stahl, J., Wobus, A.M., Ihrig, S., Lutsch, G., and Bielka, H. (1992) The small heat shock protein Hsp25 is accumulated in P19 embryonal carcinoma cells and embryonic stem cells of line BLC6 during differentiation, *Differentiation* **61**: 33-37.

Sun, J., Blaskovich, M.A., Jove, R., Livingstone, S.K., Coppola, D., and Sebti, S.M. (2005) Cucurbitacin Q: a selective STAT3 activation inhibitor with potent antitumor activity, *Oncogene* **24**: 3236-3245.

Tai Y. and Svendsen, O.N. (2004) Stem cells as potential treatment for neurological disorders, *Curr. Opin. Pharmacol.* **4**: 98-104.

Tielens, S., Verhasselt, B., Liu, J., Dhont, M., Van Der Elst, J. and Cornelissen, M. (2006) Generation of embryonic stem cell line from mouse blastocysts developed *in vivo and in vivro* : relation to Oct-4 expression, *Reproduction* **132**: 59-66.

Travers, S.A.A., and Fares, M.A. (2007) Functional coevolutionary network of Hsp70-Hop-Hsp90 system revealed through computational analyses Oxford University Press page 1-39.

Turkson, J. and Jove, R. (2000) STAT proteins: Novel molecular targets for cancer drug discovery, *Oncogene* **19**: 6613-6626.

Wegele, H., Wandinder S.K., Schimid, A.B., Reinstein. J. and Buchner J. (2006) Substrate transfer from chaperone Hsp70 to Hsp90, *J. Mol. Biol.* **356**: 802-811.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A. and Gough, N.M (1998) Myeloid leukaemia inhibitory factor maintains the development of embryonic stem cells, *Nature* **336**: 684-687.

Wobus, A.M., and Boheler, K.R. (2005) Embryonic stem cells: Prospects for developmental biology and cell therapy, *Physiol. Rev.* **85**: 635-678.

Yamamoto, T., Sato, N., Sekine, Y., Yumioka, T., Imoto, S., Junicho, A., Fuse, H., and Matsuda, T. (2003) Molecular interaction between STAT3 and protein inhibitor of activated STAT3, and androgen receptor, *Biochem. Biophys. Res. Commun.* **306**: 610-615.

Yin, X., Mayr, M., Xao, Q., Wang, W. and Xu, Q. (2006) Proteomic analysis reveals higher demand for antioxidant protection in embryonic stem cell-derived smooth muscle cells, *Proteomics* **6**: 6437-6446.

Younes, A., and Georgakis, G.V. (2005) Heat shock protein 90 inhibitors in cancer therapy: 17AAG and beyond, *Future Oncology* **1**: 273-281.

Young, J.C., Moarefi, I, and Hartl, F.U. (2001) Hsp90: a specialized but essential protein-folding tool, *J. Cell. Biol.* **154**: 267-273.

Zhang, T, Kee,W.H, Seow, K.T, Fung, W and Cao, X. (2000) The coiled-coil domain of STAT3 is essential for its SH2 domain-mediated receptor binding and subsequent activation induced by epidermal growth factor and interleukin-6, *Mol. Cell. Biol.* **20**: 7132-7139.

Zhiyuan, Y. and Kone, B.C. (2004) The STAT3 DNA-binding domain mediates interaction with NF-κB p65 and inducible Nitric oxide synthase transrepression in mesangial cell *J. Am. Soc. Nephrol.* **15**: 585-591.

APPENDIX

A1 MEDIA AND SOLUTION PREPARATION

1. PREPARATION OF FIXING SOLUTION

4.0 % paraformaldehyde

2. PREPARATION OF COATING SOLUTION

0.1 % Gelatin

3. PREPARATION OF BLOCKING SOLUTION

10.0 % (V/V) GOAT SERUM AND 0.1 % (V/V) TRITON-X100

10.0 % Goat serum and 0.1 % Triton X-100

4. 10 X PHOSPHATE BUFFERED SALINE (PBS); PH 7.4

To make a 1X working solution, dilute the stock 10X and then autoclave

Reagent	**Grams**	**Concentration**
NaCl	80	1.37 M
KCl	2.0	0.03 M
Na_2HPO_4	11.5	0.16 M
KH_2PO_4	2.0	0.02 M
dH_2O	Add up to a liter	

5. LYSIS BUFFER (HYPOTONIC HEPES BUFFER) One liter

Reagent	**Formula weight**	**Grams**	**Concentration**
HEPES	238.30	8.80	0.037M
Sucrose	342.30	17.11	0.05M
KF	58.10	5.81	0.10M

Adjust pH to 7.5 with HCl or NaOH

Sodium Cholate	415.56	6.00	0.60 % (w/v)
Protease inhibitor cocktail	Sigma Cat no. P8340		

Protease inhibitor cocktail added according to the vendor's instruction (1.0 % v/v)

A2 SDS-PAGE LOADING AND SDS-PAGE SAMPLE TREATMENT BUFFER PREPARATIONS

1: SDS-PAGE loading buffer

	Ingredient	volume
1.	Deionised water	3.55 ml
2.	0.5 M Tris-HCl, pH 6.8	1.25 ml
3.	Glycerol	2.5 ml
4.	10.0 % (w/v) SDS	2.0 ml
5.	0.5 % (w/v) bromophenol blue	0.2 ml
6.	Total volume	9.5 ml

2: SDS-PAGE sample treatment buffer (to be made prior to use)

1.	β-mercapto ethanol	50.0 µl
2.	SDS-PAGE loading buffer	950.0 µl

Dilute the sample 1:2 with SDS-PAGE sample treatment buffer

A3 SDS-PAGE GEL PREPARATION

12.0 PERCENT RESOLVING GEL

Reagent	Volume
1. Distilled water	3.35 ml
2. 1.5 M: Tris-HCl (pH 8.8)	2.5 ml
3. 10.0 % SDS	100.0 µl
4. Bis-Acrylamide	4.0 ml
5. 10.0 % Ammonium persulphate	100.0 µl
6. TEMED	20.0 µl

4.0 STACKING GEL

Reagent	Volume
1. Distilled water	3.05 ml
2. 0.5 M: Tris-HCl (pH 6.8)	1.25 ml
3. 10.0 % SDS	50.0 µl
4. Bis-Acrylamide	665.0 µl
5. 10.0 % Ammonium persulphate	100.0 µl
6. TEMED	20.0 µl

A4 PREPARATION OF 10.0 X SDS RUNNING BUFFER

10.0 X SDS running buffer

1	Glysine	144.0 g
2	SDS	10.0 g
3	Tris-base	30.3 g

Dissolve and bring total volume to 1.0 L with deionised water. For use, 50.0 ml of 10.0 X SDS running buffer was diluted with 450.0 ml deionised water.

A5 REAGENTS, CHEMICALS AND SOURCES

Reagents	**Vendor**
Cell culture freezing medium	Invitrogen
DPBS (with or without Ca^{2+} and Mg^{2+})	Invitrogen
L-glutamine	Invitrogen
10X Trypsin-EDTA	Sigma
Bradford reagent	Sigma Aldrich
Sodium Pyruvate	Invitrogen
B-Mercapto ethanol	Gibco®
FBS ES-Grade	PAA Laboratorios
Non-essential amino acids	Invitrogen
Advanced DMEM	Invitrogen
Mytomycin-C	Sigma
LIF	Chemicon ESGROW
Paraformaldehyde	Sigma
Triton X-100	Sigma
DAPI	Invitrogen
Gelatin from Porcine skin, Type A	Sigma
Trizma base	Sigma
Bovine, chicken and goat serum for immune-blocking	Sigma
Naphthol AS-MX Phosphate	Sigma
Protease inhibitor cocktail	Sigma
Fast Red TR Salt (Hemi zinc chloride salt)	Sigma
HCl	AnalaR®
Glycerol	AnalaR®
NaCl	ICH Biomedicals
KF	Fluka
Sodium deoxy cholate	Sigma-Aldrich
HEPES	BDH-Biochemicals
Bromophenol blue	Sigma

Sodium dodecyl sulphate	Sigma
SDS-PAGE sample treatment buffer	Invitrogen
SDS-PAGE loading buffer	Invitrogen
NuPAGE® MOPS SDS Running Buffer	Invitrogen
NuPAGE® Transfer Buffer	Invitrogen
Kodak GBX Developer and Replenisher	Sigma-Aldrich
Kodak BioMax Light Film	Sigma-Aldrich
NuPAGE 4-12 % Bis-Tris Gel (1.0mm x 10 well)	Invitrogen
Nitrocellulose membrane filter sandwich (0.46 µm pore size)	Invitrogen

A6 MOUSE EMBRYONIC STEM CELL MEDIA COMPOSITION

Reagent	Stock concentration	Media concentration
ES-grade FBS	100%	2.0 %
L-Glutamine	2.0 mM	0.02 mM
2-mercaptoethanol	5.0 µM	0.001 µM
LIF	10^7 U/ml	1000 U/ml
Advanced DMEM reduced serum medium	-	

A7 STO MEDIA COMPOSITION

Reagent	Stock concentration	Media concentration
ES-grade FBS	100%	10.0 %
L-Glutamine	2.0 mM	0.02 mM
Sodium pyruvate	1.0 mM	0.01 mM
Non Essential amino acids	100X	1.0 %
2-mercaptoethanol	50 mM	0.001 mM
DMEM medium	-	-

A8 PRIMARY ANTIBODIES FOR WESTERN BLOTTING, IMMUNOPRECIPITATION AND IMMUNOHISTOCHEMISTRY

Antibodies	**Vendor**
Mouse monoclonal anti-human STAT3 antibody: sc-8019 (200µg/ml)	Santa Cruz Biotechnology
Mouse monoclonal anti-human pYSTAT3: sc-8059 (200µg/ml)	Santa Cruz Biotechnology
Mouse monoclonal ant-human Hsp90 β from Dr. Toft, D.O	(Department of Biochemistry and Molecular Biology, Mayo Clinic, USA)
Mouse monoclonal anti- human Hsp90β antibody 37-9400 (0.1mg/ml)	Zymed® Laboratories
Mouse monoclonal anti-chicken Hop antibody (P60) (1.0 mg/ml)	Stressgen
Rabbit polyclonal anti-mouse β-actin	Cell Signaling
Rabbit polyclonal anti- human STAT3 antibody : sc-482 (200µm/ml)	Santa Cruz Biotechnology
Mouse monoclonal anti-human Oct-3/4 antibody: sc-5279 (200µg/ml)	Santa Cruz Biotechnology
Rabbit polyclonal anti-human Nanog antibody (0.2mg/ml)	AbCam

A9 SECONDARY ANTIBODIES FOR WESTERN BLOTTING AND IMMUNOHISTOCHEMISTRY

Antibodies	**Vendor**
Goat anti- rabbit IgG-HRP (sc-2004) 200µg/0.5 ml	Santa Cruz Biotechnology
Goat anti-mouse IgG_1-HRP (sc-2060) 200µg/0.5 ml	Santa Cruz Biotechnology
Goat anti-mouse IgG_{2b} Alexa fluor-594	Invitrogen
Chicken anti-rabbit Alexa fluor-488	Invitrogen

A10 INSTRUMENTS AND SOURCES

Instruments	**Vendor**
3.5 cm and 6.0 cm culture dishes	Nunc
Microscope slides	VWR International
Cover slips	VWR International
Fluorescent mounting medium	Dako Cytomation
Leica DMIL fluorescent microscope	Leica
Leica TCS-SP2 confocal microscope	Leica
Nikon DIPHOT	Nikon
MSE micro centaur centrifuge	-
8000r centrifuge	Senturion Scientific LTD
E 132 and E321 electrophoresis power pack	Consort