

**Establishment of human OCT4 as a putative HSP90 client protein:
A case for HSP90 chaperoning pluripotency**

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By

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Abstract

The therapeutic potential of stem cells is already being harnessed in clinical trials. Of even greater therapeutic potential has been the discovery of mechanisms to reprogram differentiated cells into a pluripotent stem cell-like state known as induced pluripotent stem cells (iPSCs). Stem cell nature is governed and maintained by a hierarchy of transcription factors, the apex of which is OCT4. Although much research has elucidated the transcriptional regulation of OCT4, OCT4 regulated gene expression profiles and OCT4 transcriptional activation mechanisms in both stem cell biology and cellular reprogramming to iPSCs, the fundamental biochemistry surrounding the OCT4 transcription factor remains largely unknown. In order to analyze the biochemical relationship between HSP90 and human OCT4 we developed an exogenous active human OCT4 expression model with human OCT4 under transcriptional control of a constitutive promoter. We identified the direct interaction between HSP90 and human OCT4 despite the fact that the proteins predominantly display differential subcellular localizations. We show that HSP90 inhibition resulted in degradation of human OCT4 via the ubiquitin proteasome degradation pathway. As human OCT4 and HSP90 did not interact in the nucleus, we suggest that HSP90 functions in the cytoplasmic stabilization of human OCT4. Our analysis suggests HSP90 inhibition inhibits the transcriptional activity of human OCT4 dimers without affecting monomeric OCT4 activity. Additionally our data suggests that the HSP90 and human OCT4 complex is modulated by phosphorylation events either promoting or abrogating the interaction between HSP90 and human OCT4. Our data suggest that human OCT4 displays the characteristics describing HSP90 client proteins, therefore we identify human OCT4 as a putative HSP90 client protein. The regulation of the transcription factor OCT4 by HSP90 provides fundamental insights into the complex biochemistry of stem cell biology. This may also be suggestive that HSP90 not only regulates stem cell biology by maintaining routine cellular homeostasis but additionally through the direct regulation of pluripotency factors.

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy of Rhodes University. It has not been submitted previously for any degree or examination at any other university.

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Table of Contents:

Abstract.....	ii
Declaration	iii
Table of Contents:.....	iv
List of Figures:	vii
List of Tables:	ix
List of Abbreviations	x
List of Symbols.....	xiv
Acknowledgements	xv
Chapter 1. Review of Literature	1
1.1. Heat Shock Protein 90 (HSP90)	2
1.1.1. Molecular chaperones.....	2
1.1.2. Structure and Cytosolic Function of HSP90	2
1.1.3. HSP90 co-chaperones	6
1.1.4. Phosphorylation of HSP90.....	7
1.1.5. HSP90 functioning in organelles.....	9
1.1.5.1. Endoplasmic Reticulum and Mitochondrial HSP90 isoforms..	9
1.1.5.2. Cytosolic HSP90 in the nucleus	10
1.1.6. HSP90 and multi-protein complexes.....	12
1.1.7. HSP90 as a drug target	12
1.2. Octamer-binding transcription factor 4 (OCT4).....	14
1.2.1. POU transcription factor family and structure of POU domain	14
1.2.2. Structure and function of OCT4.....	15
1.2.3. OCT4: post-translational modifications	19
1.3. Stem Cells.....	20
1.3.1. Stem cell-related signaling pathways and HSP90	21
1.4. Motivation for study.....	27
1.5. Research Question	28
1.6. Aims.....	28
Chapter 2. Methodology.....	29
2.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	30
2.2. Chemiluminescence-based Western blot analysis.....	30
2.3. Restriction endonuclease verification of lentiviral-based gene delivery system.....	31
2.4. Routine mammalian cell culture.....	32
2.5. Transfection and generation of lentiviral-transgene infectious particles.	32
2.6. Transduction and Generation of polyclonal HEK-hOCT4.	32
2.7. Co-immunoprecipitation of human OCT4 and HSP90 α/β	33
2.8. Effect of HSP90 inhibition on human OCT4 and known HSP90 client proteins in HEK-hOCT4.....	33
2.9. Effect of proteasome inhibition and co-inhibition of proteasome and HSP90 on human OCT4 protein levels in HEK-hOCT4 cells.....	34

2.10. Effect of specific kinase inhibition on human OCT4 and HSP90 α/β in HEK-hOCT4 cells.....	34
2.10.1. Human OCT4 co-immunoprecipitation with kinase inhibition....	34
2.10.2. Analysis of kinase inhibition on human OCT4 protein levels.....	35
2.10.3. ERK2i inhibitor kinase time course analysis on human OCT4 protein levels.....	35
2.10.4. Effect of MEK1/2 inhibition on human OCT4 protein levels.....	35
2.11. Immunofluorescence and Confocal microscopy.....	36
2.12. Subcellular fractionation of HEK-hOCT4 cells using NE-PER extraction kit.....	36
2.13. Subcellular fractionation of HEK-hOCT4 cells treated with HSP90 inhibitor GA using REAP fractionation.....	36
2.14. Effect of HSP90 inhibition on human OCT4 transactivation using OCT responsive luciferase reporter assay.....	37
2.15. Flow cytometry analysis of pluripotent stem cell transcription factors.....	38
2.16. Feeder layer-dependent culture and maintenance of induced Pluripotent Stem Cells (iPSCs).....	38
2.17. Feeder layer-free culture and maintenance of induced Pluripotent Stem Cells (iPSCs).....	39
Chapter 3. Generation of a constitutive human OCT4 expressing cell line.....	40
3.1. Introduction.....	41
3.2. Results.....	42
3.2.1. Verification of lentiviral gene delivery vectors.....	42
3.2.2. Generation of infectious lentiviral particles.....	46
3.2.3. Generation of the HEK-hOCT4 stable cell line.....	50
3.2.4. Analysis of exogenous human OCT4 expression and activity in the HEK-hOCT4 cell line.....	51
3.3. Conclusion.....	56
Chapter 4. Biochemical analysis of the relationship between HSP90 and human OCT4.....	57
4.1. Introduction.....	58
4.2. Results.....	58
4.2.1. Analysis of conservation of OCT4 across mammalian species.....	58
4.2.2. Comparison of the subcellular localization of human OCT4 and HSP90.....	61
4.2.3. Human OCT4 interacts with HSP90 α/β	64
4.2.4. Effect of HSP90 inhibition on human OCT4 protein levels in HEK-hOCT4 cells.....	66
4.2.5. Effect of proteosomal inhibition with HSP90 inhibition on human OCT4 protein.....	70
4.2.6. Effect of HSP90 inhibition on human OCT4 subcellular localization in HEK-hOCT4 cells.....	76
4.2.7. Analysis of the human OCT4 and HSP90 α/β interaction.....	82
4.2.8. Effect of HSP90 inhibition using GA on human OCT4 activity in HEK-hOCT4 cells.....	84
4.2.9. Phosphorylation inhibitors and interaction between human OCT4 and HSP90 α/β	88
Chapter 5. Discussion and Conclusion.....	94

5.1. Discussion.....	95
5.1.1. Alternative model to address the biochemical relationship between human OCT4 and human HSP90.....	97
5.1.2. Human OCT4 is an HSP90 client	101
5.1.2.1. Human OCT4 directly interacts with HSP90 α/β	101
5.1.2.2. Inhibition of HSP90 causes loss of OCT4 protein levels	105
5.1.2.3. Inhibition of HSP90 reduces human OCT4 activity	109
5.1.3. Human OCT4 and HSP90 interaction is affected by phosphorylation.....	111
5.1.4. Evidence supporting an additional indirect relationship between human OCT4 and HSP90.....	113
5.1.5. Functional relevance of the human OCT4 and HSP90 complex..	114
5.2. Concluding remarks and Future Prospects.....	117
Chapter 6. References.....	119
Chapter 7. Appendix: Development of FRET vectors to study HSP90-OCT4 interactions.	158
7.1. Methodology.....	158
7.2. Generation of pECFP-C1-HSP90AB1 and pEYFP-C1-hOCT4 fluorescent fusion protein vectors and FRET control vector pEYFP-C1-ECFP.....	160

List of Figures:

Figure 1 HSP90 domain structure and phosphorylation sites with known catalyzing kinases.....	5
Figure 2 OCT4A domain structure.	17
Figure 3 Conservation and species-specific differences in signaling pathways controlling self-renewal, pluripotency and lineage-specific differentiation.	22
Figure 4 HSP90 functioning in stem cell-related signaling pathways.	23
Figure 5 Verification of lentiviral envelope vector and lentiviral packaging vector for third generation lentiviral gene delivery by restriction endonuclease digestion.....	44
Figure 6 Verification of lentiviral transgene vectors for third generation lentiviral gene delivery by restriction endonuclease digestion.	45
Figure 7 Validation of lentiviral particle production procedure using pLV-TH control lentiviral transgene vector.	48
Figure 8 Cell line screening for endogenous pluripotent stem cell transcription factor expression.	49
Figure 9 Generation of HEK293FT cell line constitutively expressing OCT4 (HEK-hOCT4).	52
Figure 10 Linear representations of luciferase vectors used for analyzing effect of HSP90 inhibition on human OCT4 activity in HEK-hOCT4 cells.....	54
Figure 11 Transcriptional activity of exogenous human OCT4 in HEK-hOCT4 cells.	55
Figure 12 OCT4 multiple protein sequence alignment showing high degree of sequence conservation across species.	59
Figure 13 Subcellular localization of human OCT4 and HSP90 α/β in HEK-hOCT4 cells.....	63
Figure 14 Human OCT4 interacts with HSP90 α/β	65
Figure 15 Effects of HSP90 inhibition on human OCT4 protein levels in HEK-hOCT4 cells.....	68
Figure 16 Comparison of effect of HSP90 inhibition on known HSP90 client protein CDK4 and human OCT4 in HEK-hOCT4 cells.	69
Figure 17 Effects of HSP90 inhibition on human OCT4 protein levels with proteasome inhibition using MG132 in HEK-hOCT4 cells.....	72
Figure 18 Increasing concentrations of MG132 block GA induced loss of human OCT4 in HEK-hOCT4 cells.	75
Figure 19 Subcellular localization of human OCT4 with HSP90 inhibition in HEK-hOCT4 cells.....	77
Figure 20 HSP90 inhibition effect on human OCT4 and HSP90 colocalization using confocal microscopy.	80
Figure 21 Effect of GA on the interaction between human OCT4 and HSP90 α/β	85

Figure 22 Effect of HSP90 inhibition on human OCT4 activity in HEK-hOCT4 cells.	87
Figure 23 Comparison of phosphorylation conservation between human and murine OCT4.	89
Figure 24 Effect of kinase inhibition on the interaction between human OCT4 and HSP90 α/β	91
Figure 25 Effect of kinase inhibitors on human OCT4 protein levels in HEK-hOCT4 cells.	93
Figure 26 Summary model for the relationship between human OCT4 and HSP90.	96
Figure 27 Optimization of human iPSC culture for HSP90-OCT4 analysis in context of pluripotency and differentiation.	100
Figure 28 Proportional analysis of kinase, ligase and transcription factor HSP90 client proteins.	103
Figure A1 Generation of pEYFP-C1-hOCT4 vector.	165
Figure A.2 Generation of pECFP-C1-HSP90AB1 vector.	166

List of Tables:

Table 1 Comparison of OCT4 protein and DNA sequence identity from multiple mammalian species to human (<i>Homo sapiens</i>) OCT4.....	60
Table 2 Effect of HSP90 inhibition on human OCT4 and HSP90 α/β colocalization.	81

List of Abbreviations

17AAG	17-N-allylamino-17-demethoxygeldanamycin
6W	Octamer monomer binding motif (x6)
ADP	Adenosine diphosphate
AHA1	Activator of 90 kilodalton heat shock protein ATPase homolog 1
AKT	Protein kinase B
ATP	Adenosine triphosphate
BMP	Bone morphogenic proteins
CDK4	Cyclin dependent kinase 4
CK1	Casein kinase 1
CK2	Casein kinase 2
Co-IP	Co-immunoprecipitation
c-Src	Proto-oncogene tyrosine-protein kinase Src
D4476	4-(4-(2,3-dihydrobenzol[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNA-PK	DNA-dependent protein kinase
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK1	Extracellular signal-regulated kinase 1
ERK2	Extracellular signal-regulated kinase 2
ERK2i	3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride
ESC	Embryonic stem cell
Esrrb	Estrogen-related receptor β

FGF	Fibroblast growth factor
GA	Geldanamycin
Grp94	Glucose-related protein 94
GSK3 β	Glycogen synthase kinase 3 β
H7	1-(5isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride
HEK	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
HOP	HSP70-HSP90 organizing protein
HRP	Horse radish peroxidase
HSP	Heat shock protein
HSP40	Heat shock protein 40 kilodaltons
HSP70	Heat shock protein 70 kilodaltons
HSP90	Heat shock protein 90 kilodaltons
HSP90 α	Heat shock protein 90 α isoform
HSP90 α/β	Heat shock protein 90 α and β isoforms
HSP90 β	Heat shock protein 90 β
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
JAK	Janus kinase
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MEK1	Mitogen-activated protein kinase kinase 1
MG132	Carbobenzoxymethyl-Leu-Leu-leucine proteasome inhibitor
MORE	More palindromic Oct factor recognition element
mRNA	Messenger ribonucleic acids
mTOR	Mammalian target of rapamycin
MYC	c-MYC proto-oncogene protein

MyoD1	Myogenic differentiation 1
NES	Nuclear export signal
NE-PER	Nuclear and cytoplasmic extraction
NLS	Nuclear localization signal
NOV	Novobiocin
OCT3	Octamer-binding transcription factor 3 (POU5F1)
OCT4	Octamer-binding transcription factor 4 (POU5F1)
OCT4B	Octamer-binding transcription factor 4 isoform B
OCT6	Octamer-binding transcription factor 6 (POU3F1)
p23	Prostaglandin E synthase 3 (cytosolic)
p53	Transformation-related protein 53 (TRP53)
PBS	Phosphate-buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin chlorophyll-Cyanine5.5
PI3K	Phosphatidylinositol 3-kinase
PIP ₂	Phosphatidylinositol 4,5-biphosphate
PIP ₃	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PORE	Palindromic Oct factor recognition element
POU	Pit-1, Oct-1, Unc
POU _H	POU-homeo
POU _S	POU-specific
REAP	Rapid, efficient and practical
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

siRNA	Small interfering ribonucleic acids
Sox2	SRY (sex determining region Y)-box 2
Sp1	Specificity protein 1
Smad	Small body size/Mothers against decapentaplegic homolog
snoRNP	Small nucleolar RNAs
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator 5
Swe1 ^{Wee1}	<i>Saccharomyces cerevisiae</i> homolog of Wee1 serine/threonine nuclear kinase
TBS	Tris-buffered saline
TBST	Tris-buffered saline/Tween-20
TGF β	Transforming growth factor β
TGF β R2	Transforming growth factor β receptor 2
TPR	Tetratricopeptide repeat
TRAP1	TNF receptor-associated protein 1
Wnt	Wingless/integrated-1
WWP1	WW domain containing protein 1
WWP2	WW domain containing protein 2

List of Symbols

α	Alpha
β	Beta
$^{\circ}\text{C}$	Degree Celsius
g	Grams
kDa	Kilodaltons
L	Litres
μg	Micrograms
μl	Microlitres
μM	Micromolar
mg	Milligrams
ml	Millilitre
mM	Millimolar
M	Molar
nM	Nanomolar
%	Percent or g/100 ml
v/v	Volume/volume
w/v	Weight/volume

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Chapter 1. Review of Literature

1.1. Heat Shock Protein 90 (HSP90)

1.1.1. Molecular chaperones

Molecular chaperones are proteins that directly interact with, stabilize or assist another protein to acquire its active functional conformation (Hartl, 1996; Hartl and Hayer-Hartl, 2009). Functions of molecular chaperones include *de novo* protein folding; protein aggregation suppression and re-folding; protein trafficking; assistance in proteolytic degradation, and oligomeric protein assembly (Lindquist and Craig, 1988; Becker and Craig, 1994). Heat shock proteins (HSPs) form a major family of molecular chaperones, initially discovered due to up-regulation under heat shock stress conditions hence the name 'heat shock' (Tissieres *et al*, 1974). HSPs are classified according to molecular weight (e.g. HSP90 referring to HSPs of approximately 90 kilodalton), although a revised nomenclature has been proposed in which classification is based on the presence of conserved domain structures (Kampinga *et al*, 2009). Although some HSPs are up-regulated during stress conditions that increase the propensity for protein aggregation, many HSPs are constitutively expressed and therefore play vital roles in protein homeostasis under both physiological and stress conditions (Hartl *et al*; 2011).

1.1.2. Structure and Cytosolic Function of HSP90

Heat shock protein 90 (HSP90; HSPs of approximately 90 kilodaltons) represents the most abundant cellular protein, comprising between 2 to 3% of total cellular proteins (Mahalingham *et al*, 2009). There are four HSP90 isoforms, namely the cytosolic isoforms HSP90 α and HSP90 β ; mitochondrial isoform TRAP1; and the endoplasmic reticulum isoform GRP94 (Sreedhar *et al*, 2004). The two highly homologous cytosolic isoforms are encoded by two different genes, with HSP90 β showing constitutive and ubiquitous expression levels across all tissue types, while HSP90 α is heat-inducible and displays tissue-specific expression patterns (Sreedhar *et al*, 2004). Additionally HSP90 α and HSP90 β knockout mice display differential phenotypes. HSP90 β knockout in mice is embryonic lethal (Voss *et al*, 2000), while HSP90 α knockout has no abnormal effects on development in mice however these mice are sterile due to meiotic arresting of spermatogenesis (Grad *et al*, 2010). The functions

of this highly ubiquitous molecular chaperone include the regulation of protein conformation stability, stability of protein-protein quaternary structures and protein trafficking (Trepel *et al*, 2010; Hartl *et al*, 2011). Numerous reports have additionally implicated HSP90 as being required for the degradation of some proteins through the proteasome degradation system (Immamura *et al*, 1998; Goasduff and Cederbaum, 2000; Fuller and Cuthbert, 2000; Gusarova *et al*, 2001; Theodoraki and Caplan, 2012). The main difference between HSP90 and other molecular chaperones is the nature of its target substrates. HSP90 does not interact with nascent or unfolded polypeptides, but rather interacts with partially or fully folded protein substrates (Hartl *et al*, 2011; Rohl *et al*, 2013). Protein substrates that are processed by HSP90 are termed ‘client proteins’. Heat shock protein 90 is currently understood to have approximately 300 client proteins (a comprehensive and regularly updated list can be found at www.picard.ch/downloads). Many of these clients are key proteins in a vast array of essential cellular processes and as a result HSP90 is a master regulator of protein homeostasis under both physiological and stress conditions.

HSP90 functions as a homodimer and is comprised of three domains, namely the N-terminal ATP binding domain (N-domain), a flexible middle domain (M-domain) and the C-terminal dimerization (C-domain) (Figure 1; Shiau *et al*, 2006). HSP90 is constitutively dimerized via the C-domain and functions as a homodimer in an ATP hydrolysis-dependent dynamic mechanistic cycle resulting in conformational changes to the homodimer configuration (Panaretou *et al*, 1998; Huai *et al*, 2005; Shiau *et al*, 2006; Ali *et al*, 2006; Richter *et al*, 2008). At the beginning of the cycle, in the absence of ATP, the HSP90 dimer assumes an ‘open’ conformation. The binding of ATP to the N-domain results in the N-terminal lid segment folding over the bound nucleotide and subsequent association between the two N-domains. The M-domain contracts resulting in a twisted conformation of the N-domain, creating a configuration termed the ‘closed’ conformation. Upon ATP hydrolysis, HSP90 reverts back to the ‘open’ conformation with the release of ADP and an inorganic phosphate (Li *et al*, 2012). The elucidation of HSP90 functioning is complicated owing to the mechanistic diversity that HSP90 exhibits in facilitating client activation or maturation. For example the obligate HSP90 client CDK4 requires cyclin D binding for complete activation of CDK4 and release from HSP90 (Stancato *et al*, 1993; Prince and Matts 2004). In contrast, AKT when bound to HSP90 is fully activated and

thus potentially phosphorylated (Sato *et al*, 2000). Therefore HSP90 is likely to elicit client-specific mechanisms for activation or maturation of client proteins.

The protein stabilization, activation and maturation function of HSP90 additionally implicates HSP90 in cancer development. HSP90 facilitates the stabilization of proteins otherwise destabilized through mutations (Blagosklonny *et al*, 1996; Whitesell *et al*, 1998; Whitesell and Lindquist, 2005; Muller *et al*, 2008; Johnson *et al*, 2013). This effect may elicit a dual propensity for cancer development. Firstly, by acting as a phenotypic ‘buffer’ HSP90 may allow for the accumulation of mutations while maintaining a normal wild-type phenotype, until such time as the extent of mutational accumulation exceeds the ‘buffering’ capacity of HSP90 (Whitesell and Lindquist, 2005). Secondly, a more obvious and characterized mode of HSP90-mediated cancer development is through the stabilization of mutant forms of proteins, thereby allowing for the manifestation of these mutations in cellular processes. One of many examples of HSP90 functioning in cancer development is through the HSP90-dependent stabilization of the mutated p53 tumour suppressor (Blagosklonny *et al*, 1996). While wild-type p53 displays a transient interaction with HSP90 (Muller *et al*, 2004), mutant p53 is highly dependent on HSP90 for activity stabilization and to prevent proteosomal degradation (Blagosklonny *et al*, 1996; Whitesell *et al*, 1998).

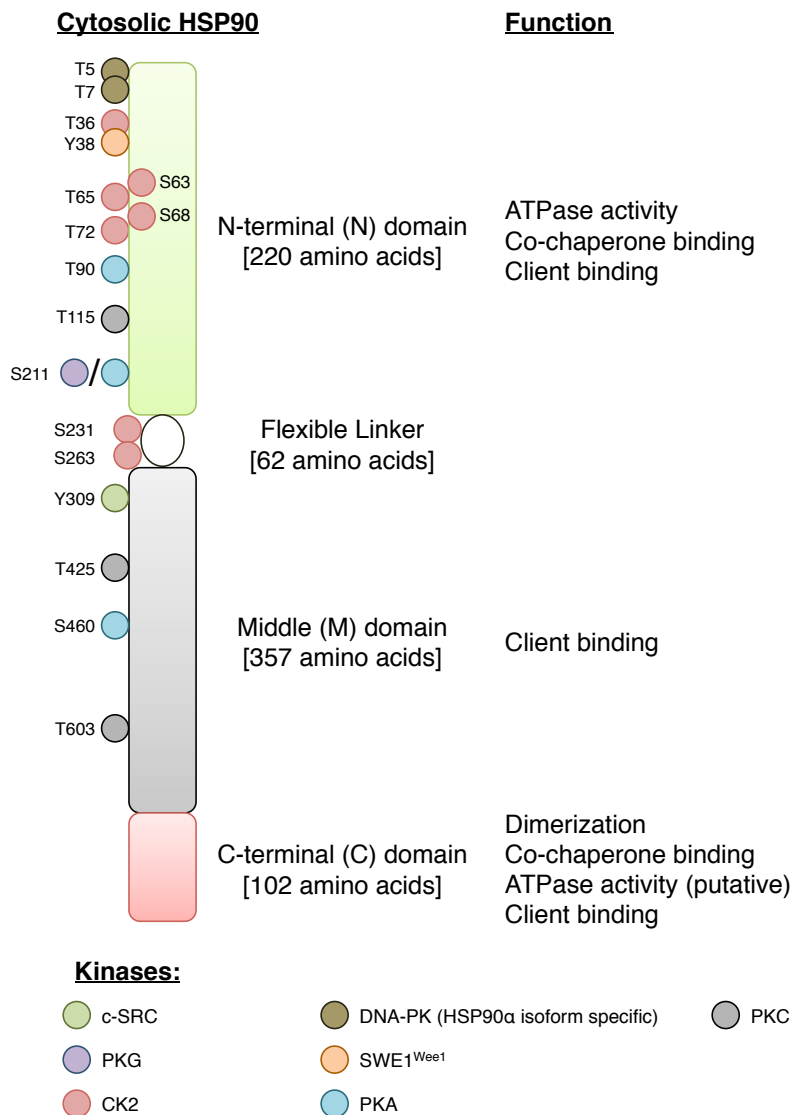


Figure 1 | HSP90 domain structure and phosphorylation sites with known catalyzing kinases. HSP90 consists of an N-terminal domain (N-domain; 220 amino acids), Middle domain (M-domain; 357 amino acids) and C-terminal domain (C-domain; 102 amino acids). Located between the N domain and M domain is a flexible linker (62 amino acids) allowing for conformational changes required for the HSP90 cycle. The N-domain is responsible for ATP hydrolysis and both co-chaperone and client binding. The middle domain is the predominant client binding domain although the N- and C-domains are involved in client binding. The C-domain is responsible for HSP90 dimerization and is involved in co-chaperone binding. Additionally studies suggest that the C-domain displays putative ATPase activity. Geldanamycin (GA) inhibits HSP90 activity through interaction with the N-domain while novobiocin (NOVO) inhibits HSP90 activity by interacting with the C-domain of HSP90. Illustrated are current characterized phosphorylation sites identified on HSP90 with respective kinases responsible for catalyzing these phosphorylations (modified from: Mollapour and Neckers, 2012). Numerous additional phosphorylated residues have been identified however the kinases responsible for phosphorylation remain unknown.

1.1.3. HSP90 co-chaperones

The ATP hydrolysis-driven dynamic HSP90 cycle is modulated and regulated by numerous HSP90 accessory proteins termed ‘co-chaperones’. These HSP90 co-chaperones interact with the HSP90 complex in a sequential, and in some cases client-specific, manner during the HSP90 complex cycle (Ratzke *et al*, 2012). Typically HSP90 co-chaperones modulate the HSP90 cycle through either linkage of the HSP70 and HSP90 cycle (e.g. HOP); promoting or preventing ATPase activity of HSP90 (e.g. p23 and Aha1); or the recruitment of specific classes of client proteins (e.g. Cdc37) (Taipale *et al*, 2010). As the association and disassociation of client proteins in the HSP90 cycle is dependent on ATP hydrolysis, HSP90 co-chaperones that prevent or inhibit HSP90 ATPase activity are seemingly involved in client association, while HSP90 co-chaperones that support or enhance HSP90 ATPase activity suggests the involvement in client disassociation (Taipale *et al*, 2010). Although there have been over 20 HSP90 co-chaperones identified, not all HSP90 co-chaperones will participate in the HSP90 maturation of each HSP90 client protein nor will they all be present at any one time point within the HSP90 cycle (Li *et al*, 2012). Owing to the cycle stage specificity, client specificity and transient state of HSP90 co-chaperone interactions, the mechanistic function and role of many HSP90 co-chaperones remains largely unknown (Taipale *et al*, 2010).

The HSP90 cycle involving obligate HSP90 client proteins, such as steroid receptors, have provided some insights into sequential HSP90 co-chaperone interactions with the HSP90 complex cycle. Client proteins are transferred from the HSP70 cycle (involved in *de novo* polypeptide folding) to the HSP90 cycle via HOP, a tetratricopeptide repeat (TPR) domain containing co-chaperone that is able to interact with both HSP90 and HSP70 simultaneously through specific TPR interactions with the MEEVD and EEVD motifs located at the C-terminus of HSP90 and HSP70 respectively (Brinker *et al*, 2002). HOP interaction with HSP90 stabilizes the ‘open’ conformation and inhibits HSP90 ATPase activity allowing for binding of HSP90 to the client protein (Johnson *et al*, 1998; Prodromou *et al*, 1999; Richter *et al*, 2003). The HSP90 complex then assumes a ‘closed’ conformation over the client protein upon HOP displacement. Hydrolysis of ATP, resulting in release of mature client

protein, is inhibited transiently through the binding of p23 to the HSP90 ‘closed’ conformation, resulting in the transient stabilization of the HSP90-client ‘closed’ complex (Grenert *et al*, 1999; Chadli *et al*, 2000; Freeman *et al*, 2000; Young *et al*, 2000; Morishima *et al*, 2001). Evidence suggests that Aha1 can induce the dissociation of the matured client proteins through the activation of HSP90 ATPase activity reverting the HSP90 complex to an ‘open’ conformation (Retzlaff *et al*, 2010). The Aha1-induced activation of HSP90 ATPase activity is due to conformation changes in the ‘closed’ conformation induced upon Aha1 binding to the HSP90 complex that accelerates ATP hydrolysis (Meyer *et al*, 2004; Hessling *et al*, 2009; Retzlaff *et al*, 2010). Although this model provides insights into the sequential association and disassociation of HSP90 co-chaperones during the HSP90 cycle, it by no means describes the maturation process of all HSP90 client proteins. Additionally, some HSP90 co-chaperones appear to be client class specific, as is the case with kinase client proteins and the HSP90 co-chaperone Cdc37. Cdc37 is a kinase specific HSP90 co-chaperone that substitutes for HOP functioning within the HSP90 cycle for kinase clients (Roe *et al*, 2004). Although the functional relevance of Cdc37 interaction with HSP90 is similar to that of HOP interaction by inhibiting ATPase activity of HSP90 and presentation of client substrates for binding to HSP90, the mechanism of interaction is vastly different. Cdc37 does not contain a TPR domain and interacts with the N-domain of HSP90 while HOP interacts with the C-domain of HSP90 (Siligardi *et al*, 2002; Roe *et al*, 2004; Gaiser *et al*, 2010)

1.1.4. Phosphorylation of HSP90

There is increasing evidence illustrating that the HSP90 cycle is regulated by post-translational modifications, the best characterized of which is phosphorylation (Figure 1). The phosphorylation of specific HSP90 residues can regulate the maturation of a client protein either by impeding or facilitating the progression of the cycle (Duval *et al* 2007; Soroka *et al*, 2012; Mollapour and Neckers, 2012). Residue specific phosphorylation can additionally affect client protein interactions or target HSP90 to subcellular localizations. For example phosphorylation of Threonine 90 by PKA targets HSP90 to the cell surface and for secretion (Lei *et al* 2007; Wang *et al*, 2009). Additionally phosphorylation of specific HSP90 residues can effect co-chaperone interactions (Mollapour *et al*, 2010; Mollapour *et al*, 2011a; Mollapour *et al*, 2011b;

Muller *et al*, 2013). Phosphorylation can additionally target HSP90 for ubiquitination as the case with cell cycle stage and nuclear specific kinase Swe1^{Wee1} which phosphorylates Tyrosine 38 of HSP90 resulting in translocation of HSP90 from the nucleus to the cytoplasm where it is ubiquitinated and degraded (Mollapour *et al*, 2010). Although numerous phosphorylation sites have been identified (Mollapour *et al*, 2011), the kinases catalyzing the phosphorylation of these specific residues remain largely unknown. Added to this complexity is the observation that some HSP90 clients themselves are capable of phosphorylating HSP90 (Barati *et al*, 2006; Kurkawa *et al*, 2008; Mollapour *et al*, 2011b). Evidence suggests that in some cases kinases in a complex with HSP90 auto-phosphorylate their own release from the HSP90 complex. PKC in complex with HSP90 phosphorylates HSP90 at Threonine 115, Threonine 425 and Threonine 603 resulting in HSP90 assuming an ‘open’ conformation thereby releasing PKC (Lu *et al*, 2014). Phosphorylation of HSP90 may present, at least in part, a means of selecting for the maturation of specific clients due to environmental stimuli as DNA-PK, a kinase responsible for phosphorylating HSP90 α but not HSP90 β (Lees-Miller and Anderson, 1989; Solier *et al*, 2012), phosphorylates HSP90 α during DNA stress (Quanz *et al*, 2012) and detection of apoptotic signals (Soloier *et al*, 2012).

1.1.5. HSP90 functioning in organelles

1.1.5.1. Endoplasmic Reticulum and Mitochondrial HSP90 isoforms

The Endoplasmic Reticulum (ER) specific HSP90 isoform is GRP94. Although displaying ATPase cycling conformational similarities with the cytosolic HSP90 isoforms (HSP90 α and HSP90 β), the regulation of the conformations and activity of this isoform are distinct (Gidalevitz *et al*, 2004; Frey *et al*, 2007; Dollins *et al*, 2007). The most striking distinction of ER HSP90 cycle compared to the cytosolic version is the absence of known co-chaperones (Eletto *et al*, 2010). This suggests that GRP94 ATP hydrolysis is un-regulated. Indeed GRP94 displays little binding affinity disparities between ADP and ATP, unlike cytosolic HSP90 isoforms that have a 10-fold greater affinity for ADP (Prodromou *et al*, 1997; McLaughlin *et al* 2004; Ge *et al*, 2007). Additionally, upon ATP binding, GRP94 displays a relatively open conformation and does not encase the ATP molecule within the nucleotide binding cleft (Immormino *et al*, 2004; Dollins *et al* 2005). Potentially due to the lack of co-chaperones, GRP94 displays client selectivity not interacting with as many clients as cytosolic HSP90. Additionally in most cases these so-called ‘clients’ do not display absolute GRP94 dependence and can operate GRP94-independently unlike the majority of cytosolic HSP90 clients (Randow and Seed, 2001; Yang *et al*, 2005; Morales *et al*, 2009). GRP94 functions in the late stage maturation and assembly of secreted and membrane-bound proteins; the ER-associated degradation of unfolded or mis-folded proteins (ERAD); and as a calcium ion binding protein thereby serving as a calcium reservoir (Melnick *et al*, 1994; Reddy *et al*, 1999; Christianson *et al*, 2008; Lee, 2014). Through promoting the maturation and assembly of secreted or membrane-bound proteins, GRP94 modulation effects signaling pathways including IGF stimulation of ERK and PI3K/AKT signaling pathways (Wanderling *et al*, 2007); Wnt/ β -catenin signaling (Liu *et al*, 2013) as well as HER2 receptor signaling (Patel *et al*, 2013).

In comparison to GRP94, the 75 kDa mitochondrial specific HSP90 isoform TRAP1 displays reduced homology to the cytosolic HSP90 isoforms (Song *et al*, 1995; Chen *et al*, 1996). Similarly to GRP94, TRAP1 is unable to bind cytosolic co-chaperones

p23 and HOP (Felts *et al*, 2000). The inability to bind to HOP is most likely attributed to the absence of the MEEVD C-terminus motif, as such TRAP1 is unlikely to bind any TPR-containing co-chaperones. Upon ATP binding TRAP1 assumes a cytosolic isoform-like ‘closed’ conformation with the N-domain lid section encasing the ATP molecules within the N-domain nucleotide binding site however, unlike cytosolic isoforms, the ‘closed’ conformation displays asymmetry (Leskivar *et al*, 2008; Lavery *et al*, 2014). Studies suggest that the TRAP1 chaperone cycle is comprised of a two step mechanism whereby ATP hydrolysis causes configuration changes in the client binding site resulting in conformational changes to the client protein. The chaperone then sequentially releases the client protein first prior to ADP release (Leskivar *et al*, 2008; Lavery *et al*, 2014). TRAP1 is predominantly localized in the mitochondrial matrix however a minor fraction of TRAP1 resides in the intermembrane space of mitochondria (Cechetto and Gupta, 2000; Pridgeon *et al*, 2007). Although TRAP1 functioning is relatively uncharacterized, TRAP1 has been shown to be required for maintenance of mitochondrial integrity protection from oxidative stress (Hua *et al*, 2007; Pridgeon *et al*, 2007; Takamura *et al*, 2012), and mitophagy and cell death signaling (Kang *et al*, 2007). Additionally there is increasing evidence suggesting that TRAP1 is linked to cancer development possibly mediating the Warburg effect in which cancers display an increased dependency on the glycolysis pathway (Kang *et al*, 2007; Leav *et al*, 2010; Zhao *et al*, 2013; Rasola *et al*, 2014).

1.1.5.2. Cytosolic HSP90 in the nucleus

HSP90 is commonly understood to be present in the nucleus of cells. This despite the fact that there is no distinct HSP90 nuclear isoform, as the case with mitochondrial HSP90 (TRAP1) and endoplasmic reticulum HSP90 (Grp94), and the HSP90 α and HSP90 β cytosolic isoforms lack a nuclear localization signal (NLS). Nuclear HSP90 represents approximately 3% of the total HSP90 level within a cell (Sawarkar and Paro, 2012). Although not extensively understood and a relatively new field of study, nuclear HSP90 plays regulatory functions in transcription, DNA replication and telomere biology through telomere complex assembly (DeZwaan and Freeman, 2010; Sekimoto *et al*, 2010; Zelin *et al*, 2012; Zhao and Houry, 2005). Evidence suggests

that HSP90 can directly influence gene expression through stabilizing and activating either transcription factors [e.g. Sp1 (Hung *et al*, 2005); STAT5 (Moullick *et al*, 2011); MyoD1 (Shaknovich *et al*, 1992)] or epigenetic regulators [e.g. Trithorax (Tariq *et al*, 2009)]. However in the case of transcription factor stabilization or activation, it remains to be determined whether these interactions are nucleus specific and whether HSP90 directly participates in the transcriptional activation process. In the case of STAT5, both STAT5 and HSP90 bind to the MYC promoter implying that HSP90 participates as a STAT5 transcriptional activator in the nucleus (Moullick *et al*, 2011). Nuclear-specific HSP90 functions include facilitating telomere complex assembly and chromosome ends through mediating the interplay between the telomere capping complex and the telomere extension complex (Holt *et al*, 1999; Keppler *et al*, 2006; Lee and Chung, 2009); and nucleosome removal to allow transcription factors and co-factors to access DNA sequences (Floer *et al*, 2008). Additionally, HSP90 assists in transcription through assembly of the RNA polymerase II complex by binding to RNA polymerase assembly protein RPAP3 (Cloutier *et al*, 2009; Boulon *et al*, 2010). Although the complex functions in the nucleus, the assembly occurs in the cytoplasm and is therefore not necessarily a function of nuclear-specific HSP90. HSP90 therefore plays a vital role in the homeostatic regulation in the nucleus not only through chromatin associated functions including assembly and disassembly of transcriptional complexes and maintenance of RNA polymerase II pausing (Sawarkar *et al*, 2012), but also through classical client maturation of structural and enzymatic proteins such as histones (Schneider *et al*, 1999) and telomerase (Forsythe *et al*, 2001; Keppler *et al*, 2006). Two questions still remain: how HSP90 is targeted to the nucleus? And what are the cohort of co-chaperones present in the nucleus responsible for regulating nuclear HSP90? It is likely that post-translational modifications participate in the nuclear translocation of HSP90 as isoform specific phosphorylation of HSP90 α by DNA-PK correlates to nuclear accumulation (Boulon *et al*, 2010; Mollapour and Neckers, 2012); while evidence in yeast indicates that specific phosphorylation of HSP90 at Tyrosine 38 by SWE1^{Wee1} targets the translocation of nuclear HSP90 to the cytoplasm (Mollapour *et al*, 2010). The possibility of HSP90 ‘piggy-backing’ into the nucleus via NLS containing client proteins (e.g steroid receptors) or co-chaperones (e.g. HOP) remains the most widely accepted notion. However, not all co-chaperones contain NLS motifs

which may indicate their absence in the nucleus and therefore the potential that nuclear localized HSP90 displays a different activity cycle.

1.1.6. HSP90 and multi-protein complexes

Not only does HSP90 modulate protein monomer stability but also multi-protein complex formation. Recent studies provide evidence for HSP90-dependent multi-protein complex assemblies. In most cases the actual mechanistic role of HSP90 in the formation of the complex remains to be determined. Two generalized HSP90 functions in protein complex formation have been proposed: 1) HSP90 stabilizing unstable subunits thereby facilitating incorporation into the protein complex, or 2) HSP90 promotes and additionally stabilizes changes in the composition of the complex to an active form for functional composition (Makhnevych and Houry, 2012). Implications of HSP90 in the assembly of functional protein complexes have been described for the C/P snoRNP complex (Zhao *et al*, 2008), RNA polymerase II complex (Boulon *et al*, 2010), telomere complex assembly (Holt *et al*, 1999; Keppler *et al*, 2006; Lee and Chung, 2009), kinetochore assembly (Davies *et al*, 2010), 26S Proteasome assembly (Imai *et al*, 2003; Yamano *et al*, 2008), RNA induced silencing complexes (RISC; Tahbaz *et al*, 2004; Johnston *et al*, 2010) and phosphatidylinositol-3 kinase-related protein kinase complexes (Gray *et al*, 2008; Takai *et al*, 2010).

1.1.7. HSP90 as a drug target

The inhibition of HSP90, mediated by small molecules, has proven indispensable to elucidating and analyzing the mechanism of HSP90 client protein maturation and activity (Prodromou *et al*, 1997; Neckers and Workman, 2012). These small molecules have additionally highlighted the importance of HSP90 in maintaining the stability of proteins involved in numerous cancer pathways identifying HSP90 as a key cancer drug target (Mahalingam *et al* 2009; Holzbeierlein *et al*, 2010). The first small molecule HSP90 inhibitor identified was the benzoquinone ansamycin antibiotic geldanamycin (GA) (De Boer *et al*, 1970) identified through the screening of natural compound libraries for anti-proliferative effects on numerous cancer cell lines (Uehara *et al*, 1986; Jove *et al*, 1987). Although the anti-proliferative effects of GA were thought to result from direct inhibition of v-Src (Jove *et al*, 1987),

subsequent studies revealed that the v-Src inhibition mediated anti-tumour effects of GA were as a result of GA inhibiting the HSP90 chaperoning of v-Src (Whitesell *et al*, 1994; Neckers *et al*, 1999). Later studies elucidated that the mechanism by which GA inhibits HSP90 is through GA binding to the N-domain ATP binding pocket thereby preventing the ATP-dependent cycling mechanism of HSP90 activity (Roe *et al*, 1999). Although displaying anti-proliferative effects against numerous cancer cell lines, GA has failed to proceed in clinical trials due to its hepatotoxicity, which is unrelated to HSP90 inhibition (Sausville *et al*, 2003; Banerji, 2005). However, GA has served as a scaffold for drug design and several analogs (for example 17-AAG) have subsequently been generated that display less toxicity in animal studies (Neckers and Workman, 2012). Nevertheless GA is the most common HSP90 inhibitor used for mechanistic studies and for HSP90 client protein identification and has proved to be an invaluable tool for our understanding of HSP90 mechanics (Eleuteri *et al*, 2002). Other antibiotic compounds have been identified as HSP90 inhibitors, including the coumarin antibiotic novobiocin (NOV) (Marcu *et al*, 2000; Allan *et al*, 2006). Interestingly NOV binds to the C-domain of HSP90 via a putative nucleotide-binding pocket (Marcu *et al*, 2000). Through interaction with the C-domain of HSP90 NOV prevents HSP90 dimerization (Matts *et al*, 2011). However NOV displays a weaker affinity for HSP90 binding compared to GA (Yu *et al*, 2005) as such analogs using NOV as a scaffold are being generated in an attempt to improve HSP90 affinity (Marcu *et al*, 2000).

The inhibition of HSP90 with GA or other N-domain inhibitors however presents complications due to the HSP90 inhibition mediated induction of the heat shock response (Eskew *et al*, 2011). Heat shock factor-1 (HSF-1) is a client protein of HSP90 (Ali *et al*, 1998). During physiological conditions HSF1 is maintained in an inactive form in a complex with HSP90 (Bishop *et al*, 2007). The inhibition of HSP90 (though N-domain inhibitors such as GA) or physiological stress (for example heat shock) results in the release of HSF1 and subsequent trimerization leading to nuclear translocation. Activated trimeric HSF1 binds to heat shock response elements located in the promoter regions of numerous heat shock protein chaperones and co-chaperones including HSP90, resulting in their up-regulation (Kim *et al*, 1999; Hu *et al*, 2003; Workman and de Billy, 2007). This mechanism allows for the re-establishment of protein homeostasis under stress conditions including HSP90

inhibition. It is interesting to note that the induction of the heat shock response is not induced with HSP90 inhibition with C-domain inhibitors such as NOV (Holzbeierlein *et al*, 2010). A possible mechanism behind the absence of a heat shock response upon inhibition of HSP90 with C-domain inhibitors is the finding that Novobiocin causes the dissociation of ‘early’ stage co-chaperones involved in client binding to HSP90 while ‘late’ stage co-chaperones involved maintaining the HSP90-client complex remain intact (Yun *et al*, 2004), this may maintain the HSP90-HSF1 complex therefore maintaining HSF1 in an inactive state.

1.2.Octamer-binding transcription factor 4 (OCT4)

1.2.1. POU transcription factor family and structure of POU domain

The Pit, Oct, UNC (POU) domain containing transcription factor family share in common the conserved 135 amino acid (approximately) POU DNA interaction domain (Herr *et al*, 1988; Rosenfeld, 1991, Botfield *et al*, 1992). Although termed a domain, the POU domain is comprised of two structurally independent subdomains that although each capable of binding to DNA (Klemm *et al*, 1994), cooperatively function as a single DNA binding unit. As such, the ‘POU domain’ refers to both of these DNA binding regions as a unit (Herr and Cleary, 1995). The POU domain consists of the POU-specific (POU_S; 75 amino acid) and POU-homeo (POU_H; 60 amino acid) DNA binding structures (Figure 2; Herr *et al*, 1998). Connecting the two DNA binding structures is a hypervariable highly flexible linker region ranging from 15 to 56 amino acids (Figure 2; Herr *et al*, 1988; Greenstein *et al*, 1994). The POU transcription factors, owing in part to the two structurally independent DNA binding regions, show versatility in DNA interaction modules and DNA recognition motifs. POU domain proteins display versatility in DNA binding, being able to bind DNA in monomeric units (Ingraham *et al*, 1988) or protein complexes consisting of homodimeric, heterodimeric, or heteromeric complexes with other POU domain proteins (Voss *et al*, 1991) or other transcription factors and co-activators (Ullman *et al* 1991; Ullman *et al*, 1993; Zwilling *et al*, 1994).

1.2.2. Structure and function of OCT4

The OCT4 gene (also known as OCT3) is a highly and tightly transcriptionally regulated gene that encodes for numerous isoforms. OCT4 is comprised of three domain structures: N-terminal transactivation domain (rich in Proline amino acids residues); POU domain (consisting of POU_S and POU_H subdomains); and the C-terminal transactivation domain (rich in Proline, Serine and Threonine residues) (Figure 2; Wu and Scholer, 2014). OCT4 isoforms are generated through alternative splicing and alternative translation initiation sites, which although consisting of the POU domain and C-terminal transactivation domain, display N-terminal domain truncations (Wang and Dai, 2010). Although not implicated in direct DNA interactions, the transactivation domains are required for the regulation of OCT4 activity through mediating interactions with other transcription factors and transcription regulators, and serve as the site of post-translational modifications such as phosphorylation (Vigano and Staudt, 1996; Brehm *et al*, 1997; Brehm *et al*, 1998; Saxe *et al*, 2009; Brumbaugh *et al*, 2012). Indeed, the OCT4B isoform, although consisting of POU (POU_S and POU_H) domain and C-terminal transactivation domain, is unable to bind to or activate OCT4A target gene promoters and therefore unable to support self-renewal and pluripotency (Lee *et al*, 2006). A nuclear localization signal (NLS) is located at the amino-terminus of the POU_H subdomain allowing for the nuclear import of OCT4 (Figure 2). Recently, OCT4 has been described as a nucleocytoplasmic shuttling protein although no clear nuclear export signal (NES) has been identified. A putative NES has been identified located in the POU_H domain downstream of the NLS based on sequence identity with the OCT6 NES (Figure 2; Baranek *et al*, 2005). However mutational analyses suggest that this putative NES may not be the only mechanism by which OCT4 is exported and that OCT4 also translocates from the nucleus to the cytoplasm via alternative mechanisms (Oka *et al*, 2013). Of interest is the differential subcellular localization of the OCT4 isoforms even though all isoforms contain a NLS. OCT4A displays a nuclear localization (Pan *et al*, 2004), while OCT4B displays a predominant cytoplasmic localization (Cauffman *et al*, 2006; Lee *et al*, 2006). Other OCT4 isoforms display both cytoplasmic and nuclear localizations (Wang *et al*, 2009). Additionally only OCT4A

and not the OCT4 isoforms are capable of sustaining self-renewal and pluripotency (Lee *et al*, 2006). From this moment forward “OCT4” refers to the OCT4A isoform.

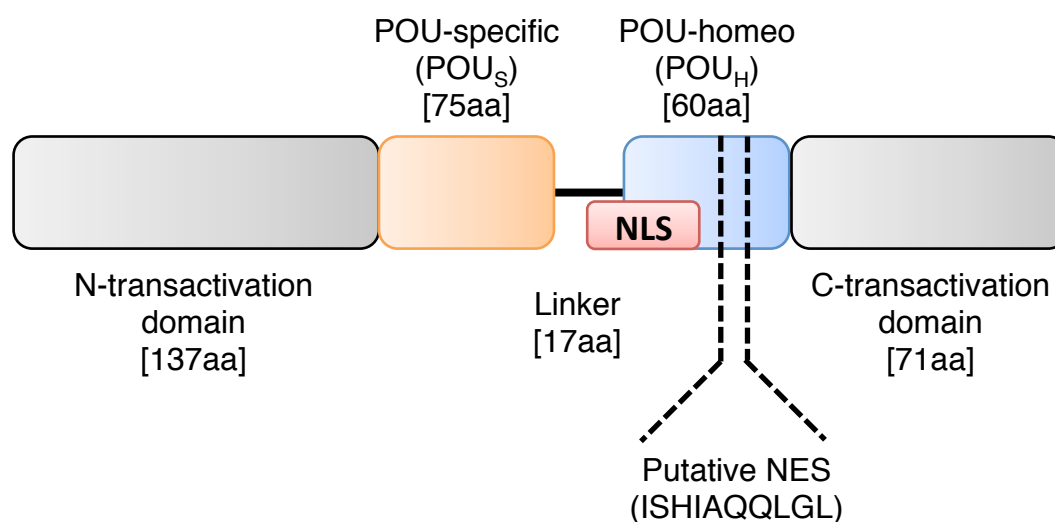


Figure 2 | OCT4A domain structure.

Domain structure of POU transcription factor OCT4 (OCT4A). OCT4 consists of an N-terminal transactivation domain (137 amino acids, aa), the POU domain consisting of the POU-specific (POU_S; 75 amino acids, aa) and POU-homeo (POU_H; 60 amino acids, aa) DNA-binding sub-domains separated by a flexible and variable linker region (17 amino acids, aa), and the C-terminal transactivation domain (71 amino acids, aa). A nuclear localization signal (NLS; RKRKR) is located at the N-terminal end of the POU_H domain. Oka and colleagues (2013) identified a putative nuclear exportation signal (NES; ISHIAQQLGL) based of sequence similarity with the nucleocytoplasmic shuttling POU family member OCT6 (Oka *et al*, 2013). Numbers in square brackets represent length of domain or region in amino acids.

OCT4 expression is tightly regulated and is limited to early-stage developmental tissues (inner cell mass of blastocysts), oocyte, germ cells and embryonic carcinomas (Scholer *et al*, 1990; Nichols *et al*, 1998). OCT4, like other POU domain transcription factors, regulates transcription of target genes through both activator and repressor functions (Latchman, 1996; Pan *et al*, 2002). Therefore OCT4 importantly regulates pluripotency by both promoting self-renewal and inhibiting differentiation through the suppression or promotion of target gene transcription (Niwa *et al*, 2000). Although capable of prompting or repressing transcription in homomeric form, the synergistic activity of direct protein-protein interactions between OCT4 and other transcription factors or transcriptional regulatory proteins drives the tightly regulated transcriptional profile mediating stem cell biology (Yuan *et al*, 1995; Ambrosetti *et al*, 1997; Okuda *et al*, 1998; Nishimoto *et al*, 1999 Tomioka *et al*, 2002). In some cases, reductions in the binding of OCT4 to promoters has been shown to affect the ability of other transcription factors such as Smad1 and STAT3 to bind the promoter in question, suggesting that OCT4 may support the stabilization of transcription factor complexes (Chen *et al*, 2008)

The loss of OCT4 induces differentiation (Nichols *et al*, 1998; Ratajczak *et al*, 2007). However, OCT4 also promotes lineage specific differentiation in a concentration-dependent manner. When OCT4 levels are higher or lower than the relative level in embryonic stem cells, differentiation occurs and thus OCT4 levels are highly and tightly regulated (Figure 3; Niwa *et al*, 2000; Karwacki-Neisius *et al*, 2013; Radzeushkaya *et al*, 2013). Therefore unlike other transcription factors that function in a deterministic binary manner, OCT4 acts in a contextualized quantitative manner whereby changes in levels results in distinct biological outcomes. A startling observation is the species-specific differences in OCT4 target genes exhibited between human and murine ECS. Loh and colleagues identified that while OCT4 binds to the promoters of 233 genes in murine ESCs, OCT4 bound 603 promoters in human ESCs (Loh *et al*, 2006). Of even greater interest is that of these genes only 33 OCT4-bound gene targets were common between murine and human ESCs (Loh *et al*, 2006)

1.2.3. OCT4: post-translational modifications

Although the POU domain is conserved for all POU transcription factors, the N- and C-terminal transactivation regions outside of this domain are variable in size and highly variable in sequence (Pan *et al*, 2002). Evidence suggests that the N- and C-terminal transactivation domain of OCT4 are important in activity regulation through post-translational modification (Xu *et al*, 2004; Zhang *et al*, 2007; Wei *et al*, 2007; Saxe *et al*, 2009; Swaney *et al*, 2009; Brumbaugh *et al*, 2012). The C-terminal transactivation domain of OCT4 is phosphorylated in a cell type specific manner illustrating the highly regulated nature of OCT4 (Vigano and Staudt, 1996; Brehm *et al*, 1997; Brehm *et al*, 1998). Ubiquitination of both murine and human OCT4, mediated by murine and human WWP2 (an E3 ubiquitin ligase) respectively, results in the proteosomal degradation of OCT4 (Xu *et al*, 2004; Xu *et al*, 2009; Liao and Jin, 2010). However the OCT4 degradation mediated by WWP2 occurs in different biological contexts between mouse and human ESCs. While WWP2 is only involved in OCT4 degradation of murine OCT4 under differentiation (Xu *et al*, 2004, Liao and Jin, 2010; Liao *et al*, 2013), human WWP2 functions in OCT4 degradation under undifferentiated conditions (Xu *et al*, 2009). Regular degradation of murine OCT4 in undifferentiated cells is mediated by Itch in murine ESCs (Liao *et al*, 2013). The most observed post-translational modification to OCT4 is phosphorylation. Phosphorylation modifications within the POU_S or POU_H domains disrupt interaction of OCT4 with DNA through steric hindrance of the DNA binding domains with DNA (Swaney *et al*, 2009; Saxe *et al*, 2009; Brumbaugh *et al*, 2012). However phosphorylation within the N- and C- transactivation domains generally increase OCT4 activity and in some cases, selectively enhances sequence-specific activity (Kang *et al*, 2009; Saxe *et al*, 2009; Brumbaugh *et al*, 2012; Lin *et al*, 2012; Campbell and Rudnicki, 2013). Interestingly, there is evidence suggesting that OCT4 and OCT4-binding proteins, including many transcription factors required for self-renewal and pluripotency, are all phosphorylated and hence co-regulated by common kinases including the FGF stimulated ERK (Brumbaugh *et al*, 2012). Analysis of total phosphorylation events in human ESCs has revealed that CDK1/2 mediates 26% of all phosphorylation events that occur in human ESCs (van Hoof *et al*, 2009). Human ESCs show additional enrichment in MAPK, TGF β R2 and GSK3 β kinase activity (van Hoof *et al*, 2009). Together this suggests that in order to maintain homeostasis

within an ESC environment, precise signaling transductions and target activations are required and that homeostasis is tightly regulated by post-translational modifications.

1.3.Stem Cells

Studies on murine embryonal carcinomas led to the conclusion and identification of a developmentally supportive population of cells present during early stages of development (Stevens and Little, 1954; Kleinsmith and Pierce, 1964; Evans and Kaufman, 1981; Martin, 1981; Thomson *et al*, 1998). Stem cells are accepted to display two vitally important cellular properties, that of self-renewal and that of differentiation (Martello and Smith, 2014). Self-renewal is the propensity to upon division generate progeny that display the equivalent proliferative and developmental potential, or simply an identical copy of the parent cell. Differentiation is the developmental program that results in the generation of all specialized cell types within the body. As differentiation occurs, a cell becomes more specialized gaining specific cellular traits of a given cell type. The capacity for self-renewal and differentiation is indefinite in stem cells (Martello and Smith, 2014). Although self-renewal and potential for differentiation are shared features of stem cells, there is a difference in differentiation potential between stem cells populations. Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts and are capable of differentiating into every specialized cell within the body and as such are termed pluripotent stem cells (Nichols *et al*, 2009). Whereas somatic or adult stem cells are tissue specific stem cells compartmentalized within particular organs that, although capable of self-renewal, are responsible for the generation of only the cell types within that given tissue system (Wagers and Weissman, 2004). These somatic stem cells are generally termed multipotent stem cells. Whereas embryonic stem cells function in development, somatic stem cells are responsible for tissue turnover and repair functions thereby supporting tissue homeostasis (Simons and Clever, 2011).

Self-renewal and pluripotency, and differentiation are balanced programs. As cells undergo differentiation to become more specialized, so the ability to self-renew reduces and there is a loss in pluripotency potential of the cells. Cellular developmental progression from pluripotent stem cells to differentiated cells was previously hypothesized to be a uni-directional program. However, seminal studies by

Gurdon (Gurdon, 1962), Davis and colleagues (Davis *et al*, 1987), and Schneuwly and colleagues (Schneuwly *et al*, 1987) lead to the groundbreaking discovery that cellular developmental progression can be forced into bi-directionality (Takahashi and Yamanaka, 2006; Takahashi *et al*, 2007; Yu *et al*, 2007). Differentiated cells can be reprogrammed by the forced expression of master transcription factors into pluripotent stem cells known as induced pluripotent stem cells (iPSCs) that display similar identities of ESCs, certainly in the case of pluripotency potential (Takahashi and Yamanaka, 2006; Takahashi *et al*, 2007; Yu *et al*, 2007). Although numerous combinations of transcription factors have been used to produce iPSCs, OCT4 remains the pre-eminent transcription factor required for both ESC maintenance (Okamoto *et al*, 1990; Scholer *et al*, 1990) and iPSC reprogramming (Takahashi and Yamanaka, 2006; Takahashi *et al*, 2007; Yu *et al*, 2007).

1.3.1. Stem cell-related signaling pathways and HSP90

The pluripotent phenomenon is a highly regulated and balanced state of homeostasis requiring finely tuned molecular signals. As such, signaling pathways are vitally important in maintaining this balance (Martello and Smith, 2014). Although the full description and understanding of how many signaling pathways affect stem cell nature remains to be elucidated, several studies have identified signaling nodes vital to stem cell maintenance. Interestingly, although some signaling pathways have similar mechanisms of action across murine and human pluripotent stem cell maintenance, several display species-specific influences (Figure 3.A and B; Schnerch *et al*, 2010). Many of the key signaling intermediates of these pathways are HSP90 client proteins, including but not limited to AKT, STAT3 (Signal Transducer and Activator of Transcription 3), β -catenin and Smad, suggesting that HSP90 plays an important role in mediating signaling events required for maintenance of the pluripotent stem cell state (Figure 3 and 4).

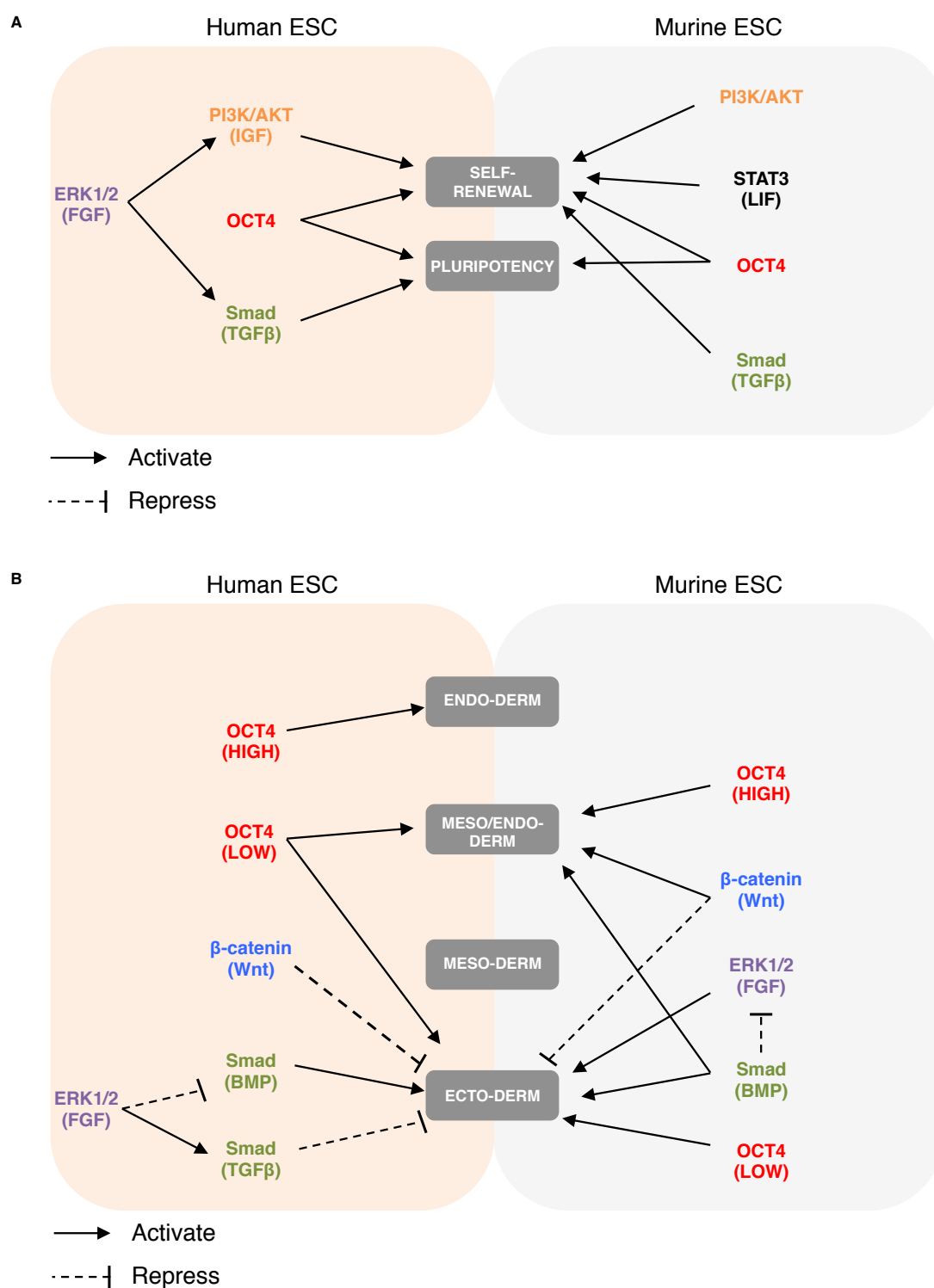


Figure 3 | Conservation and species-specific differences in signaling pathways controlling self-renewal, pluripotency and lineage-specific differentiation.

Illustration of conserved and altered signaling pathways effecting self-renewal and pluripotency (A), and lineage-specific differentiation (B), in human and mouse embryonic stem cells (modified from: Schnerch *et al*, 2010). Solid lines represent activation, dotted lines represent repression. Bracketed are the stimulating ligands responsible for activation of signaling pathway. For OCT4, brackets represent relative expression levels.

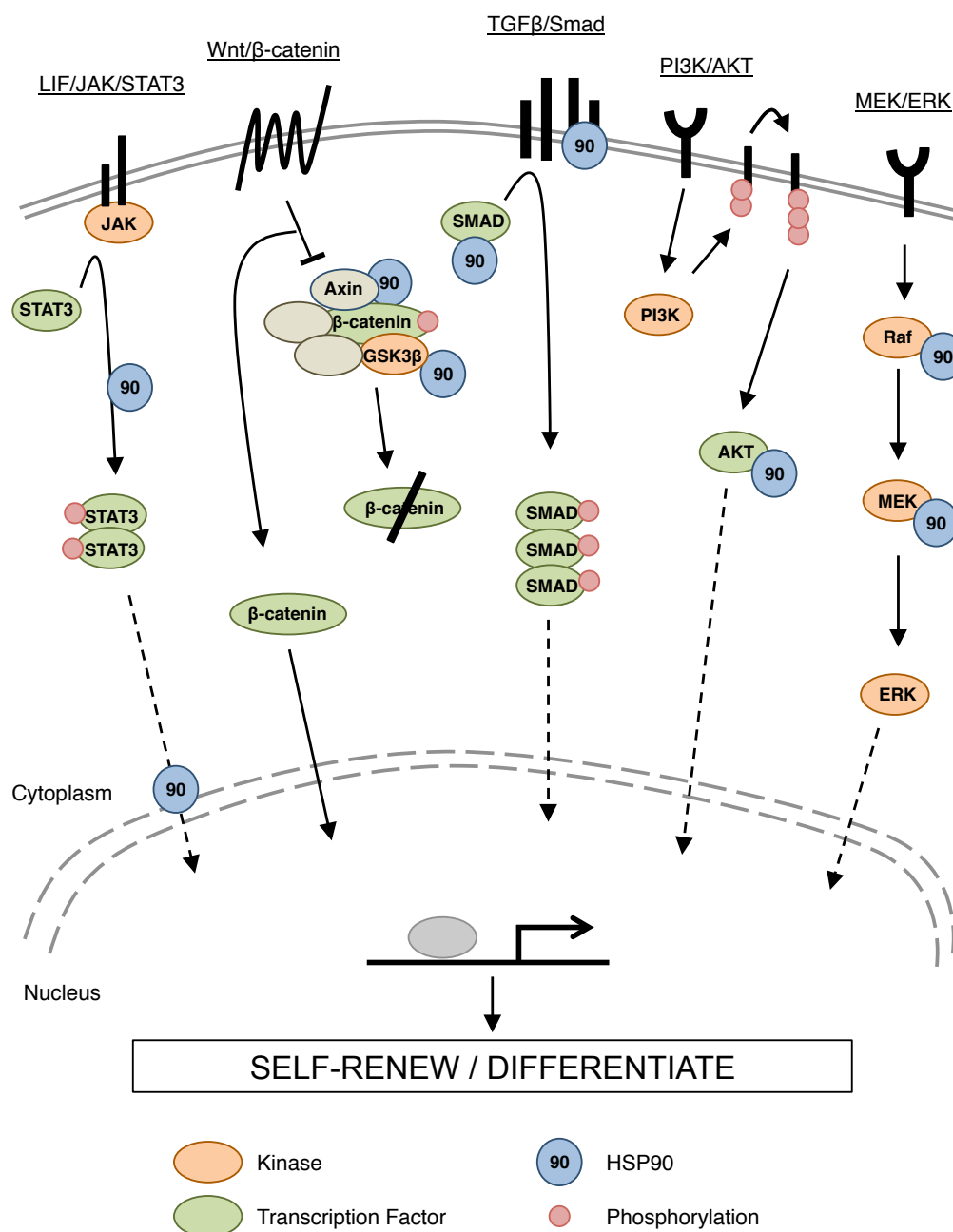


Figure 4 | HSP90 functioning in stem cell-related signaling pathways.

Numerous signaling intermediate proteins within stem cell-related signaling pathways are client proteins of HSP90. These clients rely on HSP90 function for stability and hence activity. HSP90 client proteins within stem cell-related signaling pathways include: STAT3 (LIF/JAK/STAT3 pathway; phosphorylation, dimerization and nuclear translocation of STAT3); Axin and GSK3β (Wnt/β-catenin pathway; protein stability and activity); Smad (TGFβ/Smad pathway; protein stability); AKT (PI3K/AKT pathway; AKT stability and phosphorylation therefore activity); Raf and MEK (MEK/ERK pathway; protein stability).

The LIF/JAK/STAT3 pathway (Figure 4), the most well understood of the stem cell-related signaling pathways, is one example of species-specific signaling pathway differences between murine and human ESCs. LIF/JAK/STAT3 signaling is mediated via the leukemia inhibitory factor (LIF) ligand. LIF binds to the LIF receptor/gp130 cell surface receptor complex resulting in the stimulation and activation of the HSP90 client Janus Kinase (JAK). Activated JAK phosphorylates STAT3 resulting in STAT3 dimerization and subsequent nuclear localization to activate the transcription of genes (Burdon *et al*, 2002). HSP90 is required for both STAT3 dimerization upon phosphorylation and nuclear translocation, whereby HSP90 facilitates in the shuttling of phosphorylated dimeric STAT3 into the nucleus via importin α (Liu *et al*, 2005; Ushijima *et al*, 2005; Okumura *et al*, 2011). Inhibition of HSP90 results in the reduction of phosphorylated STAT3 protein levels (Sato *et al*, 2003; Setati *et al*, 2010). This signaling pathway is essential in maintaining murine ESCs self-renewal and pluripotency (Figure 3.A; Boeuf *et al*, 1997; Matsuda *et al*, 1999; Raz *et al*, 1999; Niwa *et al*, 2000). Interestingly, the LIF/JAK/STAT3 pathway is unable to promote self-renewal and maintain pluripotency in human ESCs, with evidence suggesting that this particular pathway is non-functional in human ESCs (Humphrey *et al*, 2004; Wei *et al*, 2005).

The TGF β /Smad signaling pathway (Figure 4) is stimulated through interaction between cell surface TGF β type II receptors and a member of the TGF β superfamily of ligands which include, but are not limited to, Bone Morphogenetic Proteins (BMPs), TGF β s, Activin and Nodal (Attisano and Wrana, 2002). Stimulation of the pathway sees the formation heteromeric Smad complexes due to phosphorylation of Smad proteins, and subsequent nuclear translocation. Heteromeric Smad complexes function as transcription factors activating the expression or repression of target genes (Shi and Massague, 2003). Smad proteins and TGF β type II receptors are HSP90 client proteins, with HSP90 prevention of ubiquitin mediated degradation of Smads resulting in the promotion of TGF β /Smad signaling events (Wrighton *et al*, 2008; Shang *et al*, 2014). The TGF β /Smad signaling pathway functions in a balance with LIF/JAK/STAT3 signaling to maintain pluripotency in murine ECSs (Ruzinova and Benezra, 2003). BMP ligand activation of TGF β /Smad signaling has been shown to induce differentiation in human ESCs (Figure 3.B; Schuldiner *et al*, 2000). While the

repression of TGF β /Smad signaling through BMP ligands and activation of TGF β /Smad through TGF β sustains long-term human ECS self-renewal (Figure 3.A; Xu *et al*, 2005).

The canonical Wnt/ β -catenin signaling pathway (Figure 4) signals conversely to most signaling pathways whereby the effector protein is phosphorylated in the unstimulated state. In the absence of the Wnt ligand, β -catenin is phosphorylated by Glycogen Synthase Kinase 3 (GSK3) and Casein Kinase 1 (CK1), both kinases form part of a 'destruction complex' made up of numerous proteins including Axin (Ikeda *et al*, 1998; Dajani *et al*, 2003). Phosphorylated β -catenin is constantly degraded through the ubiquitin-proteasome system. Upon Wnt ligand stimulation of the Frizzled receptor, the 'destruction complex' disassembles, preventing phosphorylation of and subsequent stabilization of β -catenin, which translocates into the nucleus (Logan and Nusse, 2004). In the nucleus, β -catenin associates with and activates the TCF/LEF transcription factor complex resulting in the expression of Wnt/ β -catenin target genes (Reya and Clevers, 2005; MacDonald *et al*, 2009). β -Catenin has additionally been shown to interact with and modulate OCT4 activity (Takao *et al*, 2007). HSP90 is required for the stabilization and hence activity of GSK3 β (Lohead *et al*, 2006; Banz *et al*, 2009, Taipale *et al*, 2012). Additionally HSP90 has been shown to interact with phosphorylated β -catenin in the 'destruction complex' suggesting that HSP90 may be required for the 'destruction complex' mediated phosphorylation of β -catenin (Cooper *et al*, 2011). The functional role of Wnt/ β -catenin signaling in ESCs remains controversial. There is evidence suggesting that Wnt/ β -catenin signaling both promotes differentiation of murine ESCs (Dravid *et al*, 2005; Pereira *et al* 2006; He *et al*, 2008) and promotes self-renewal and pluripotency of murine ESCs and human ESCs (Figure 3.A and B; Sato *et al*, 2004; Singla *et al*, 2006; Hai *et al*, 2006; Takao *et al*, 2007;).

Activation of the PI3K/AKT signaling pathway (Figure 4) is induced by numerous growth factors including EGF, FGF and IGF. Growth factor binding to respective receptor tyrosine kinases activates PI3K, resulting in the phosphorylation of PIP2 to PIP3. Inactive AKT is recruited to PIP3, where PIP3 assists in AKT phosphorylation and stabilization of active AKT by PDK1 (Cantley, 2002). Activated AKT modulates

the activity of several client proteins including mTOR (Inoki *et al*, 2002) and GSK3 β (Rokutanda *et al*, 2009), therefore PI3K/AKT signaling modulates the activity of other signaling pathways including Wnt/ β -catenin signaling pathway. AKT is a kinase client protein of HSP90 (Haendeler *et al*, 2003; Taipale *et al*, 2012; Zhang *et al*, 2013) and conversely HSP90 is a substrate for AKT (Barati *et al*, 2006). The PI3K/AKT pathway positively regulates the self-renewal and pluripotency of both murine and human ESCs (Figure 3.A and B; Paling *et al*, 2004; Kim *et al*, 2005; Takahashi *et al*, 2005; Armstrong *et al*, 2006).

The MAPK/ERK signaling pathway (Figure 4) transduces extracellular signals to the nucleus through a series of phosphorylation activating intermediate kinases. Stimulation of the pathway, by various growth factors, most notably FGF, catalyzes the nucleotide exchange of inactive Ras-GDP to active Ras-GTP. Activated Ras binds to Raf kinase, promoting the phosphorylation of numerous kinases including MEK, with MEK subsequently activating ERK1/2. Activated ERK1/2 modulates and activates numerous transcription factors through phosphorylation (Ehrhardt *et al*, 2002; Lee and McCubrey, 2002). The MAPK/ERK signaling pathway displays species-specific effects. In murine ESCs, activation of the pathway promotes differentiation (Yoshida-Koide *et al*, 2004), with ERK inhibition required for the derivation of ESCs from blastocysts (Burdon *et al*, 1999). However in human ESCs, FGF stimulation of the MAPK/ERK pathway is required for maintaining pluripotency, and FGF is used to supplement human ESCs culture media to prevent differentiation (Xu *et al*, 2005). Although FGF stimulates the MAPK/ERK pathway, the growth factor additionally activates the PI3K/AKT pathway suggesting that the pluripotency promoting effects result from activation of both signaling pathways (Bottcher and Niehrs, 2005). Numerous intermediate proteins involved in the MAPK/ERK pathway are HSP90 client proteins including Raf, (Stancato *et al*, 1993; Grammatikakis *et al*, 1999), MEK (Stancato *et al*, 1997; Bouwmeester *et al*, 2004; Shinozaki *et al*, 2006) and numerous MAPK intermediate kinases (Taipale *et al*, 2012). Although HSP90 inhibition results in the degradation of these kinases, GA has been shown to transiently activate MAPK kinases (Donze *et al*, 2001).

Recently evidence has been provided for the importance of HSP90 in the maintenance and regulation of ESCs. In this study Bradley and colleagues (2012) analyzed the effect of HSP90 inhibition (using the GA derivative 17-AAG) and HSP90 knockdown (using miRNA targeting HSP90 α or HSP90 β) on pluripotency of murine ESCs. With both inhibition and knockdown of HSP90 they found a loss in phenotypic pluripotency characteristics including morphology changes, *in vivo* teratoma formation and pluripotency markers namely OCT4, Nanog and phosphorylated STAT3 (Bradley *et al*, 2012). These characteristics were partially restored with the overexpression of HSP90 β . They illustrated that HSP90 α and HSP90 β directly interact with both murine OCT4 and Nanog and that inhibition of HSP90 resulted in loss of murine OCT4, phosphorylated STAT3 and Nanog (Bradley *et al*, 2012). Interestingly inhibition of HSP90 additionally resulted in the decrease of murine OCT4 mRNA, this together with the reduction of phosphorylated STAT3 may be suggestive of murine ESCs differentiation (Niwa *et al*, 2000; Bradley *et al*, 2012). HSP90 inhibition has been shown to prevent STAT3 phosphorylation and hence activation (Sato *et al*, 2003; Setati *et al*, 2010). As STAT3 activity is essential for maintaining murine ESC pluripotency (Niwa *et al*, 2000), the loss of murine OCT4 protein levels and murine OCT4 mRNA levels with HSP90 inhibition may be caused by the induction of differentiation rather than OCT4 requiring HSP90 for stability. Indeed, Bradley and colleagues (2012) observed depletion of phosphorylated STAT3 at both a lower concentration of 17-AAG and earlier time point of 17-AAG treatment than murine OCT4. Although this study illuminates the importance of HSP90 activity for maintenance of pluripotency in ESCs, the mechanisms surrounding this maintenance may be confounded by the murine ESCs model used.

1.4.Motivation for study

The molecular chaperone HSP90 plays a pivotal role in cellular homeostasis and modulating cell-specific biological processes. The regulatory role of HSP90 within the biological context of pluripotency and differentiation remains relatively unknown, although there is evidence suggesting that HSP90 is required for murine ESC maintenance. Additionally there is relatively little data on the biochemistry of human OCT4 since most biochemical analyses of OCT4 have been based on the murine version. While there is some evidence implying a role for HSP90 in OCT4

biochemistry in the mouse system, there is no information on HSP90 and OCT4 in the human system. This is particularly relevant due to differences observed between pathways and pathway outputs governing pluripotency and differentiation in human and murine systems. The initial assessment of the relationship between HSP90 and key stem cell master regulator proteins, such as OCT4, *ex vivo* may provide clues to HSP90 functioning within the biological context of human stem cells.

1.5. Research Question

Is human OCT4 a client protein of HSP90, and if so what is the biochemical relationship between HSP90 and human OCT4?

1.6. Aims

- Generation of a constitutive human OCT4 expressing cell line.
- Biochemical analyses of the relationship between HSP90 and human OCT4.

Chapter 2. Methodology

2.1.Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Cell lysates from equal cell numbers were prepared in 5 x SDS-PAGE sample buffer without bromophenol blue [0.05M Tris-HCL, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2% (w/v) SDS] and stored at -20°C until analysis. Prior to analysis lysates were incubated at 95°C for 5 minutes twice. Lysates were mixed with equal volumes of 5x SDS-PAGE sample buffer containing 1% (w/v) bromophenol blue. Proteins were separated by SDS-PAGE using the protocol established by Laemmli (1970) using a 4% (v/v) stacking (0.5M Tris-HCl, pH 6.8) and 12% (v/v) resolving gel (1.5M Tris-HCl, pH 8.8). Gels were run at 65 V for 20 minutes followed by 100 V for approximately 1.5 hours in SDS-PAGE running buffer [0.25mM Tris, 192mM glycine, 1% (w/v) SDS].

2.2.Chemiluminescence-based Western blot analysis.

Nitrocellulose membranes were pre-treated in transfer buffer [13 mM Tris-HCL, 100 mM glycine and 20% (v/v) methanol] for 10 minutes. Cellular proteins separated using SDS-PAGE were transferred onto pre-treated nitrocellulose membrane using a semi-dry Transblot (LASEC) at constant amplitude of 400 mA for 50 minutes (Towbin *et al*, 1979). Protein transfer onto nitrocellulose was confirmed by Ponceau staining [0.5% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. Nitrocellulose membranes with transferred proteins were blocked with 1% (w/v) Blotto [non-fat dry milk (Santa Cruz Biotechnology) in Tris-buffered saline-Tween [TBST; 50 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Tween20] for 30 minutes and incubated with primary antibody (Appendix Table 2) overnight at 4°C with agitation, diluted to the manufacturer's recommended dilution in 1% (w/v) Blotto in TBST. Membranes were rinsed four times in TBST for 8 minutes each replacing the TBST each rinse. The membrane was incubated with species-specific secondary antibodies conjugated to horseradish peroxidase (HRP) at 1:5000 dilution in 1% (w/v) Blotto in TBST for 45 minutes with agitation at room temperature (Appendix Table 3). After secondary antibody incubation, membranes were rinsed four times with TBST for 8 minutes each, replacing the TBST at each rinse. Detection of protein bands was performed using the Clarity Western ECL Substrate kit (Biorad; UK) with visualization using

XRS Chemidoc (Biorad; UK). Histone H3 was used as a protein loading control in each case and densitometry was performed using ImageJ software (NIH).

2.3. Restriction endonuclease verification of lentiviral-based gene delivery system.

The lentiviral delivery system consisting of pMD2.G (lentiviral capsid vector), psPAX2 (lentiviral enzyme vector) and pLVTH (lentiviral transgene vector) were kind gifts from Prof Weinberg (WITS University). pSIN-hOCT4-Puro was a kind gift from Dr Janine Scholefield (CSIR Biosciences). Plasmid DNA (pMD2.G, psPAX2 and pLVTH) was extracted from filter paper by soaking the filter paper in 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, HCl, pH 8) for 1 hour. All plasmid constructs were transformed into chemically competent JM109 and Stbl2 *E.coli* cells. Fifty microlitres of competent cells and 1 µg of plasmid DNA were incubated on ice for 2 minutes prior to heat shock at 42°C. After heat shock, cells were incubated on ice for a further 5 minutes. Cells were mixed with 500 µl of 2 x YT broth (16 g/l Tryptone, 10 g/l Yeast extract, 5 g/l NaCl) with JM109 cells incubated at 37°C for 30 minutes or Stbl2 cells incubated at 30°C for 90 minutes, before 150 µl of the transformation was plated on 2 x YT agar plates [16 g/l Tryptone, 10 g/l Yeast extract, 5 g/l NaCl, 100 µg/ml Ampicillin (Sigma-Aldrich)] and incubated overnight at 37°C for JM109 cells and 30°C for Stbl2 cells. Single colony transformants were used to inoculate 5 ml 2 x YT broth overnight at 37°C with shaking (200 rpm) for JM109 cells, and at 30°C with 220 rpm agitation for Stbl2 cells. Lentiviral vectors were extracted using the GeneJET Plasmid Miniprep kit (Fermentas) according to manufacturer's instructions. Restriction digest analyses were used to confirm the identification of the vectors based on DNA patterning. Vectors were verified using restriction endonuclease digestion at 37°C for 1 hour as follows: *HindIII* (NEB) and *NotI* (NEB) for pMD2.G; *NheI* (NEB) and *SacII* (NEB) for psPAX2; *BamHI* (NEB) and *SalI* (Promega) for pLVTH; and *SpeI* (NEB) and *SalI* for pSIN-hOCT4-Puro. Resulting digestion patterns were analyzed with Agarose Gel Electrophoresis (AGE) utilizing a 0.8% (w/v) agarose gel in Tris-Acetate-EDTA buffer (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) containing 0.5 µg/ml ethidium bromide. Digestions were electrophoresed at 100 V for approximately 1 hour with DNA bands detected using

UV illumination (XRS Chemidoc, Biorad). Approximate DNA band sizes (in base pairs, bp) were determined using the KAPA Universal ladder (KAPA Biosystems).

2.4. Routine mammalian cell culture.

HEK293FT human embryonic kidney cell line (kind gift from Prof Sharon Prince, UCT) was cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco, Invitrogen) supplemented with 1% (v/v) GlutaMAX (Gibco, Invitrogen), 10% (v/v) FBS (BioWest), 0.1 mM Non-Essential Amino Acids (Gibco, Invitrogen) 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml PSA (Sigma-Aldrich) and 500 µg/ml G418 (Santa Cruz Biotech) (HEK media) at 37°C and 10% CO₂. The HEK-hOCT4 cell line developed as part of this study was routinely maintained in HEK media additionally supplemented with 2 µg/ml puromycin (Sigma-Aldrich). Cells were monitored for mycoplasma contamination using 1 µg/ml Hoechst 33342 (Santa Cruz Biotech) with immunofluorescence images taken using the Zeiss LSM510 meta confocal microscope (Zeiss).

2.5. Transfection and generation of lentiviral-transgene infectious particles.

HEK293FT cells were seeded 24 hours before transfection into 6-well culture plates in HEK media at a concentration of 2×10^4 cells per well. Twenty-four hours post seeding, cells were transfected with lentiviral packaging vector psPAX2 (450 ng per 6-well well), lentiviral capsid vector pMD2.G (50 ng per 6-well well) and pSIN-EF2-OCT4-Pur (500 ng per 6-well well) using X-treme GENE HP Transfection Reagent (ROCHE). Virus particle-containing supernatant was collected 24 and 48 hours post-transfection and filtered using 0.45 µm filters. Filtered viral supernatant was stored at -80°C until transduction procedure.

2.6. Transduction and Generation of polyclonal HEK-hOCT4.

HEK293FT cells were seeded at a concentration of 2×10^4 cells per well into 6-well culture plates 24 hours prior to transduction. Cells were transduced at 70 - 90% confluence using pre-warmed viral supernatant supplemented with 8 µg/ml

hexadimethrine bromide (Sigma-Aldrich) for 24 hours at 37°C and 10% CO₂. Positive transduced cells were selected for using HEK media supplemented with 2 µg/ml puromycin (Sigma-Aldrich). Puromycin-resistant colonies were pooled after 5 weeks for selection pressure to create polyclonal HEK-hOCT4 with constitutive OCT4 expression confirmed by Western analysis. HEK-hOCT4 cells were subsequently expanded and maintained in HEK media supplemented with 2 µg/ml puromycin.

2.7.Co-immunoprecipitation of human OCT4 and HSP90α/β.

HEK-hOCT4 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EGTA/EDTA, 1 mM Na₃VO₄, 1% (v/v) NP40, 1 mM sodium deoxycholate, 1 mM PMSF, 1 mM protease inhibitor cocktail (Sigma-Aldrich)] and stored at -20°C until co-immunoprecipitation. Co-immunoprecipitation of human OCT4 was performed using MagReSyn protein A beads (ReSyn Biosciences) as per manufacturer's instructions at a ratio of 1 µg αOCT4 rabbit (AbCAM) capture antibody: 1 mg RIPA lysate loaded. For HSP90α/β co-immunoprecipitation, co-immunoprecipitation was performed using MagReSyn protein G beads (ReSyn Biosciences, RSA) as per manufacturer's instructions at a ratio of 5 µg αHSP90α/β goat (Santa Cruz Biotech) capture antibody: 1 mg RIPA lysate loaded. Capture antibodies were immobilized onto protein A or protein G MagReSyn beads for 30 minutes at 25°C in binding/wash buffer [50mM Tris-HCl (pH7.4), 150 mM NaCl, 0.05% (v/v) Tween 20] followed by three wash steps using binding/wash buffer for 5 minutes each. RIPA cell lysates were incubated with capture antibody-immobilized protein A or protein G MagReSyn beads at 4°C for 24 hours with agitation followed by three wash steps using binding/wash buffer for 5 minutes each. The capture antibody-target protein complex was eluted from protein A and protein G MagReSyn beads using denaturing elution buffer (5 x SDS-PAGE sample buffer). Corresponding negative control co-immunoprecipitation reactions were performed with all listed reagents and steps excluding addition of a capture antibody.

2.8.Effect of HSP90 inhibition on human OCT4 and known HSP90 client proteins in HEK-hOCT4.

HEK-hOCT4 cells were seeded at a concentration of 0.8×10^4 or 0.4×10^4 cells per well into 12-well or 24-well culture plates respectively for 24 hours prior to treatment. HEK-hOCT4 cells were treated with varying concentrations (0 – 1000 nM) geldanamycin (GA) or novobiocin (NOV; 0 – 1000 μ M) [GA dissolved in dimethyl sulphoxide (DMSO, final treatment concentration of 0.002%), NOV stocks dissolved in dH₂O]. Subsequent dilutions of GA and NOV were performed in fresh HEK media with cells being treated for 24 hours prior to cell lysis in 5x SDS-PAGE sample buffer. Protein levels of human OCT4, HSP90 α/β and known HSP90 client proteins AKT and CDK4 were determined using Western blot analyses. Histone H3 was used a protein loading control to account for protein loading.

2.9. Effect of proteasome inhibition and co-inhibition of proteasome and HSP90 on human OCT4 protein levels in HEK-hOCT4 cells.

HEK-hOCT4 cells were seeded at a concentration of 0.8×10^4 cells per well 24 hours prior to treatment with inhibitors diluted in fresh HEK media. Cells were treated for 24 hours with increasing MG132 (Sigma-Aldrich; MG132 stock dissolved DMSO, final DMSO treatment concentration of 0.01%) concentrations (0 – 10 μ M) alone; co-treatment with a constant concentration of MG132 (1 and 10 μ M) and increasing concentrations of GA (0 – 1000 nM); or co-treatment with constant GA (100, 250 or 1000 nM) and increasing MG132 (0 – 10 μ M) concentrations. All subsequent dilutions of MG132 and GA were performed using HEK media. For MG132 co-treatments with GA, cells were additionally pre-treated for 2 hours with MG132 at specified concentrations. Post treatments cells were lysed in 5 x SDS-PAGE sample buffer and human OCT4 and HSP90 α/β protein levels analyzed by Western blot analysis.

2.10. Effect of specific kinase inhibition on human OCT4 and HSP90 α/β in HEK-hOCT4 cells.

2.10.1. Human OCT4 co-immunoprecipitation with kinase inhibition.

Kinase inhibitors [H7 (Sigma Aldrich), D4476 (Sigma Aldrich) and ERK2i (Sigma Aldrich)] were dissolved in DMSO. Subsequent dilutions were performed in HEK

media. HEK-hOCT4 cells (1×10^6 cells) were seeded into T75 culture flasks 24 hours prior to treatments. Cells were treated with 6 μ M H7, 0.3 μ M D4476, 25 μ M ERK2i or DMSO (as a vehicle control, final concentration of 0.0625%) for 24 hours using fresh HEK media. Treated cells were lysed in RIPA buffer after which human OCT4 co-immunoprecipitation was performed. Human OCT4 and HSP90 α/β protein levels were analyzed by Western blot.

2.10.2. Analysis of kinase inhibition on human OCT4 protein levels.

HEK-hOCT4 cells were seeded at a concentration of 0.8×10^4 cells per well into 12-well culture plates 24 hours prior to treatment. Cells were treated for 24 hours with 5-fold decreased and increased kinase inhibitor concentrations relative to co-immunoprecipitation working concentrations as follows: H7 (1.2, 6 and 30 μ M); D4476 (0.06, 0.3 and 1.5 μ M); ERK2i (5, 25 and 125 μ M); and DMSO (0.0625% final concentration). Dilutions of kinase inhibitors performed in HEK media. Cells were lysed in 5 x SDS-PAGE sample buffer with human OCT4 protein levels analyzed by Western blot.

2.10.3. ERK2i inhibitor kinase time course analysis on human OCT4 protein levels.

HEK-hOCT4 cells were seeded at a concentration of 0.8×10^4 cells per well into 12-well culture plates 24 hours prior to treatment. Cells were treated with 25 μ M ERK2i diluted in fresh HEK media or DMSO (0.0625%) for increasing time intervals (0, 2, 4, 8, 16 and 24 hours). Cells were lysed in 5 x SDS-PAGE sample buffer with human OCT4 protein levels analyzed by Western blot.

2.10.4. Effect of MEK1/2 inhibition on human OCT4 protein levels.

HEK-hOCT4 cells were seeded at a concentration of 0.8×10^4 cells per well into 12-well culture plates respectively for 24 hours prior to treatment. Cells were treated with increasing concentrations of MEK inhibitor U1026 diluted in HEK media (0 – 12 μ M; Cell Signaling Technologies) or DMSO control (0.008%) for 24 hours prior to cell

lysis in 5 x SDS-PAGE sample buffer and Western blot analyses for human OCT4 and HSP90 α/β protein levels.

2.11. Immunofluorescence and Confocal microscopy.

HEK-hOCT4 cell samples were seeded onto Attachment Factor (Gibco, Invitrogen) coated glass coverslips and treated as required. Cell samples were fixed with flash incubation in ice-cold methanol. Fixed cells were permeabilized with 0.3% (v/v) Triton X-100 in PBS for 5 minutes at room temperature with agitation. Prior to primary antibody incubation fixed cells were incubated in blocking buffer [1% (w/v) BSA/PBS] for 1 hour at room temperature with agitation. Primary antibody [diluted in 1% (w/v) BSA/PBS] incubation was carried out at 4°C for 24 hours with agitation followed by three 5 minute washes with 0.1% (w/v) BSA/PBS. Samples were incubated with secondary antibodies [diluted in 1% (w/v) BSA/PBS] at room temperature for 1 hour in the dark followed by three 5 minute washes with 0.1% (w/v) BSA/PBS. Nuclei were counterstained with a flash incubation with PBS containing 1 μ g/ml Hoechst 33342 (Santa Cruz Biotech). Coverslips were mounted onto glass slides using Dako mounting media (Dako). Immunofluorescence images were taken using the Zeiss AxioVert.A1 FL-LED fluorescence microscope (Zeiss) or Zeiss LSM510 meta confocal microscope (Zeiss).

2.12. Subcellular fractionation of HEK-hOCT4 cells using NE-PER extraction kit.

HEK-hOCT4 cells (2.5×10^6 cells) were pelleted by centrifugation at 500x g for 3 minutes. Cytoplasmic and nuclear protein fractions were isolated using the NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific) as per manufacturer's instructions. Human OCT4 and HSP90 α/β protein levels from equal volumes of resulting cytoplasmic and nuclear fractions were analyzed by Western blot. Histone H3 and GAPDH were used to determine the purity of nuclear and cytoplasmic fractions respectively.

2.13. Subcellular fractionation of HEK-hOCT4 cells treated with HSP90 inhibitor GA using REAP fractionation.

HEK-hOCT4 cells (0.8×10^6) were seeded into 100 mm culture dishes 24 hours prior to treatment. Cells were treated with 100 nM and 1000 nM GA for 24 hours prior to REAP subcellular fractionation using the protocol established by Suzuki and colleagues (Suzuki *et al*, 2010). Untreated and DMSO treated samples were used as controls. Cells were washed in ice-cold PBS twice prior to mechanical lifting of cells, cell collection and centrifugation of cells at 13 000x g for 30 seconds. Cell pellets were resuspended in 0.1% (v/v) NP40-PBS by trituration (whole cell lysate fraction) before centrifugation at 13 000x g for 30 seconds. The resulting supernatant was further processed as the cytoplasmic fraction while the resulting pellet was resuspended in 0.1% (v/v) NP40-PBS and further processed as the nuclear fraction. Human OCT4 and HSP90 α/β protein levels were analyzed by Western blot. Histone H3 was used to determine the purity of the nuclear fractions.

2.14. Effect of HSP90 inhibition on human OCT4 transactivation using OCT responsive luciferase reporter assay.

The pTATA-37Tk-LUC control vector, pTATA-6W-37Tk-LUC, pTATA-PORE-37Tk-LUC and pTATA-MORE-37Tk-LUC OCT4 responsive luciferase vectors were a kind gift from Prof. Dr. Hans Scholer (Max Planck Institute for Molecular Biomedicine, Germany). HEK-hOCT4 cells were seeded at a concentration of 0.2×10^4 cells per well in triplicate into 24-well culture plates 24 hours prior to reporter vector transfections. Cells were transfected with pTATA-37Tk-LUC control vector, pTATA-6W-37Tk-LUC, pTATA-PORE-37Tk-LUC or pTATA-MORE-37Tk-LUC together with pECFP-C1 as a transfection control vector, using X-treme GENE HP transfection reagent (ROCHE) for 24 hours. Post transfection, cells were treated in triplicate with 100 nM, 500 nM or 1000 nM GA for 24 hours, with DMSO treated and untreated samples used as controls. Following treatments cell were lysed in 1% (v/v) Triton X-100 and resuspended in 2x FLAR buffer [400 mM Tricine, 200 μ M EDTA, 5.14 mM MgSO₄, 34 mM DTT, 250 μ M ATP and 100 μ M D-luciferin (Promega)] (Siebring-van Olst *et al*, 2012). Each resuspension was divided into three separate wells of a black-walled 96-well plate with ECFP fluorescence recorded at 485nm (excitation at 436nm) (accounting for transfection control using the pECFP-C1

vector) using the SynergyMX spectrophotometer. Samples were then transferred into a white-walled 96-well plate and luminescence was recorded using the SynergyMX spectrophotometer (BioTek) with sensitivity set to 160.

2.15. Flow cytometry analysis of pluripotent stem cell transcription factors.

The expression levels of Nanog, OCT4 and SOX2 in cell lines were analyzed using the Human pluripotent stem cell transcription factor analysis kit (BD) as per manufacturer's protocol. Cells (1×10^6) were lifted using trypsin and washed twice with PBS. Cells were permeabilized with 1x BD Perm/Wash buffer and resuspended to a final concentration of 1×10^7 cells/ml. Test and isotype control samples were incubated with test antibodies (PE-Nanog, PerCP-Cy5.5 OCT4 or Alexa Fluor647 SOX2) or fluorophore matched isotype control antibodies (PE isotype control, PerCP-Cy5.5 isotype control or Alexa Fluor 647 isotype control) for 30 minutes at room temperature in the dark. For compensation controls negative and anti-mouse beads were incubated with all specified test antibody for 30 minutes at room temperature in the dark. Samples were then analyzed using a FACS Aria III fluorescence-activated cell sorter and FACSDiva software. The compensation controls were used to generate compensation values attributing to fluorescence spectra overlap between channels. For each test and isotype sample 50 000 live events were recorded and data analyzed using FlowJo software (NIH). Isotype controls were used to establish fluorescence threshold gates to determine the number of events showing positive expression of each of the stem cell transcription factors. Results are displayed as histograms with gates accounting for the percentage of events displaying positive expression of each transcription factor.

2.16. Feeder layer-dependent culture and maintenance of induced Pluripotent Stem Cells (iPSCs).

The induced Pluripotent Stem Cell (iPSC) cell line was a kind gift from Dr Janine Scholefield and Dr Musa Mhlana (CSIR Biosciences). Mouse embryonic fibroblasts (MEFs) Passage 0 (CF-1; ATCC) were maintained and expanded to Passage 6 (at an expansion ratio of 1:3) in MEF media [DMEM supplemented with 15% (v/v) FBS,

3.7 g/L sodium bicarbonate (Sigma-Aldrich), 0.1 mM sodium pyruvate and 100 U/ml PSA at 37°C and 10% CO₂]. At Passage 6 MEFs were mitotically inactivated with MEF media supplemented with 0.01 mg/ml Mitomycin C (Sigma-Aldrich) for 2 hours at 37°C. Mitotically inactivated MEFs (iMEFs) were cryopreserved in liquid nitrogen using MEF media supplemented with 40% (v/v) FBS and 10% (v/v) DMSO. iMEFs were plated at a concentration of 1.5×10^5 cells per culture area of 9.5 cm² on Attachment Factor coated plates with MEF media and incubated at 37°C and 10% CO₂ 24 hours prior to iPSC culturing. iPSCs were cultured on iMEF feeder layers in iPSC media [Knockout DMEM/F-12 (Gibco, Invitrogen), 20% (v/v) Knockout Serum Replacement (Gibco, Invitrogen), 0.1 mM Non-Essential Amino Acids, 4 ng/ml basic Fibroblast Growth Factor (bFGF; Sigma-Aldrich), 2 mM L-glutamine, 0.1 mM 2-Mercaptoethanol (Sigma-Aldrich), and 100 U/ml PSA] at 37°C and 10% CO₂. The iPSC media was replaced daily. Mechanical dissociation was performed for iPSC expansion and routine maintenance using UV sterilized glass pipettes visualized using a dissecting microscope with dark field illumination.

2.17. Feeder layer-free culture and maintenance of induced Pluripotent Stem Cells (iPSCs).

Induced pluripotent stem cells were cultured feeder layer-free on 1% (v/v of DMEM/F-12) Geltrex (Life Technologies) coated dishes and flasks in StemPro media [DMEM/F-12 (Gibco), 2% (v/v) StemPRO growth supplement (Gibco), 1.8% (w/v) bovine serum albumin (BSA; Gibco), 8 ng/ml bFGF, 0.1 mM 2-Mercaptoethanol, and 100 U/ml PSA] at 37°C and 10% CO₂. The StemPro media was replaced daily. Enzymatic dissociation was performed for expansion and routine maintenance of feeder-free iPSCs using Accutase (Sigma-Aldrich) at a splitting ratio of 1:10 to 1:20.

Chapter 3. Generation of a constitutive human OCT4 expressing cell line.

3.1.Introduction

Self-renewal and pluripotency, the ability to differentiate into cell types of all three germ layers, are key and distinguishing characteristics of pluripotent stem cells. A cohort of transcription factors, of which OCT4 is vitally important, regulates these cellular properties (Nichols *et al*, 1998; Niwa *et al*, 2000; Mitsui *et al*, 2003). Previous research has suggested a relationship between OCT4 and the molecular chaperone HSP90 in murine embryonic stem cells (Bradley *et al* 2012). Although this study illustrated the importance of HSP90 function in murine embryonic stem cells (Bradley *et al*, 2012), the direct relationship between OCT4 and HSP90 remains unclear. It was not possible from this study to accurately determine whether the loss in OCT4 upon HSP90 inhibition was due to OCT4 being an obligate HSP90 client, or due to the fact that HSP90 inhibition induces differentiation, which is accompanied by a concomitant loss of OCT4 protein levels. There is, additionally, growing evidence describing key fundamental differences between human pluripotent stem cells and murine pluripotent stem cells. Although the pluripotency effector molecules (OCT4, SOX2 and Nanog) are conserved between human and murine systems, the signaling pathways modulating the stem cell state and molecular signals targeted by the pluripotency effector molecules exhibit species-specific differences (Figure 2; Sato *et al*, 2003; Ginis *et al*, 2004; Rao *et al*, 2004; Loh *et al*, 2006; Okita and Yamanaka, 2006; Schnerch *et al*, 2010). Differences are observed in the differentiation profiles created by modulation of pluripotency effector molecules. For instance, increased OCT4 expression causes differentiation to mesoderm and endoderm lineages in murine ESCs (Niwa *et al*, 2000) while in human ESCs only endoderm lineage differentiation is observed (Rodrigues *et al*, 2007). While reduced OCT4 expression levels causes trophoctoderm lineage differentiation in both murine and human ESCs, human ESCs also display mixed mesoderm/endoderm lineage differentiation (Niwa *et al*, 2000; Hay *et al*, 2004; Matin *et al*, 2004; Ivanova *et al*, 2006; Rodrigues *et al*, 2007). Cytokine expression profiles exhibit large differences between human ESCs and murine ESCs (Ginis *et al*, 2004). We therefore analysed the biochemical relationship between HSP90 and human OCT4 using an exogenous expression system in which human OCT4 expression was under transcriptional control of a constitutive promoter. This would negate influences on OCT4 expression resulting from HSP90 inhibition mediated changes to cell fate or differentiation. In this chapter, we describe

the development a model for the constitutive overexpression of human OCT4 with which to analyze the biochemical relationship between human OCT4 and HSP90. Without the indirect effects of off-target changes to HSP90 client proteins modulating stem cell biology and affecting OCT4 protein levels.

3.2.Results

3.2.1. Verification of lentiviral gene delivery vectors

A third generation lentiviral gene delivery system was used to introduce human OCT4 coding region under transcriptional control of the constitutive promoter EF1 α into a human cell line showing no human OCT4 endogenous expression. The third generation lentiviral gene delivery system utilized the envelope vector pMD2.G (Addgene: 12259), the packaging vector psPAX2 (Addgene: 12260) with the transgene vector encoding hOCT4 pSIN-hOCT4-Puro (Addgene: 16579; Yu *et al*, 2007), while the transgene vector pLV-TH encoding GFP (Addgene: 12262; Wiznerowics and Trono, 2003) was used as a control for optimization. All vectors were verified using restriction digestion analyses. For pMD2.G, the vector was digested with *HindIII* and *NotI* restriction endonucleases, with pMD2.G encoding a single recognition site for both restriction endonucleases (Figure 5.A). Uncut DNA sample of pMD2.G showed the presence of two vector conformations (lower band being supercoiled conformation and upper band being the open circular conformation; Figure 5.C: lane 2). Restriction digestion with *HindIII* (Figure 5.C: lane 3) and *NotI* (Figure 5.C: lane 4) alone linearized the vector producing a band of approximately 5824 bp. The presence of both endonucleases generated two bands corresponding to the expected sizes of 3046 bp and 2778 bp (Figure 5.C: lane 5). The psPAX2 vector was verified using *NheI* and *SacII* restriction endonucleases, both endonucleases were predicted to have only a single recognition site on psPAX2 (Figure 5.B). The uncut psPAX2 showed the presence of two vector conformations, namely linear (Figure 5.D: lane 2, lower band) and open circular (Figure 5.D: lane 2, upper band) conformations. Single endonuclease digestion using *NheI* linearized the psPAX2 vector generating a single band of approximate size 10 703 bp (Figure 5.D: lane 3). The single endonuclease digestion using *SacII* however generated two DNA bands unexpectedly, the upper band displaying approximate size to that of the original

vector (10 703 bp; Figure 5.D: lane 4, upper band) while the lower band showing approximate size of 500 bp (Figure 5.D: lane 4, lower band). Digestion using both endonucleases generated three bands with the middle and upper bands approximately the predicted size of DNA fragments generated by *NheI* and *SacII* endonuclease activity on the psPAX2 vector, 3749 bp and 6954 bp respectively (Figure 5.D: lane 5, middle and upper band). The lower band generated (Figure 5.D: lane 5, lower band) was the approximate size of the lower band present for psPAX2 digestion using *SacII* alone (Figure 5.D: lane 4, lower band). Digestion of psPAX2 with *NheI* and *SacII* was carried out with an endonuclease buffer that, although compatible with both *NheI* and *SacII*, was prone to *SacII* star activity therefore the likely cause of the lower bands visible with the presence of *SacII* (Figure 5.D, lanes 4 and 5, lower bands).

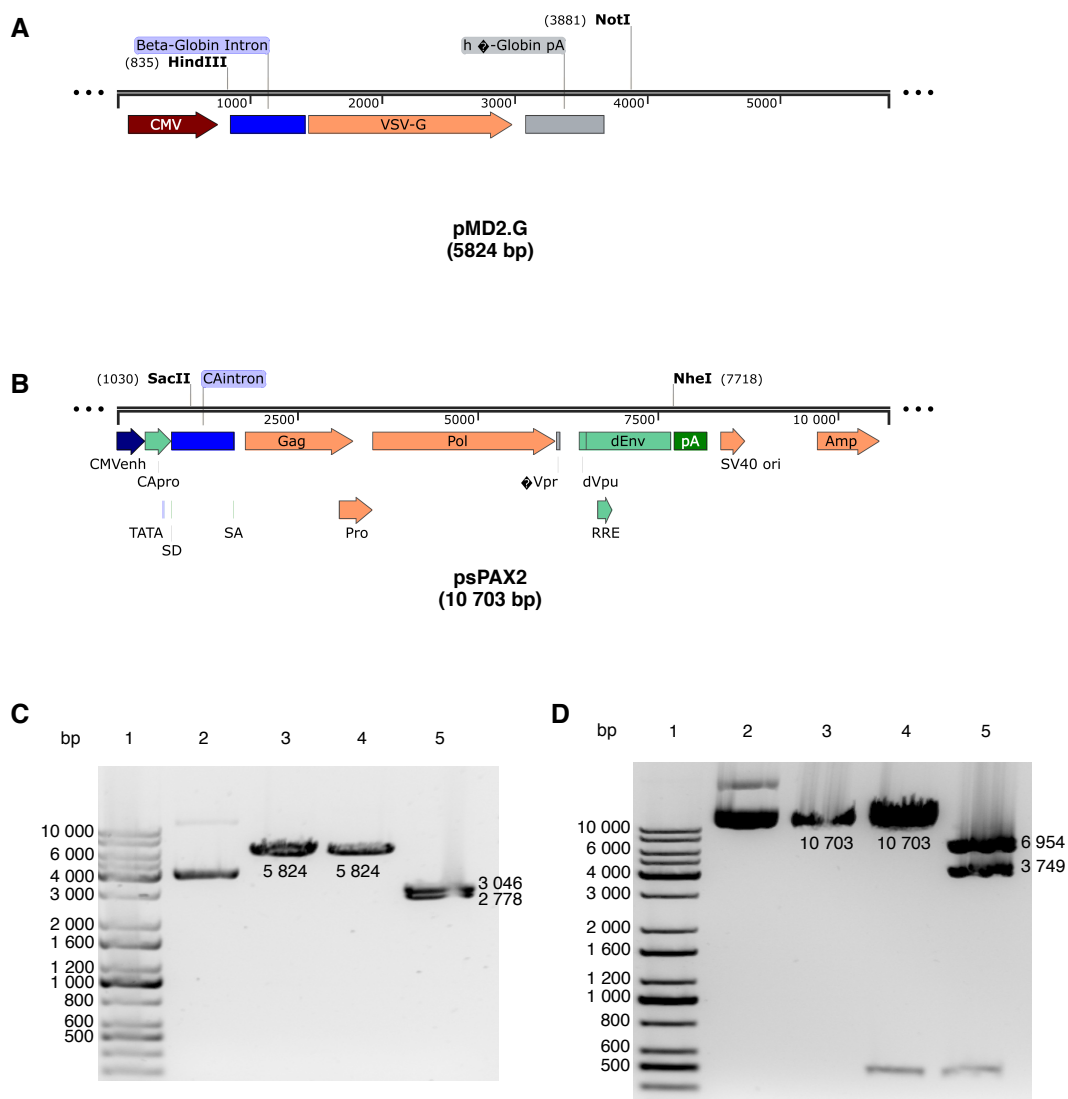


Figure 5 | Verification of lentiviral envelope vector and lentiviral packaging vector for third generation lentiviral gene delivery by restriction endonuclease digestion. Plasmid maps for lentiviral envelope vector pMD2.G (A) and lentiviral packaging vector psPAX2 (B) showing restriction enzyme recognition sites used for vector verification. (C) Restriction digestion verification by agarose gel electrophoresis of lentiviral envelope vector pMD2.G using *Hind*III and *Not*I restriction enzymes. Lane 1: KAPA universal DNA ladder. Lane 2: uncut pMD2.G. Lane 3: *Hind*III digestion. Lane 4: *Not*I digestion. Lane 5: *Hind*III and *Not*I double digestion. (D) Restriction digestion verification by agarose gel electrophoresis of lentiviral packaging vector psPAX2 using *Nhe*I and *Sac*II restriction enzymes. Lane 1: KAPA universal DNA Ladder. Lane 2: uncut psPAX2. Lane 3: *Nhe*I digestion. Lane 4: *Sac*II digestion. Lane 5: *Nhe*I and *Sac*II double digestion.

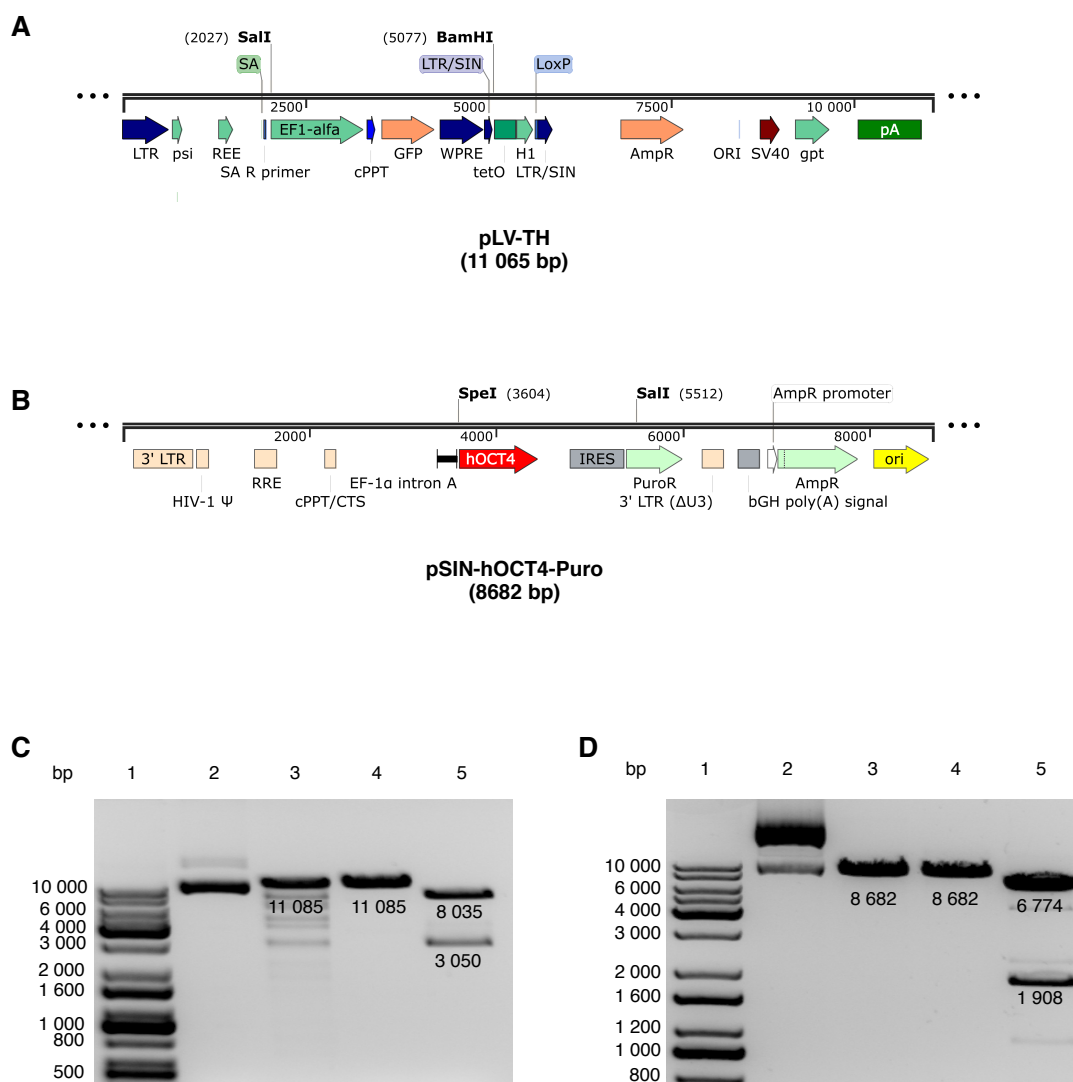


Figure 6 | Verification of lentiviral transgene vectors for third generation lentiviral gene delivery by restriction endonuclease digestion.

Plasmid maps for lentiviral transgene vectors pLV-TH (A) and pSIN-hOCT4-Puro (B) showing restriction enzyme recognition sites used for vector verification. (C) Restriction digestion verification by agarose gel electrophoresis of lentiviral transgene vector pLV-TH using *Bam*HI and *Sal*I restriction enzymes. Lane 1: KAPA universal DNA ladder. Lane 2: uncut pLV-TH. Lane 3: *Bam*HI digestion. Lane 4: *Sal*I digestion. Lane 5: *Bam*HI and *Sal*I double digestion. (D) Restriction digestion verification by agarose gel electrophoresis of lentiviral transgene vector pSIN-hOCT4-Puro using *Sal*I and *Spe*I restriction enzymes. Lane 1: KAPA universal DNA Ladder. Lane 2: uncut pSIN-hOCT4-Puro. Lane 3: *Sal*I digestion. Lane 4: *Spe*I digestion. Lane 5: *Sal*I and *Spe*I double digestion.

The pLV-TH transgene control vector was verified using *Bam*HI and *Sal*I restriction endonucleases with both endonucleases predicted to have a single recognition site (Figure 6.A). Uncut pLV-TH showed both supercoiled and open circular vector conformations (lower and upper respectively; Figure 6.C: lane 2). Digestion of pLV-TH with *Bam*HI (predominant band; Figure 6.C: lane 3) and *Sal*I (Figure 6.C: lane 4) produced bands of corresponding approximate size to pLV-TH, 11 085 bp. pLV-TH digestion using both *Bam*HI and *Sal*I endonucleases generated as predicted two DNA bands of approximate sizes 3050 bp and 8035 bp (Figure 6.C: lane 5, lower band and upper band). Numerous staggered bands were observed for the *Bam*HI endonuclease digestion (Figure 6.C: lane 3), these were likely the result of star activity in this reaction as these bands were not observed for the digestion of pLV-TH using *Bam*HI and *Sal*I (Figure 6.C: lane 5). The restriction endonuclease verification of pSIN-hOCT4-Puro, the hOCT4 encoding transgene vector, was performed using *Sal*I and *Spe*I restriction endonucleases recognizing single sites for pSIN-hOCT4-Puro (Figure 6.B). Two vector conformations were evident for the uncut pSIN-hOCT4-Puro sample, the lower band representing linear conformation and the upper band representing open circular vector conformation (Figure 6.D: lane 2). Digestion of pSIN-hOCT4-Puro using *Sal*I and *Spe*I alone linearized the vector generating single DNA band of approximate size 8682 bp (Figure 6.D: lanes 3 and 4 respectively). The digestion using both *Sal*I and *Spe*I generated two, as predicted, predominant DNA bands of approximate sizes 1908 bp and 6774 bp (Figure 6.D: lanes 5, predominant lower and upper bands respectively). For the pSIN-hOCT4-Puro double endonuclease digestion several other lower concentrated DNA bands were observed (Figure 6.D: lane 5) however as these bands were not detected for the digestions using *Sal*I and *Spe*I alone (Figure 6.D: lanes 3 and 4 respectively) these were likely star activity in this reaction.

3.2.2. Generation of infectious lentiviral particles

To generate transgene containing lentiviral particles HEK293FT cells were transfected with the envelope vector (pMD2.G; Figure 5.A), the packaging vector (psPAX2; Figure 5.C), as well as a transgene vector [either pLV-TH (Figure 6.A) or pSIN-hOCT4-Puro (Figure 6.C)]. The pLV-TH vector, encoding GFP (Figure 6.A) served as the control transgene vector to determine the transfection efficiency,

transgene containing lentiviral particle generation efficacy and transduction efficiency. GFP fluorescence was used as the read-out for positive transfection and transduction (with transduction resulting from successful transgene containing lentiviral particle generation). For transfection efficiency analysis HEK293FT cells were transfected with pMD2.G, psPAX2 and pLV-TH plasmids using a concentration ratio of 1:9:10 using X-tremeGENE HP transfection reagent. Fluorescence microscopy imaging was performed 24 hours post-transfection. Greater than 80% transfection efficiency was observed with GFP fluorescence detected in the majority of cells (Figure 7.A). The pMD2.G and psPAX2 vectors contained no fluorescent protein markers thus the transfection efficiency of these vectors specifically was unable to be visualized using fluorescence microscopy.

The pLV-TH lentiviral particles were generated by transfection of HEK293FT cells with pMD2.G, psPAX2 and pLV-TH using a concentration ratio of 1:9:10 respectively. Supernatants containing pLV-TH lentiviral particles were harvested 24 hours and 48 hours post-transfection and pooled. For viral transduction, HEK293FT cells were treated with increasing volumes of supernatant containing pLV-TH lentiviral particles. The efficacy of pLV-TH lentiviral particle generation and transduction efficiency was analyzed based on GFP fluorescence using fluorescence microscopy 72 hours post-transduction (Figure 7.B). The negative control comprised of un-transduced HEK293FT cells, while the presence of GFP expression in transduced HEK293FT cells signified the successful generation of pLV-TH lentiviral particles (Figure 7.B: GFP channel). A volume dependent increase in GFP fluorescence was observed for transductions using 100, 400 and 800 μ l of supernatant containing pLV-TH lentiviral particles in HEK293FT cells (Figure 7.B). A transduction volume of 1000 μ l was subsequently selected for future transductions as the greater concentration of lentiviral particles (arising from a greater transduction volume) had no negative effects on transduction efficiency.

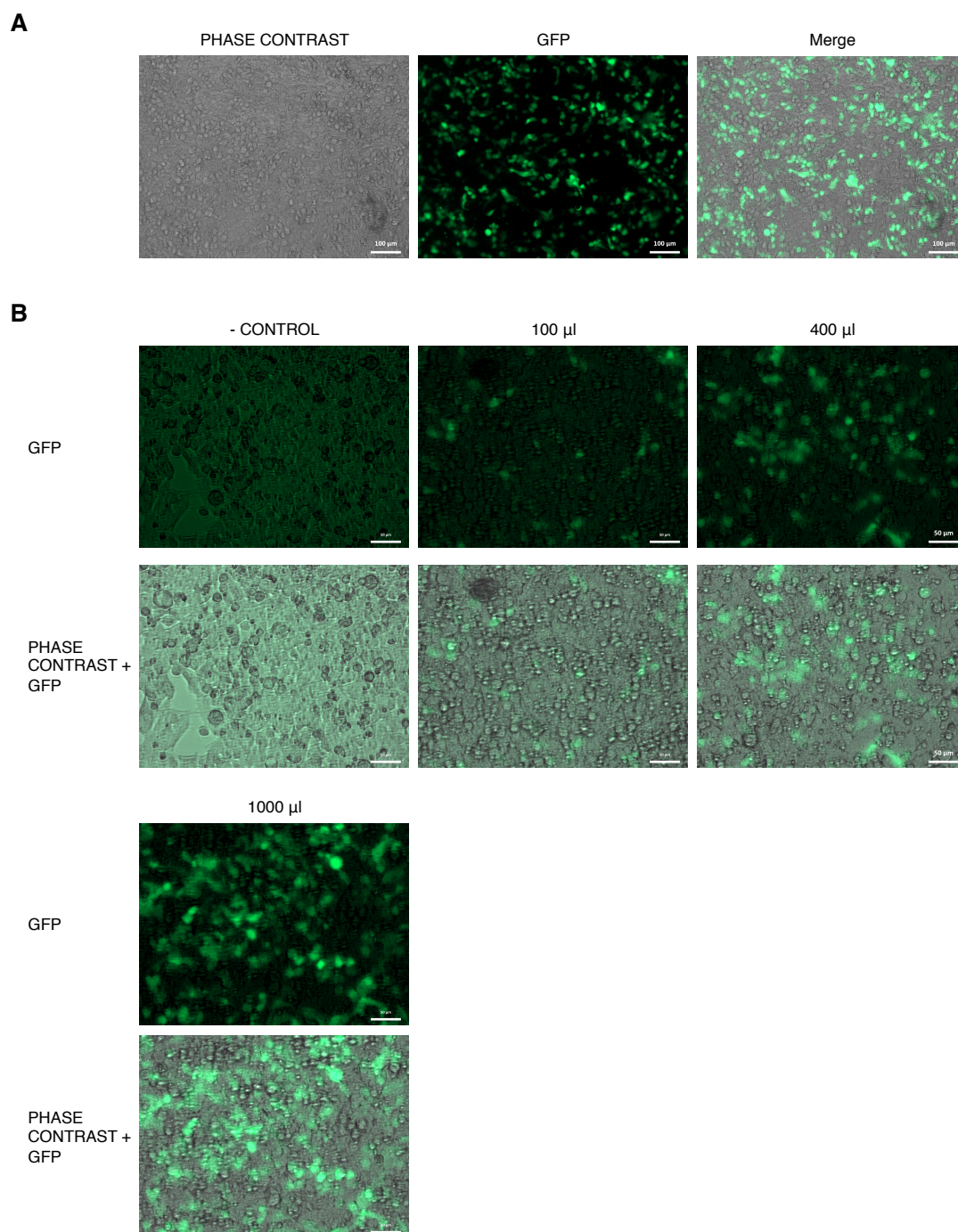


Figure 7 | Validation of lentiviral particle production procedure using pLV-TH control lentiviral transgene vector.

(A) Analysis of transfection efficiency of lentiviral gene delivery vectors pMD2.G, psPAX2 and pLV-TH into HEK293FT cells using fluorescence microscopy for GFP fluorescence (pLV-TH). (B) HEK293FT cells were co-transfected with lentiviral gene delivery vectors pMD2.G, psPAX2 and pLV-TH (ratio of 1:9:10) using X-tremeGENE HP (Roche). pLV-TH viral particle containing supernatants was harvested 24 and 48 hours post transfection and pooled. Validation of lentiviral particle production and transduction efficiency was analyzed using GFP fluorescence 72 hours post virus particle transduction into HEK293FT. Volumes (μ l) indicate volume of viral supernatant used for transduction procedure. Images taken using Zeiss AxioVert.A1 FL-LED fluorescence microscope (20x objective). Scale bar: 50 μ m. n = 2.

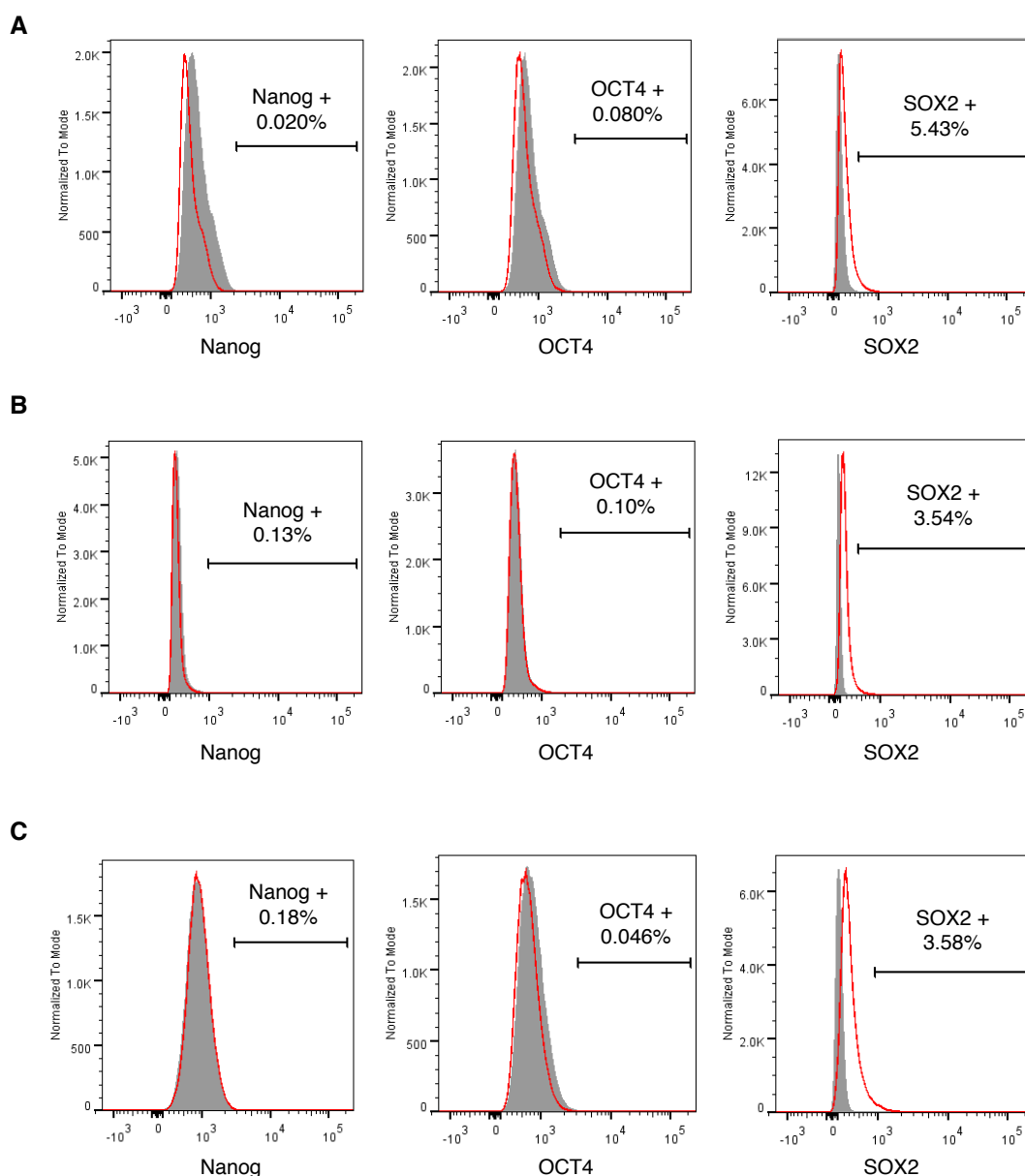


Figure 8 | Cell line screening for endogenous pluripotent stem cell transcription factor expression.

The MCF-7 (A), A549 (B) and HEK293FT (C) cell lines were screened for endogenous expression of the pluripotent stem cell transcription factors Nanog, OCT4 and SOX2 using flow cytometry using the BD Human Pluripotent Stem Cell Transcription Factor Analysis Kit. A total of 1×10^6 cells for each cell line were harvested and permeabilized and stained as per the manufacturer's recommendations with isotype controls included. Compensation controls accounting from overlapping of fluorescence spectra were performed using negative and anti-mouse IgG beads. For both isotype (grey shading) and test samples (red line) for each cell line 50 000 events were recorded using a FACS Aria III flow cytometer and FACSDiva software (BD). Histogram graphs were generated using FlowJo software with gates recording events showing expression presence of the specific transcription determined using the isoform controls in each case.

3.2.3. Generation of the HEK-hOCT4 stable cell line

We initially screened our available human cell lines for the expression of endogenous Nanog, OCT4 and SOX2 transcription factors (Figure 8). In order to create a model in which human OCT4 expression was not under transcriptional regulation of outside factors, we needed to use a parent cell line in which OCT4 and factors regulating endogenous expression of OCT4 (such as Nanog and SOX2) were absent. Using a commercially available stem cell transcription factor analysis kit we analyzed the expression of Nanog, OCT4 and SOX2 in MCF-7, A549 and the HEK293FT cell lines. The MCF-7 breast carcinoma cell line showed no expression of Nanog and OCT4 (Figure 8.A). The MCF-7 cell line contained a small percentage of cells displaying positive SOX2 expression (5.43%; Figure 8.A). Both the A549 lung carcinoma and the HEK293FT cell lines showed negligible expression of Nanog and OCT4 (Figure 8.B and C). Like the MCF-7 cell line, both the A549 and HEK293FT cell line showed minor populations of cells displaying positive expression of SOX2 (3.54% and 3.58% respectively; Figure 8.B and C). Both the MFC-7 and A549 cell line are derived from cancer tissue while the HEK293FT cell line, although transformed, is derived from non-cancerous tissue. It is for this reason together with the negligible expression of stem cell transcription factors and high transfection efficiencies achievable with this cell line, that the HEK293FT cell line was chosen as the parent cell line for the production of an OCT4 constitutive expression model.

In order to generate the cell line with constitutive expression of human OCT4, a transgene lentiviral vector encoding for hOCT4 under control of the EF1 α constitutive promoter, pSIN-hOCT4-Puro (Figure 7.A), was used to produce lentiviral particles according to the protocol previously optimized with the pLV-TH plasmid. The EF1 α promoter additionally transcriptionally regulated the puromycin resistance gene allowing for selection of pSIN-hOCT4-Puro transduced cells. To produce lentiviral particles, HEK293FT cells were transfected with pMD2.G, psPAX2 and pSIN-hOCT4-Puro vectors at a concentration ratio of 1:9:10. Supernatant containing pSIN-hOCT4-Puro lentiviral particles were harvested 24 and 48 hours post-transfection and pooled. The pSIN-hOCT4-Puro lentiviral particle containing supernatant was then used to transduce HEK293FT cells for 72 hours prior to puromycin selection for transduced cells using 2 μ g/ml puromycin. Both non-

transduced HEK293FT cells, and HEK293FT cells transduced for 72 hours with pSIN-hOCT4-Puro lentiviral particles, were treated with 2 $\mu\text{g/ml}$ puromycin for 5 days (Figure 9.A: I and II). HEK293FT cells treated for 5 days with 2 $\mu\text{g/ml}$ puromycin had lost their adherence and were detached [Figure 9.A: I (inset, black arrows)], a feature of cells having undergone apoptosis [Figure 9.A: I (inset, black arrows)]. After 5 days of 2 $\mu\text{g/ml}$ puromycin treatment, HEK293FT cells transduced with pSIN-hOCT4-Puro resulted in the formation of puromycin resistant colonies [Figure 9.A: II (inset, black arrows and blue line highlighting colony border)]. The puromycin selection was maintained until the puromycin resistant colonies could be pooled and expanded to generate the HEK-hOCT4 polyclonal cell line.

3.2.4. Analysis of exogenous human OCT4 expression and activity in the HEK-hOCT4 cell line

The presence of hOCT4 expression in the HEK-hOCT4 polyclonal cell line was assessed by Western blot analysis. Western blot of control HEK293FT cell lysate detected no hOCT4 protein (Figure 9.B: lane 1) however relatively high levels, relative to the protein loading control HSP90 α/β , of hOCT4 protein was observed in HEK-hOCT4 lysate (Figure 9.B: lane 2). To determine the stability of hOCT4 expression the presence of hOCT4 protein was initially assessed 6 weeks and 6 months post-transduction. Relatively high hOCT4 protein levels, relative to the protein loading control HSP90 α/β , were consistently observed at both 6 weeks and 6 months post-transduction (Figure 9.C: lane 1 and 2 respectively). HEK293FT control cell line lysate was used as a negative control with no hOCT4 protein level observed (Figure 9.C: lane 3). Together with the overexpression of hOCT4 (Figure 9.B and C), the HEK-hOCT4 cells displayed a different cell morphology (Figure 9.D: II) compared to that of the HEK293FT control cell line (Figure 9.D: I). The HEK293FT control cells grew in characteristic colony morphology with cellular extensions observed at colony borders [Figure 9.D: I (inset, black arrows)]. The HEK-hOCT4 cells displayed a greater propensity for non-colony growth with cells displaying a mesenchymal-like morphology with numerous cellular extensions [Figure 9.D: II (inset, black arrows)].

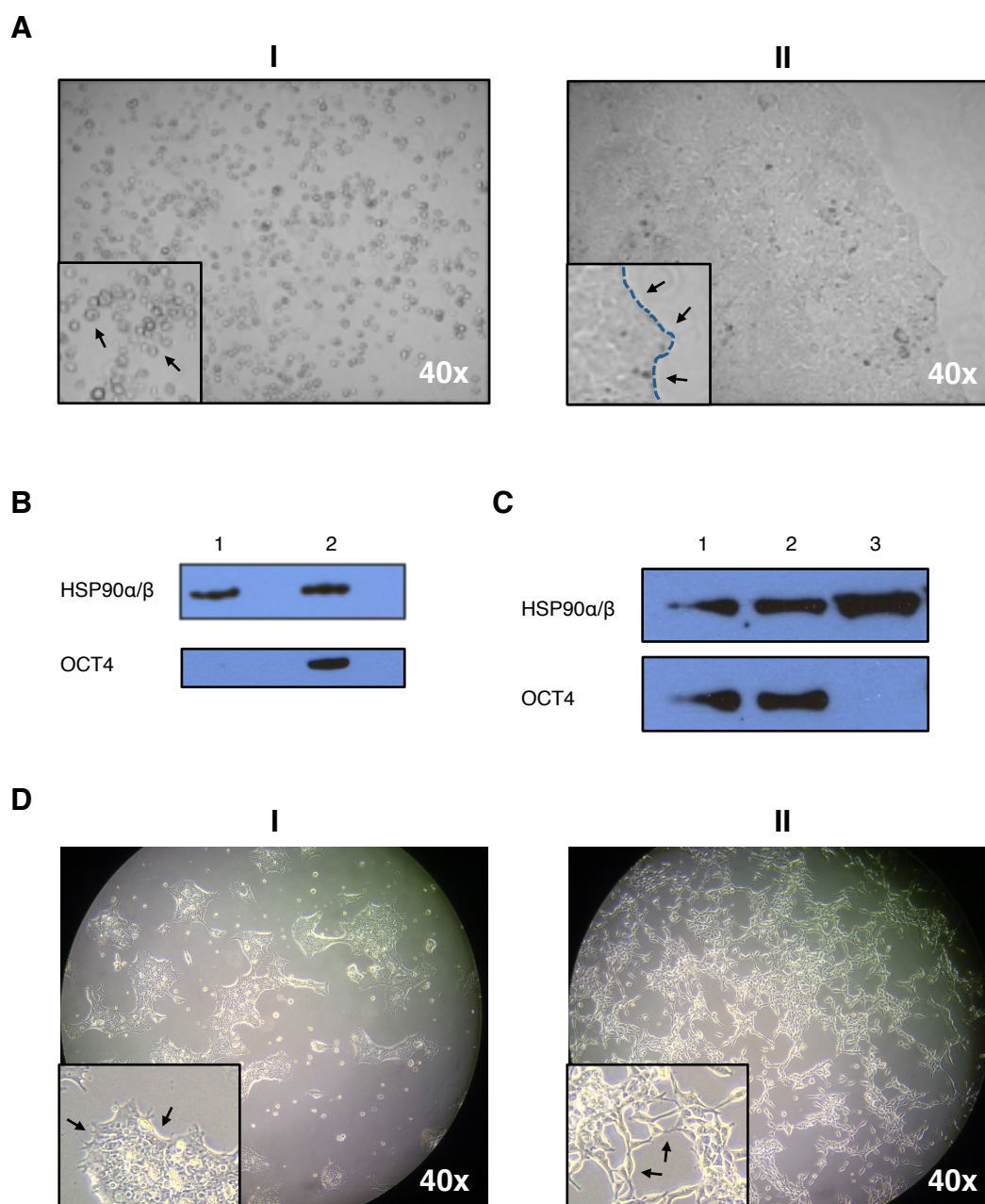


Figure 9 | Generation of HEK293FT cell line constitutively expressing OCT4 (HEK-hOCT4). (A) Non-transduced (I) and pSIN-hOCT4-Puro transduced (II) HEK293FT cells treated for 5 days with 2 μ g/ml puromycin. (II) Representative image of pSIN-hOCT4-Puro transduced HEK293FT cell colony after 5 days of 2 μ g/ml puromycin selection. pSIN-hOCT4-Puro transduced HEK293FT colonies were pooled to form the HEK-hOCT4 polyclonal transgenic cell line after 5 weeks of 2 μ g/ml puromycin selection. Confirmation of constitutive OCT4 expression in HEK-hOCT4 cells was determined by Western blot analysis (B, C). (B) Western blot detection of OCT4 expression in HEK293FT cell line (lane 1) and HEK-hOCT4 polyclonal cell line (lane 2). HSP90 α/β used as a protein loading control. (C) Western blot validation of stable constitutive OCT4 expression several months post transduction. Lane 1: 6 weeks post transduction with pSIN-hOCT4-Puro lentiviral particles. Lane 2: 6 months post transduction with pSIN-hOCT4-Puro lentiviral particles. Lane 3: Untransduced HEK293FT cell line. HSP90 α/β used as protein loading control. (D) Morphological differences observed between the HEK293FT cell line (I) and HEK-hOCT4 polyclonal cell line (II). Phase contrast images captured at 100x magnification using smartphone attached to Olympus CKX41 microscope. Scale bar: 100 μ m

To determine whether the exogenous human OCT4 constitutively expressed in the HEK-hOCT4 cell line displayed transcription activation activity, we transfected HEK-hOCT4 cells with luciferase reporter vectors. The luciferase reporter vectors encoded for the firefly luciferase gene under transcriptional control of various OCT4 responsive promoters (Figure 10). A control reporter vector (pTATA-37Tk-Luc; Figure 10.A) was used to account for the baseline level of transcriptional activation of the firefly luciferase gene with the Herpes simplex virus (HSV) thymidine kinase (Tk) TATA-box containing promoter. The transcriptional activity of monomeric OCT4 was assessed using the pTATA-6W-37Tk-Luc vector containing the octamer motif (ATGCAAT) located upstream of the HSV Tk TATA-box containing promoter (Figure 10.B). OCT4 is able to activate transcription in two distinct homodimer configurations (Remenyi *et al*, 2001). These homodimers interact with DNA sequences in two distinct configurations that are capable of recognizing two motifs comprised of different configurations of the octamer sequence, namely the Palindromic Oct factor Recognition Element (PORE; ATTTGAAATGCAAAT) sequence and the More PORE (MORE; ATGCATATGCAT) sequence. The pTATA-PORE-37Tk-Luc vector (Figure 10.C) was used to analyze OCT4 homodimer transcriptional activation of PORE sequences, while the pTATA-MORE-37Tk-Luc vector (Figure 10.D) was used to analyze OCT4 homodimer transcriptional activation of MORE sequences.

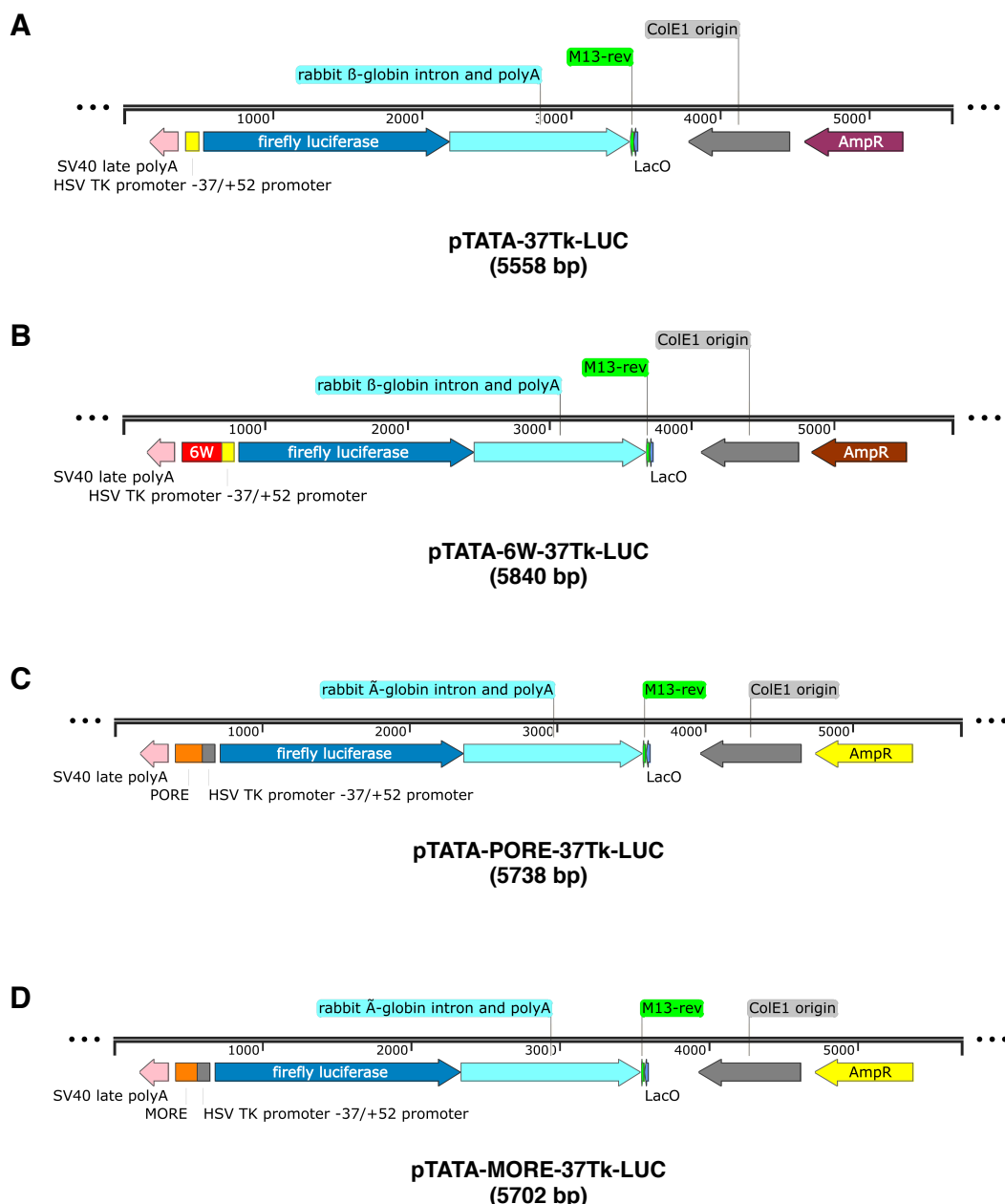


Figure 10 | Linear representations of luciferase vectors used for analyzing effect of HSP90 inhibition on human OCT4 activity in HEK-hOCT4 cells.

(A) pTATA-37Tk-LUC control vector accounting for basal level transcriptional activity of Herpes simplex virus (HSV) thymidine kinase (Tk) TATA-containing promoter. (B) pTATA-6W-37Tk-LUC vector containing the octamer binding motif (6W; CTGAGCAAAAACACCACCTGGGTAATTTGCATTTCTAAAATAAGTCGA). (C) pTATA-37Tk-PORE-LUC vector containing the Palindromic OCT factor Recognition Element (PORE) repeat sequence (ATTTGAAAGGCAAAT). (D) pTATA-37Tk-MORE-LUC vector containing the MORE palindromic OCT factor Recognition Element (MORE) repeat sequence (ATGCATATGCAT).

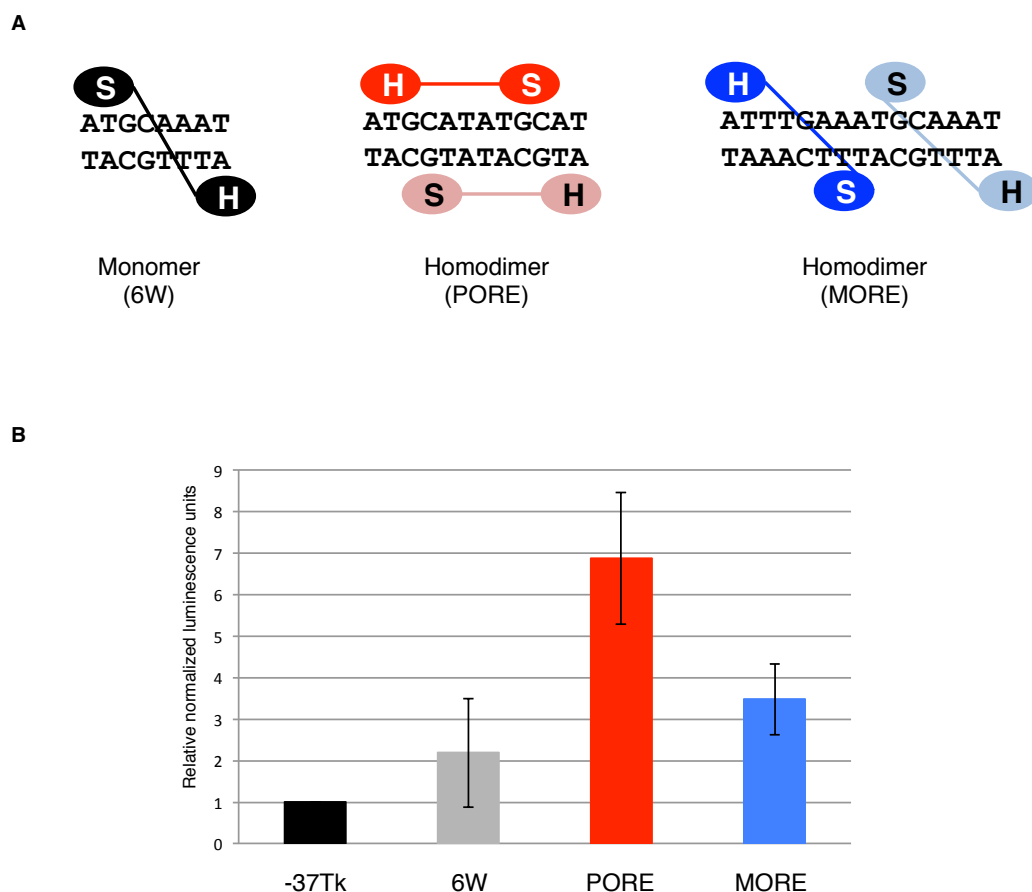


Figure 11 | Transcriptional activity of exogenous human OCT4 in HEK-hOCT4 cells. HEK-hOCT4 cells were transfected with pTATA-37Tk-Luc, pTATA-6W-37Tk-Luc (6W), pTATA-PORE-37Tk-Luc (PORE) or pTATA-MORE-37Tk-Luc (MORE) together with pECFP-C1, used as a transfection control, for 48 hours. Luminescence and fluorescence readings were recorded and data analyzed as indicated in Methodology 2.14. (A) Schematic diagrams representing OCT4 monomer and homodimer conformations (S, POU_S; H, POU_H) responsible for transcriptional activation of octamer (monomer; 6W), PORE and MORE promoter elements (Adapted from: Kang *et al*, 2009). (B) Graph of luciferase luminescence resulting from OCT4 transcriptional activity of 6W, PORE and MORE reporters generated using technical triplicates from triplicate independent transfections.

HEK-hOCT4 cells were transfected with each individual luciferase reporter vector together with pECFP-C1 as a transfection control vector. The resulting luminescence values were divided by the corresponding fluorescence values to create relative luminescence values normalized for transfection efficiency. The relative luminescence values were then normalized against the control reporter pTATA-37Tk-Luc (Figure 10.A) with the average for each biological triplicate and each technical triplicate calculated and represented in Figure 11. Exogenous human OCT4 in HEK-hOCT4 cells displayed both monomeric (6W) and homodimeric (PORE and MORE) transcription activation activity (Figure 11). Human OCT4 in HEK-hOCT4 cells showed greatest transcriptional activation of the PORE promoter elements (Figure 11.B), while transcriptional activation of 6W and MORE promoter elements displayed similar activities (Figure 11.B). This suggested that the constitutively expressed human OCT4 in HEK-hOCT4 cells displayed both monomeric and homodimeric transcriptional activation properties.

3.3.Conclusion

We screened our available human cell lines for the expression OCT4 and pluripotency genes associated with the transcriptional activation of endogenous OCT4. The HEK293FT cell line showed no endogenous OCT4 and Nanog expression and negligible expression of SOX2, and additionally displayed high transduction efficiencies. We therefore identified the HEK293FT cell line as the control cell line for our constitutive exogenous human OCT4 expression model. We successfully generated the HEK-hOCT4 cell line showing constitutive expression of human OCT4 using a lentiviral gene delivery system. We determined that the exogenous human OCT4 expressed in our model was active and able to transcriptionally activate an OCT4 activity reporter as both a monomer and homodimer. We subsequently used the HEK-hOCT4 cell line to study the biochemical relationship between HSP90 and human OCT4.

**Chapter 4. Biochemical analysis of the relationship
between HSP90 and human OCT4.**

4.1.Introduction

A given protein can be classified as an HSP90 client if it directly interacts with HSP90 and is dependent on HSP90 activity to maintain the stability of the protein (Taipale *et al*, 2010; Taipale *et al*, 2012). Previously murine OCT4 has been described as a HSP90 client protein using a murine ESC model (Bradley *et al*, 2012). Co-immunoprecipitation of murine OCT4 as well as the reciprocal co-immunoprecipitation of HSP90 α and HSP90 β suggested that murine OCT4 interacts with HSP90 α/β (Bradley *et al*, 2012). HSP90 inhibition using the geldanamycin derivative 17-AAG or HSP90 knockdown using miRNA resulted in reduction in murine OCT4 protein levels, suggesting that murine OCT4 was a HSP90 client protein (Bradley *et al*, 2012). Although suggestive of a HSP90 client status for murine OCT4, the murine ESC model used for this analysis may prove to be contentious due to the highly regulated signaling networks governing the homeostasis of embryonic stem cells that directly influence OCT4 protein levels. We suggest that a model in which OCT4 expression is not influenced by signaling networks reliant on HSP90 for regulation may provide a more accurate model in which to begin to understand the biochemical interplay between HSP90 and human OCT4. In this chapter we describe the biochemical analysis of the “client” status of human OCT4 using the HEK-hOCT4 polyclonal cell line model.

4.2.Results

4.2.1. Analysis of conservation of OCT4 across mammalian species

OCT4 is highly conserved protein across mammalian species in both protein and DNA sequences (Figure 12 and Table 1). This conservation is particularly noted between human (*Homo sapiens*) and primate species [*Pan troglodytes* (chimpanzee) with 100% protein sequence identity; and *Macaca mulatta* (rhesus macaque) with 98.3% protein sequence identity] (Figure 12 and Table 1). Interestingly human OCT4 displays a greater sequence identity with *Bos taurus* (cow; 90.8%) and *Canis lupus* (dog; 90.8%) than with OCT4 from rodent species [*Mus musculus* (mouse; 84.7%) and *Rattus norvegicus* (rat; 84.9%)] (Figure 12 and Table 1). For all species, the POU_S subdomain displays the greatest sequence conservation (Figure 12; orange box). Although highly conserved, the POU_H subdomain is less conserved than the POU_S subdomain (Figure 12: blue box).

<i>Canis lupus</i>	MAGHLASDLAFSPSPGGGGDGGPGGDPGWDPRAWLSFPGPPGGPALGPGVGPGEVWGL	60
<i>Bos taurus</i>	MAGHLASDFAFSPPPGGGGDGGPGGPEPGWVDPRTWMSFQGGPPGSGIGPGVVPGEVWGL	60
<i>Homo sapiens</i>	MAGHLASDFAFSPPPGGGGDGGPGGPEPGWVDPRTWLSFQGGPPGGPGIGPGVGPGEVWGI	60
<i>Pan troglodytes</i>	MAGHLASDFAFSPPPGGGGDGGPGGPEPGWVDPRTWLSFQGGPPGGPGIGPGVGPGEVWGI	60
<i>Macaca mulatta</i>	MAGHLASDFAFSPPPGGGGDGGPGGPETGWVDPRTWLSFQGGPPGGPGIGPGVGPGEVWGI	60
<i>Mus musculus</i>	MAGHLASDFAFSPPPGGG-DGSAGLEPGWVDPRTWLSFQGGPPGGPGIGP---GSEVLGI	55
<i>Rattus norvegicus</i>	MAGHLASDFAFSPPPGGG-DGSAGLEPGWVDPRTWLSFQGGPPSGPGIGP---GSEVLGI	55
	*****:****.*** **..* :.* ***:*** ***.**.*:*** **	
<i>Canis lupus</i>	PPCPPPFEFCGGMAYCGPQVGVGLLPQGGLDTSQPEGERGAGLESGSEGASPEPCAAPP	120
<i>Bos taurus</i>	PPCPPPDLGCGMAYCAPQVGVGPVPPGGLETPQPEGEAGAGVESNSEGASDPCAAPAG	120
<i>Homo sapiens</i>	PPCPPPFEFCGGMAYCGPQVGVGLVPQGGLETSQPEGEAGVGVESNSDASPEPCTVTPG	120
<i>Pan troglodytes</i>	PPCPPPFEFCGGMAYCGPQVGVGLVPQGGLETSQPEGEAGVGVESNSDASPEPCTVTPG	120
<i>Macaca mulatta</i>	PPCPPPFEFCGGMAYCGPQVGVGLVPQGGLETSQPEGEAGAGVESNSDASPEPCTVTPG	120
<i>Mus musculus</i>	SPCPPAYEFCGGMAYCGPQVGLGLVPQVGVELQPEQGAGARVESNSEGTSSEPCADRP	115
<i>Rattus norvegicus</i>	SPCPPAYEFCGGMAYCGPQVGLGLVPQVGVELQPEQGAGARVESNSEGASSGPCTARPS	115
	*****:*****.*****: * * *:*** ***: * .:..*:*.* **:	
<i>Canis lupus</i>	VVKPDKEKLEQNPEESQDIKALQKDLEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	180
<i>Bos taurus</i>	APKLDKEKLEPNPEESQDIKALQKDLEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	180
<i>Homo sapiens</i>	AVKLEKEKLEQNPEESQDIKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	180
<i>Pan troglodytes</i>	AVKLEKEKLEQNPEESQDIKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	180
<i>Macaca mulatta</i>	AVKLEKEKLEQNPEESQDIKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	180
<i>Mus musculus</i>	AVKL--EKVEPTPEESQDMKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	173
<i>Rattus norvegicus</i>	AVKL--EKVEPSPEESQDMKALQKELEQFAKLLKQKRITLGYTQADVGLTLGVLFKGVFS	173
	. * **.* .*****:*****:*****:*****:*****:*****:*****	
<i>Canis lupus</i>	QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR	240
<i>Bos taurus</i>	QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR	240
<i>Homo sapiens</i>	QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR	240
<i>Pan troglodytes</i>	QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR	240
<i>Macaca mulatta</i>	QTTICRFEALQLSFKNMCKLRPLLQKWVEEADNNENLQEICKAETLVQARKRKRTSIENR	240
<i>Mus musculus</i>	QTTICRFEALQLSLKNMCKLRPLLEKWVEEADNNENLQEICKSETLVQARKRKRTSIENR	233
<i>Rattus norvegicus</i>	QTTICRFEALQLSLKNMCKLRPLLEKWVEEADNNENLQEICKSETLVQARKRKRTSIENR	233
	*****:*****:*****:*****:*****:*****:*****:*****	
<i>Canis lupus</i>	VRGNLENMFLOCPKPTLQQTISHIAQQLGLEKDVVRVWFNRRQKGRSSSDYSQREDFEA	300
<i>Bos taurus</i>	VRGNLESMFLOCPKPTLQQTISHIAQQLGLEKDVVRVWFNRRQKGRSSSDYSQREDFEA	300
<i>Homo sapiens</i>	VRGNLENLFLOCPKPTLQQTISHIAQQLGLEKDVVRVWFNRRQKGRSSSDYAQREDFEA	300
<i>Pan troglodytes</i>	VRGNLENLFLOCPKPTLQQTISHIAQQLGLEKDVVRVWFNRRQKGRSSSDYAQREDFEA	300
<i>Macaca mulatta</i>	VRGSLENLFLOCPKPTLQQTISHIAQQLGLEKDVVRVWFNRRQKGRSSSDYAQREDFEA	300
<i>Mus musculus</i>	VRWSLETMFLKCPKPSLQQTTHIANQLGLEKDVVRVWFNRRQKGRSSIEYSQREEYEA	293
<i>Rattus norvegicus</i>	VRWNLENMFLOCPKPSLQQTSTIAQLGLEKDVVRVWFNRRQKGRSSIEYSQREEYEA	293
	** .**.:***:***:***: * *:****:*****:*****:*****:***:***:***	
<i>Canis lupus</i>	AGSPFSGAPVSFPLAPGPHFGTPGYGGPHFTTLYSSVPLPEGEFPPSVVTLGSPMHSN	360
<i>Bos taurus</i>	AGSPFTGGPVSSPLAPGPHFGTPGYGGPHFTTLYSSVPFPEGEVFPSPVTLGSPMHAN	360
<i>Homo sapiens</i>	AGSPFSGGPVSFPLAPGPHFGTPGYGSPHFTALYSSVPFPEGEAFPPSVVTLGSPMHSN	360
<i>Pan troglodytes</i>	AGSPFSGGPVSFPLAPGPHFGTPGYGSPHFTALYSSVPFPEGEAFPPSVVTLGSPMHSN	360
<i>Macaca mulatta</i>	AGSPFSGGPVSFPLAPGPHFGTPGYGSPHFTALYSSVPFPEGEAFPPVPVTLGSPMHSN	360
<i>Mus musculus</i>	TGTPFPGGAVSFPLPPGPHFGTPGYGSPHFTLYS-VPFPEGEAFPPSVVTLGSPMHSN	352
<i>Rattus norvegicus</i>	AGKPFPGGAVSFPLPPGPHFGAPGYGSPHFTLYS-VPFPEGEAFPPSVVTLGSPMHSN	352
	:*.*.*.* ** .*****:*****.*****:*** ***:*** ***.**.*:*****:*	

-  POU_s domain
 POU_H domain
 Nuclear localization signal (NLS)
 Nuclear export signal (NES)

Figure 12 | OCT4 multiple protein sequence alignment showing high degree of sequence conservation across species.

POU_s domain residues highlighted in orange box. POU_H domain residues highlighted in blue box. NLS residues highlighted in black box. Putative NES residues highlighted in dashed black box. Sequence alignment created using ClustalW. *Canis lupus*, dog; *Bos taurus*, cow; *Homo sapiens*, human; *Pan troglodytes*, chimpanzee; *Macaca mulatta*, rhesus macaque; *Mus musculus*, mouse; *Rattus norvegicus*, rat.

Table 1 | Comparison of OCT4 protein and DNA sequence identity from multiple mammalian species to human (*Homo sapiens*) OCT4.

Species	Gene	Protein Identity vs <i>Homo sapiens</i> (%)	DNA Identity vs <i>Homo sapiens</i> (%)
<i>Pan troglodytes</i>	POU5F1	100.0	99.9
<i>Macaca mulatta</i>	POU5F1	98.3	98.1
<i>Canis lupus</i>	POU5F1	90.8	89.0
<i>Bos taurus</i>	POU5F1	90.8	88.6
<i>Mus musculus</i>	Pou5f1	84.7	82.6
<i>Rattus norvegicus</i>	Pou5f1	84.9	82.2

4.2.2. Comparison of the subcellular localization of human OCT4 and HSP90

We first investigated whether human OCT4 and HSP90 share similar subcellular distribution within the HEK-hOCT4 cell line. Immunofluorescence microscopy analysis of HEK-hOCT4 cells showed human OCT4 staining co-distributing with Hoechst 33342 stained nuclei [Figure 13.A: Hoechst (blue), OCT4 (green) and Merge channels] indicating the presence of human OCT4 in the nucleus of HEK-hOCT4 cells. Human OCT4 expression was also additionally observed to be present in the cytosol of HEK-hOCT4 cells [Figure 13.A: OCT4 channel (green)]. HEK-hOCT4 cells displayed a predominant cytosolic distribution for HSP90 α/β , although the presence of low concentrations of HSP90 α/β was observed in the nucleus [Figure 13.A: HSP90 α/β channel (red)].

We subsequently performed subcellular fractionation to determine the relative proportions of human OCT4 and HSP90 α/β within the nucleus and cytoplasm in HEK-hOCT4 cells. The nuclear and cytoplasmic fractions from HEK-hOCT4 cells were isolated using the commercially available NE-PER subcellular fractionation kit (Methodology 2.12). Western blot and densitometry was used to determine the relative proportions of human OCT4 and HSP90 α/β within the resulting nuclear and cytoplasmic fractions (Figure 13.B and C). Histone H3 and GAPDH proteins were analyzed in order to determine the purity of the nuclear and cytoplasmic fractions, respectively. Histone H3 protein was predominantly observed in nuclear fractions, although cytoplasmic fractions showed evidence of low Histone H3 protein levels suggesting minor contamination of the cytoplasmic fraction with nuclear proteins (Figure 13.B and data not shown). However, the distribution of the Histone H3 suggested that the nuclear fraction was sufficiently enriched. GAPDH protein was found to be present in both nuclear and cytoplasmic fractions, although a greater enrichment was found in the cytoplasmic fractions (Figure 13.B) suggesting the cytoplasmic fractions were sufficiently enriched. HSP90 α/β was found to be present in both the nuclear and cytoplasmic fractions from HEK-hOCT4 cells, with the greater average proportion of HSP90 α/β (64.4% of total Hsp90 α/β signal) present in the cytoplasmic fractions (Figure 13.B and C). Human OCT4 protein levels revealed a high proportion of human OCT4 present in the nuclear fractions (90.9%; Figure 13.B

and C) with a minor proportion observed in cytoplasmic fractions (9.1%; Figure 13.B and C). Human OCT4 protein analysis additionally detected lower molecular weight bands that, although present in both nuclear and cytoplasmic fractions, were observed to predominantly reside in the cytoplasmic fractions (65.5%; Figure 13.B and C).

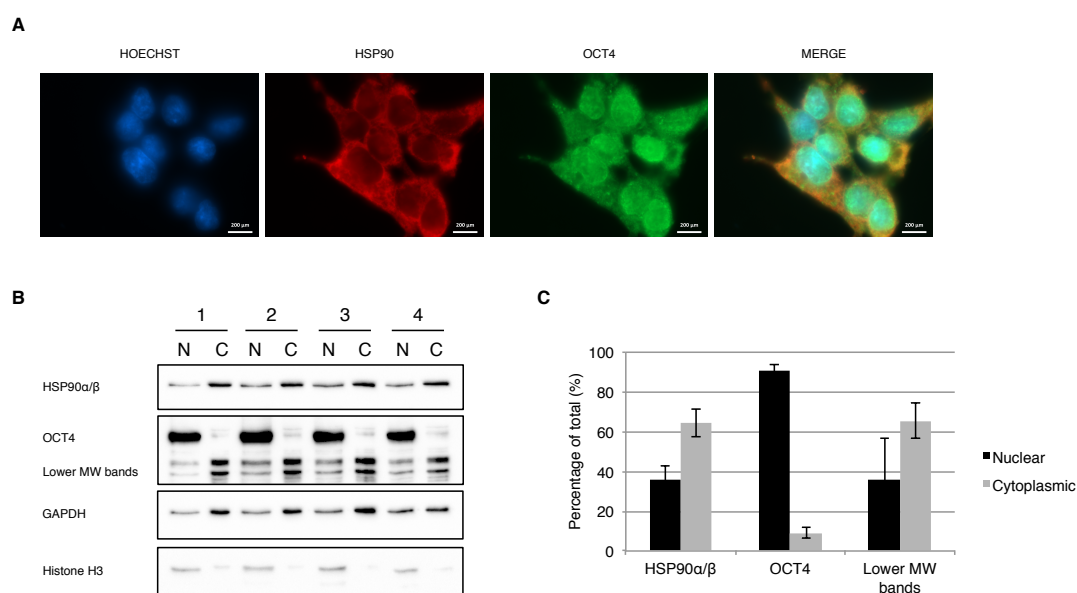


Figure 13 | Subcellular localization of human OCT4 and HSP90α/β in HEK-hOCT4 cells.
 (A) Immunofluorescence detection of HSP90α/β (red) and human OCT4 (green) in HEK-hOCT4 polyclonal cell line. Hoechst 33342 was used to stain nuclei (blue). Images captured using a Zeiss AxioVert.A1 FL-LED (100x magnification). Scale bar: 100 nm. (B, C) Nuclear and cytoplasmic fractions from HEK-hOCT4 cells were isolated by subcellular fractionation (NE-PER kit). Resulting fractions were analyzed by Western blot (B) with equal cell numbers loaded. Samples represent four independent fractionations. Histone H3 was used as a marker for the nuclear fraction and for contamination of the cytoplasmic fractions. (C) Relative proportions (%) of each protein in nuclear and cytoplasmic fractions were determined with densitometry using ImageJ software.

4.2.3. Human OCT4 interacts with HSP90 α/β

Having determined that human OCT4 and HSP90 showed some colocalization in HEK-hOCT4 cell line, albeit at different levels, we next tested for a direct interaction between the two proteins. Co-immunoprecipitation was performed on HEK-hOCT4 cells to determine if human OCT4 and HSP90 α/β directly interact (Figure 14). Human OCT4 was co-immunoprecipitated from HEK-hOCT4 cells lysed in radioimmunoprecipitation (RIPA) buffer using a rabbit anti-OCT4 capture antibody with protein A matrix. The presence of human OCT4 and HSP90 α/β was detected using goat anti-OCT4 and mouse anti-HSP90 α/β antibodies, respectively (Figure 14.A). To account for non-specific interactions between protein A and cellular proteins, a negative control co-immunoprecipitation was performed with beads alone (without the rabbit anti-OCT4 capture antibody). A band corresponding to human OCT4 was detected in both the negative control and test samples when human OCT4 was co-immunoprecipitated from HEK-hOCT4 cells (Figure 14.A). However human OCT4 detected in the test sample was observed to be more abundant than human OCT4 present in the negative control sample (Figure 14.A). Similarly HSP90 α/β protein was detected in both the negative control and test samples with HSP90 α/β displaying a greater abundance in the test sample (Figure 14.A). The greater abundances of human OCT4 and HSP90 α/β in the test sample compared to the negative control sample suggested that human OCT4 directly interacted with HSP90 α/β (Figure 14.A).

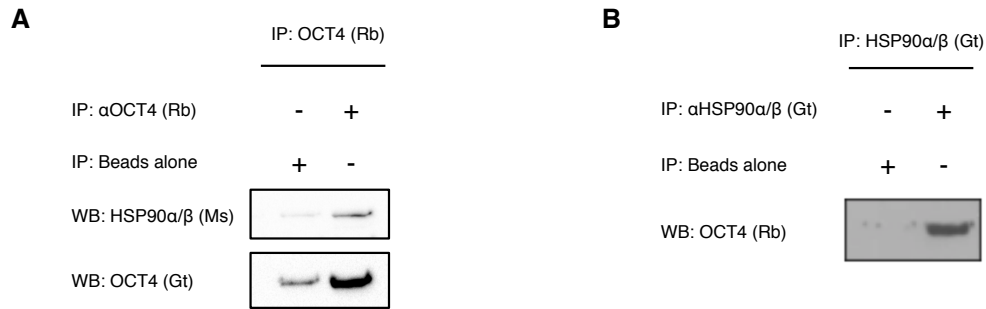


Figure 14 | Human OCT4 interacts with HSP90 α/β .

HEK-hOCT4 cells were lysed in radioimmunoprecipitation (RIPA) buffer. (A) OCT4 co-immunoprecipitation using rabbit anti-OCT4 capture antibody from HEK-hOCT4 RIPA lysates. (B) HSP90 α/β co-immunoprecipitation using goat anti-HSP90 α/β capture antibody from HEK-hOCT4 RIPA lysates. For both (A) and (B) bound proteins were analyzed using Western blot where OCT4 was probed using a goat anti-OCT4 antibody while HSP90 α/β was probed using a mouse anti-HSP90 α/β antibody. Negative control co-immunoprecipitations (A and B) included all reagents excluding capture antibodies. For (A), and (B), '-' denotes absence of specified treatment or capture antibody while '+' denotes the presence of specified treatment or capture antibody.

The reciprocal experiment was performed with the co-immunoprecipitation of HSP90 α/β from HEK-hOCT4 cells lysed in RIPA buffer using goat anti-HSP90 α/β capture antibody with protein G matrix and detection of human OCT4 protein using rabbit anti-OCT4 antibodies (Figure 14.B). To account for non-specific interactions with the protein G matrix, the co-immunoprecipitation was performed with beads alone as a negative control. Human OCT4 was detected in the test sample when HSP90 α/β was co-immunoprecipitated from HEK-hOCT4 cells lysed with RIPA buffer (Figure 14.B). The negative control co-immunoprecipitation with beads alone showed near undetectable levels of human OCT4 suggesting that human OCT4 co-immunoprecipitated with the target protein rather than as a result of interaction of human OCT4 with the protein G matrix (Figure 14.B).

4.2.4. Effect of HSP90 inhibition on human OCT4 protein levels in HEK-hOCT4 cells

Studies on canonical HSP90 α/β interactions with client protein illustrate that HSP90 α/β client proteins are dependent on HSP90 α/β activity for protein stability (Whitesell and Cook, 1996). To assess whether human OCT4 protein is dependent on HSP90 α/β activity for stability, HEK-hOCT4 cells were treated with HSP90 α/β N-terminal chemical inhibitor GA and HSP90 α/β C-terminal chemical inhibitor novobiocin (NOV) overnight prior to cell lysis in SDS-PAGE sample buffer and Western blot analyses for human OCT4 protein levels. Equal cell numbers were loaded per well and densitometry performed using ImageJ software. The response of human OCT4 was compared to the known HSP90 client proteins AKT and CDK4. Inhibition of HSP90 α/β using increasing concentrations of N-terminal HSP90 α/β inhibitor GA (0 – 1000 nM) caused degradation of known HSP90 client proteins AKT and CDK4 (Figure 15.A). In the case of both AKT and CDK4, loss of protein was observed at the highest concentration of GA (1000 nM; Figure 15.A) with no apparent loss of protein, relative to the untreated sample, observed with 100 nM GA or lower concentrations (Figure 15.A). As with AKT and CDK4, treatment of HEK-hOCT4 cells with 1000 nM GA resulted in the loss of human OCT4 protein, while lower concentrations of GA (0 – 100 nM) showed minor increases in human OCT4 protein levels (Figure 15.B). HSP90 α/β protein levels showed a dose-dependent increase between 0 and 10 nM GA followed by reduction to near untreated levels at higher GA

concentrations (Figure 15.B). Interestingly, treatment of HEK-hOCT4 cells using increasing concentrations of the C-terminal HSP90 chemical inhibitor NOV induced a similar trend to HSP90 inhibition with the N-terminal HSP90 chemical inhibitor GA (Figure 15.C) with respect to human OCT4 protein levels, albeit at higher concentrations. Human OCT4 protein levels were reduced at the highest concentration of NOV (1000 μ M; Figure 15.C). It was noted that at the highest concentrations of NOV, 1000 μ M, there was also a reduction in HSP90 α/β protein levels (Figure 15.C).

In order to determine whether the observed HSP90 inhibition-induced degradation of the human OCT4 protein was as sensitive as that of known HSP90 client protein CDK4, HEK-hOCT4 cells were treated with an expanded GA concentration range with human OCT4 and known HSP90 client protein CDK4 protein levels analyzed by Western blot (Figure 16). CDK4 protein levels were reduced at GA concentrations above 100 nM (as previously shown in Figure 15.A), with near complete loss of total CDK4 protein observed at GA concentrations of 250 nM and greater (Figure 16). Human OCT4 levels showed near complete total reduction at concentrations around 250 nM GA (Figure 16). The presence of distinct Histone H3 bands in all lanes suggested that the loss of OCT4, CDK4 and AKT were not due to cell death. In subsequent experiments with GA treatments, the effect on OCT4 levels was seen to vary between minor decreases and total loss of the protein (Figure 16). However fluctuations in total Histone H3 were noted in Figure 15, this suggests that higher HSP90 inhibitor concentrations may be influencing total protein levels possibly due to cytotoxicity of the inhibitors.

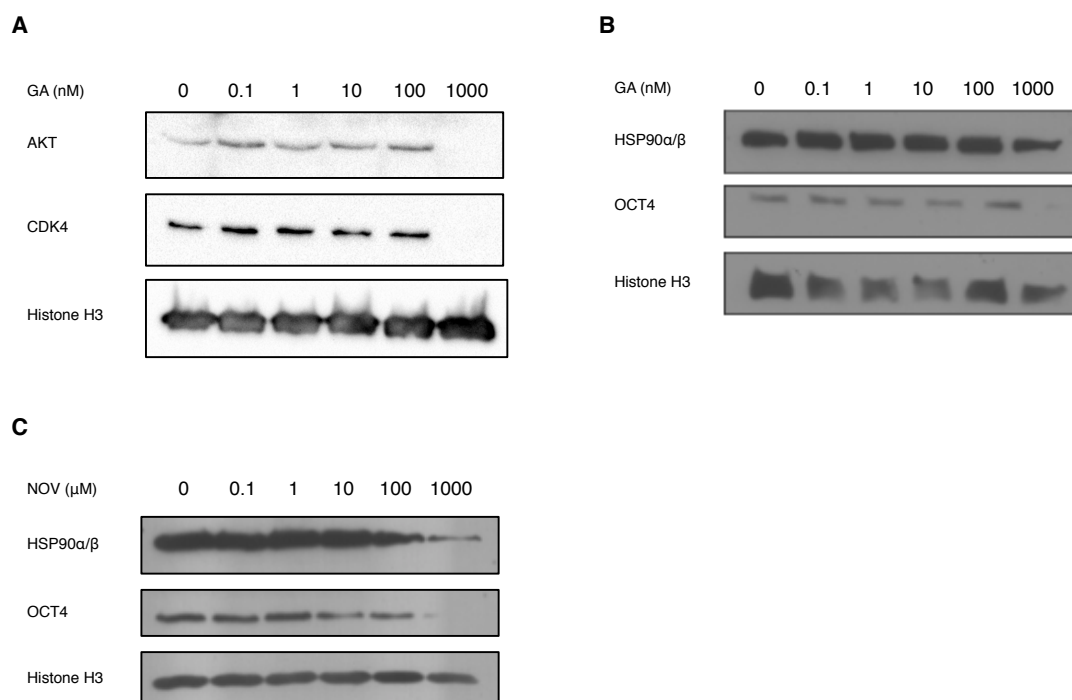


Figure 15 | Effects of HSP90 inhibition on human OCT4 protein levels in HEK-hOCT4 cells. HEK-hOCT4 cells were treated with increasing concentrations of HSP90 inhibitors for 24 hours prior to cell lysis in SDS-PAGE sample buffer and Western blot analyses. (A) Effects of increasing concentrations of N-terminal HSP90 inhibitor geldanamycin (GA; 0 – 1000 nM) on protein levels of known HSP90 client proteins AKT and CDK4. (B) Effects of increasing concentrations of N-terminal HSP90 inhibitor GA (0 – 1000 nM) on human OCT4 protein levels in HEK-hOCT4 cells. (C) Effects of increasing concentrations of C-terminal HSP90 inhibitor novobiocin (NOV; 0 – 1000 μM) on human OCT4 protein levels in HEK-hOCT4 cells. Equal cell numbers were loaded in each lane.

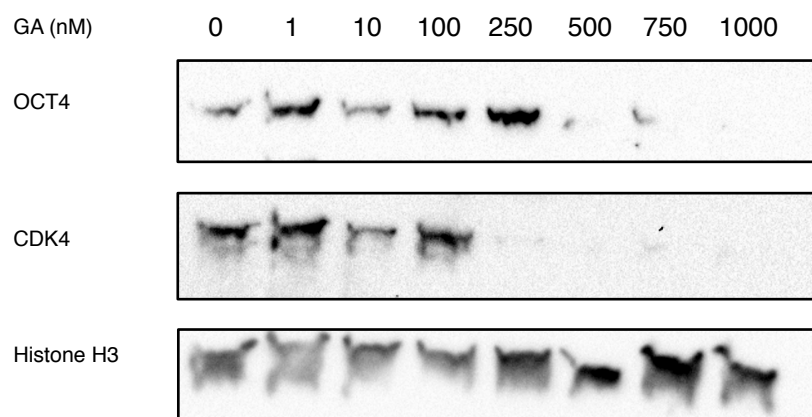


Figure 16 | Comparison of effect of HSP90 inhibition on known HSP90 client protein CDK4 and human OCT4 in HEK-hOCT4 cells.

Comparison of sensitivities of known HSP90 client protein CDK4 and human OCT4 protein levels to HSP90 inhibition. HEK-hOCT4 cells were treated with increasing concentrations of HSP90 C-terminal inhibitor GA (0 – 1000 nM) for 24 hours prior to cell lysis in SDS-PAGE sample buffer and Western blot analyses. Equal cell numbers were loaded for each lane..

4.2.5. Effect of proteosomal inhibition with HSP90 inhibition on human OCT4 protein

Initial analyses suggested that inhibition of HSP90 with GA concentrations above 250 nM induced the degradation of the human OCT4 protein in HEK-hOCT4 cells. To determine whether apparent HSP90 inhibition-induced human OCT4 degradation was via the proteasome pathway, we used a proteasome inhibitor MG132. We first tested the effect of increasing concentrations of MG132 on OCT4 levels in untreated cells. Human OCT4 protein levels remained relatively constant at MG132 concentrations 1 μ M and lower, although a reduction of human OCT4 protein levels to below that of the untreated sample was observed at 0.1 μ M MG132 (Figure 17.A). Substantial increases in human OCT4 protein levels were observed for 5 μ M and 10 μ M MG132 concentrations (Figure 17.A). Increasing concentrations of MG132 (0.1 – 10 μ M) also increased HSP90 α/β protein levels. These data suggest that turnover of both proteins is mediated through the proteasome under normal conditions in the HEK-hOCT4 cell line.

To assess whether the reductions of human OCT4 resulting from HSP90 inhibition could be overcome by inhibition of the proteasome, HEK-hOCT4 cells treated with combinations of fixed concentrations of MG132 and increasing concentrations of GA (Figure 17.B and C). Treatment with 1 μ M MG132 resulted in the reduction of human OCT4 protein levels (Figure 17.B). While we observed treatment alone with GA caused a dose-independent decrease in human OCT4 protein levels, the combination of 10 and 100 μ M GA reduced human OCT4 protein levels to below that of the corresponding GA treatments alone (Figure 17.B). However this reduction resulting from the combination of GA and MG132 was not greater than the reduction caused by 1 μ M MG132 alone (Figure 17.B). The combination of 1 μ M MG132 and 1000 nM GA reduced human OCT4 proteins levels however not to the extent of MG132 and GA treatments alone (Figure 17.B). Although we observed additive reductions in human OCT4 at combinations of 1 μ M MG132 and low concentrations of GA (10 nM and 100 nM) (Figure 17.B), this combination effect was not observed when the experiment was conducted with constant high MG132 concentration (10 μ M) (Figure 17.C). The treatment of HEK-hOCT4 cells with 1000 nM GA caused reductions in both human OCT4 and the model HSP90 client protein CDK4, however

the response of OCT4 protein levels to increasing concentrations of GA varied (Figure 17.C). CDK4 protein levels were reduced with both increasing concentrations of GA and 10 μ M MG132 alone (Figure 17.C). A rescue of CDK4 protein levels was observed for cotreatment with 10 nM GA and 10 μ M MG132, however this rescue was negated with co-treatments of 10 μ M MG132 and 100 nM or higher of GA (Figure 17.C). We observed that co-treatment of HEK-hOCT4 cells with 1000 nM GA and 10 μ M MG132 increased human OCT4 protein levels together with HSP90 α/β , but not CDK4, greater than any treatments alone or co-treatments. (Figure 17.C). The effect of higher concentrations of GA (10 and 100 nM) were able to overcome the inhibition of the proteasome and resulted in reduced protein levels of CDK4, a known HSP90 client protein. In contrast, human OCT4 levels were increased in the presence of MG132 and GA, compared to the equivalent concentration of GA alone.

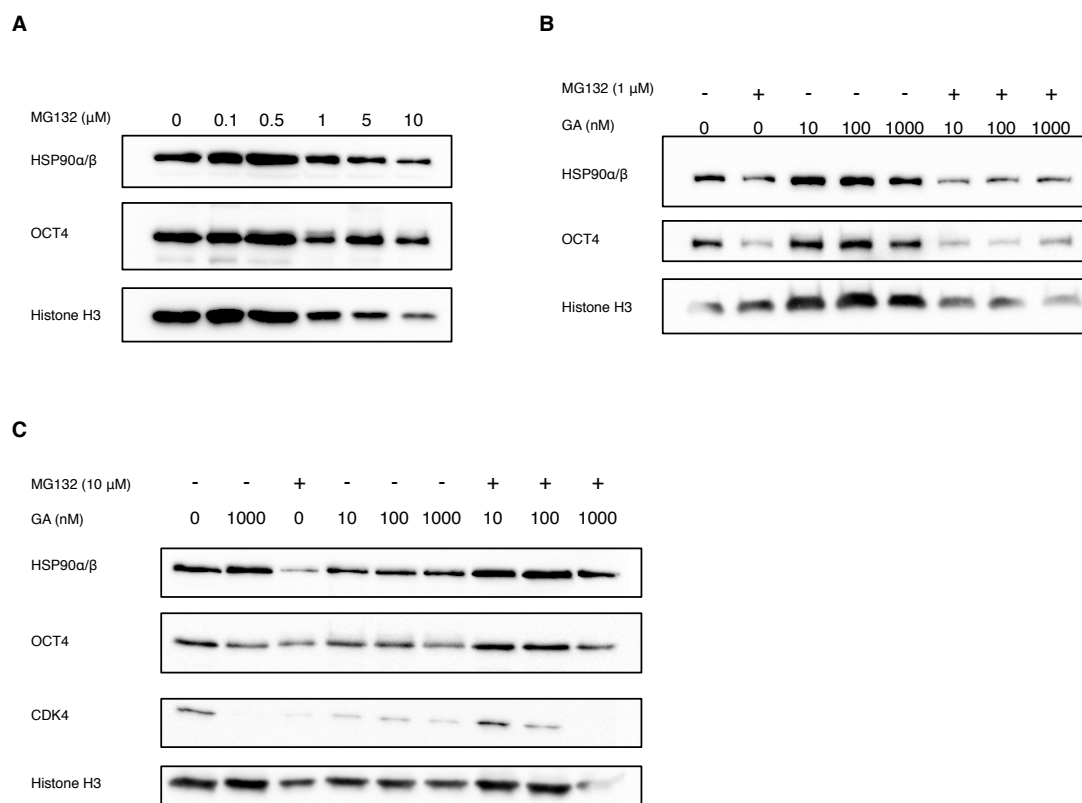


Figure 17 | Effects of HSP90 inhibition on human OCT4 protein levels with proteasome inhibition using MG132 in HEK-hOCT4 cells.

(A) HEK-hOCT4 cells were treated with increasing concentrations of proteasome inhibitor MG132 (0 – 10 μ M) for 24 hours prior to cell lysis in SDS-PAGE sample buffer and Western blot analyses of HSP90 α/β and human OCT4 protein levels. Densitometry of bands analyzed using ImageJ software. (B, C) HEK-hOCT4 cells were treated with increasing concentrations of GA (0 – 1000 nM) with or without 1 μ M MG132 (B) and 10 μ M MG132 (C) for 24 hours prior to cell lysis in SDS-PAGE sample buffer. Human OCT4 and HSP90 α/β protein levels determined using Western blot analysis. Equal cell numbers loaded with Histone H3 used as protein loading control.

To further analyze the observed human OCT4 protein reduction with the combination of GA and low concentration of MG132, HEK-hOCT4 cells were co-treated with increasing concentration of MG132 in the presence of constant concentrations of GA (Figure 18.A – C). Treatment with 100 nM GA increased human OCT4 protein levels (Figure 18.A) as was previously observed (Figure 15.B). However treatment with 100 nM GA in combination with 1 μ M MG132 resulted in reduction of human OCT4 protein levels (Figure 18.A). Co-treatment with 100 nM GA and MG132 concentrations above 1 μ M increased human OCT4 levels above that of the untreated and 100 nM GA samples. Treatment with 250 nM GA resulted in a minor decrease in human OCT4 protein (Figure 16.A and Figure 18.B). Although 250 nM GA alone had a minor effect on human OCT4 protein levels in HEK-hOCT4 cells, co-treatment with 250 nM GA in combination with 0.1 μ M MG132 resulted in decreased human OCT4 protein levels in HEK-hOCT4 cells (Figure 18.B). This decrease in human OCT4 protein levels in HEK-hOCT4 cells was additionally observed for the co-treatment of 250 nM GA with 0.5 μ M MG132 and 250 nM GA with 1 μ M MG132. Human OCT4 recovery from co-treatments with 250 nM GA and 0.1 μ M MG132 appeared dose-dependent between 0.5 and 1 μ M MG132 concentration (Figure 18.B). The loss of OCT4 upon co-treatment with 250 nM GA and MG132 was not observed at MG132 concentrations above 5 μ M (Figure 18.B). At 5 μ M and 10 μ M MG132, human OCT4 protein levels were determined to be higher than that of the untreated control, with 5 μ M showing the greatest amount of human OCT4 (Figure 18.B). HSP90 α / β protein levels followed a similar trend to that of human OCT4 protein levels although the fluctuations in HSP90 α / β protein levels observed were to a lesser extent than that observed for human OCT4 degradation (Figure 18.B).

Treatment with 1000 nM GA led to a reduction in human OCT4 protein levels. Co-treatment with 1000 nM GA and 0.1 μ M MG132 had no effect of human OCT4 protein levels in HEK-hOCT4 cells relative to 1000 nM GA treatment alone (Figure 18.C). The combination of 1000 nM GA and 0.5 μ M MG132 caused a marginal decrease in human OCT4 protein levels in HEK-hOCT4 cells (Figure 18.C). The reduction in human OCT4 protein levels resulting from 1000 nM GA was rescued upon co-treatments with 10 μ M MG132 with an increase in human OCT4 levels observed relative to 0.1 μ M and 5 μ M co-treatments with 1000 nM GA and 1000 nM

GA treatment alone (Figure 18.C). The 5 μ M and 10 μ M co-treatments resulted in human OCT4 protein levels above that of the untreated sample (Figure 18.C). The appearance of higher molecular weight bands in the OCT4 samples treated with the concentrations between 1 and 10 μ M MG132 are predicted to represent ubiquitinated forms of OCT4. Co-treatments of 1000 nM GA with 0.1 μ M, 0.5 μ M and 1 μ M showed no change in HSP90 α/β protein levels relative to 1000 nM GA treatment alone (Figure 18.C). HSP90 α/β levels were rescued to that of the untreated sample for co-treatments of 1000 nM GA with both 5 μ M and 10 μ M MG132 (Figure 18.C).

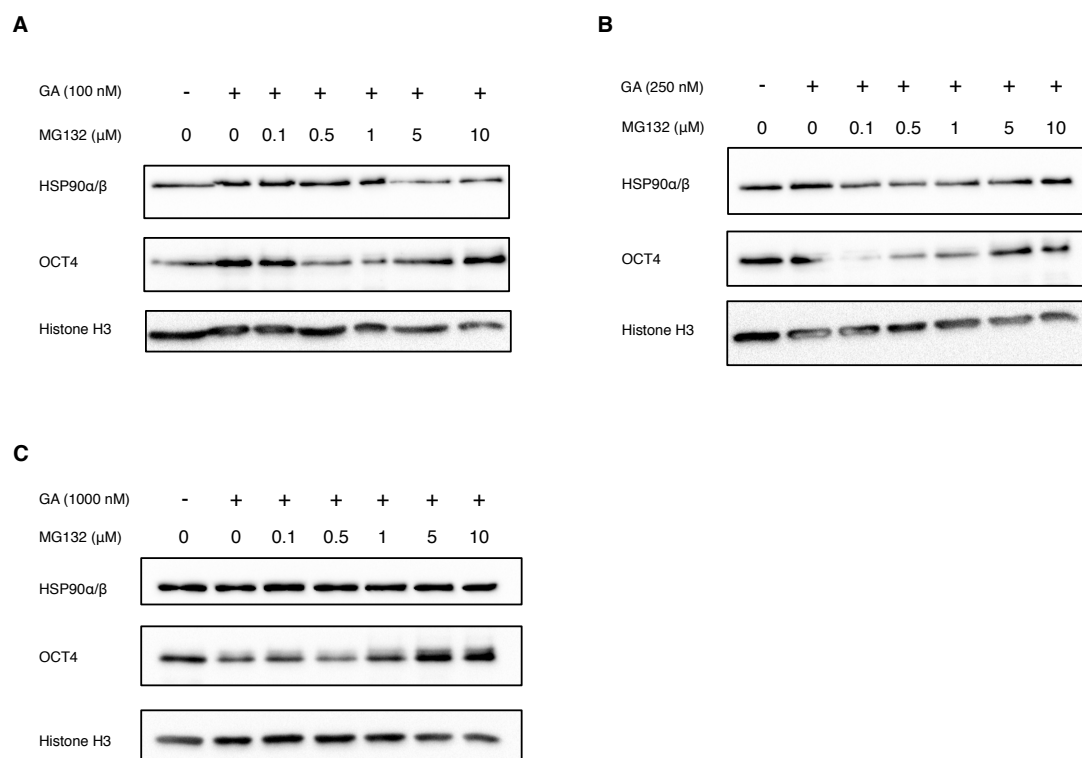


Figure 18 | Increasing concentrations of MG132 block GA induced loss of human OCT4 in HEK-hOCT4 cells.

HEK-hOCT4 cells were treated with increasing concentrations of proteasome inhibitor MG132 (0 – 10 μ M) in the presence of 100 nM (A), 250 nM (B) or 1000 nM (C) GA for 24 hours. Cells were lysed in SDS-PAGE sample buffer with human OCT4 and HSP90 α / β protein levels analyzed using Western blot. Equal cell numbers loaded with Histone H3 used as protein loading control.

4.2.6. Effect of HSP90 inhibition on human OCT4 subcellular localization in HEK-hOCT4 cells

We used a co-immunoprecipitation-responsive subcellular fractionation protocol (Methodology 2.13) to establish whether the interaction between human OCT4 and HSP90 α/β is compartmentalized, and whether HSP90 inhibition causes translocation of human OCT4 between subcellular compartments. HEK-hOCT4 cells were treated with 100 nM GA or 1000 nM GA for 24 hours prior to subcellular fractionation, with DMSO treated samples used as control treatments. HSP90 α/β and OCT4 protein levels in each fraction were analyzed using Western blot (Figure 19), with Histone H3 used to assess fraction purities. For the 100 nM GA and 1000 nM GA samples HSP90 α/β was localized predominantly in the cytoplasm (Figure 19). Although HSP90 α/β was predominantly localized in the cytoplasmic fraction, some nuclear accumulation was observed with DMSO treatment (Figure 19). The chaperone HSP70 displayed both cytoplasmic and nuclear localization, although the majority of HSP70 was confined to cytoplasmic fractions (Figure 19). It was additionally noted that HSP70 showed an apparent greater abundance than that of HSP90 α/β in the nucleus (Figure 19). Total HSP70 protein levels increased with GA treatments, with 1000 nM GA increasing the abundance of nuclear HSP70 protein levels (Figure 19). The HSP90 co-chaperone P23 was confined to cytoplasmic fractions with no evidence of nuclear localization, additionally GA treatments reduced P23 protein levels (Figure 19). Human OCT4 was shown to predominantly reside in the nuclear fractions (Figure 19). In the presence of GA treatments, there was a decrease of OCT4 from both the cytoplasmic and nuclear fractions and there was a reduction in total human OCT4 levels in the whole cell lysate (Figure 19). GA treatment with both 100 nM and 1000 nM concentrations caused a reduction in the presence of OCT4-detected lower molecular weight bands in nuclear fractions (Figure 19).

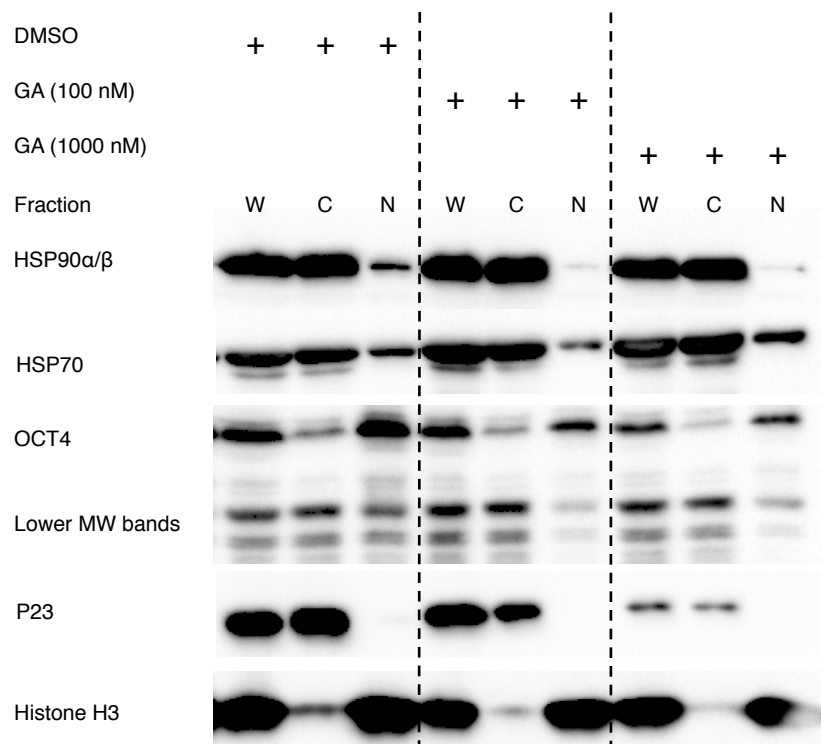


Figure 19 | Subcellular localization of human OCT4 with HSP90 inhibition in HEK-hOCT4 cells.

HEK-hOCT4 cells were treated with DMSO (vehicle control) or GA (100 nM and 1000 nM) for 24 hours prior to subcellular fractionation using the REAP fractionation protocol (Methods 2.13) to isolate cytoplasmic and nuclear fractions from respective treatments. Resulting fractions were analyzed for human OCT4, HSP90α/β, HSP70 and P23 protein using Western blot. Histone H3 served as a nuclear fraction purity control. W, whole cell; C, cytoplasmic fraction; N, nuclear fraction.

In order to determine whether GA affected colocalization between HSP90 α/β and human OCT4, confocal microscopy was performed on HEK-hOCT4 cells treated with increasing concentrations of GA (0 – 1000 nM). Post treatment cells were stained for human OCT4 and HSP90 α/β and visualized by confocal microscopy (Figure 20). The ratio of the corrected total cell fluorescence (CTCF) in the nuclear and cytoplasmic fractions for each treatment was calculated using ImageJ software and reported in Figure 20. We observed a qualitative loss of cytoplasmic OCT4 staining (as evident by the increased prominence of the nucleus) with increasing concentrations of GA (Figure 20.A). At the highest concentration of GA (1000 nM) very few cells were observed (Figure 20.A). These observed cells displayed vastly diminished cytosols and irregular shaped nuclei (Figure 20.A), morphologies suggesting the cells are undergoing apoptosis [this notion is further supported by loss of Histone H3 levels associated with high HSP90 inhibitor concentrations observed in previous experiments (Figures 15 – 18)].

The qualitative observations of loss of cytoplasmic OCT4 with increasing concentrations of GA was further supported by the quantitative analysis of nuclear and cytoplasmic OCT4 staining by calculating the CTCF of OCT4 in each subcellular location (Figure 20.B). The ratio between the CTCF values of nuclear OCT4 and cytoplasmic OCT4 increased with increasing GA concentrations suggesting that GA treatments resulted in a greater loss of cytoplasmic OCT4 than nuclear OCT4. Additionally colocalization constants, including the Pearson's coefficient (Rr) (Manders *et al*, 1992), intensity correlation quotient (ICQ) (Li *et al*, 2004) and channel coefficients M1 and M2 (Manders *et al*, 1992), were calculated using ImageJ software and recorded in Table 2. Pearson's coefficient (Rr) measures the linear relationship between two channels and assess whether the variability in one channel is resultant by the variability in the second channel. Values of 1 or -1 are indicative of perfect colocalization or perfect exclusion respectively, while a value of 0 suggests random (and hence no) colocalization (Adler and Parmryd, 2013). The ICQ determines the degree of dependency between two stainings based on pixel-to-pixel measurements. It measures the ratio of the number of positive pixels to the total number of pixels displaying paired staining. For the ICQ, 0.5 or -0.5 represent

dependent staining or segregated staining respectively, while a value of 0 represents random staining (Li *et al*, 2004). The M1 and M2 coefficients measures signal coincidences between two channels by calculating the ratio of the intensities of pixels from one channel, which display a second channel intensity above 0, to the total intensity of the first channel (Manders *et al*, 1992). These coefficients detail the coincidence of the signal from one channel with the signal of a second channel, with 0 representing a lack of overlap between signals and 1 representing co-localization. Treatment with GA decreased the Rr values between human OCT4 and HSP90 α/β compared to that of the untreated sample (Table 2). A decrease was additionally noted in the case of the ICQ values compared to the untreated sample (Table 2). In both cases Rr and ICQ values showed only slight decreases for 10 nM GA compared to the untreated sample, while decreases observed for 1 nM, 100 nM and 1000 nM compared to the untreated were greater than those observed for 10 nM GA (Table 2). It was however noted that in the case of 1000 nM GA, the number of cells in each frame was lower than that of the other GA concentrations.

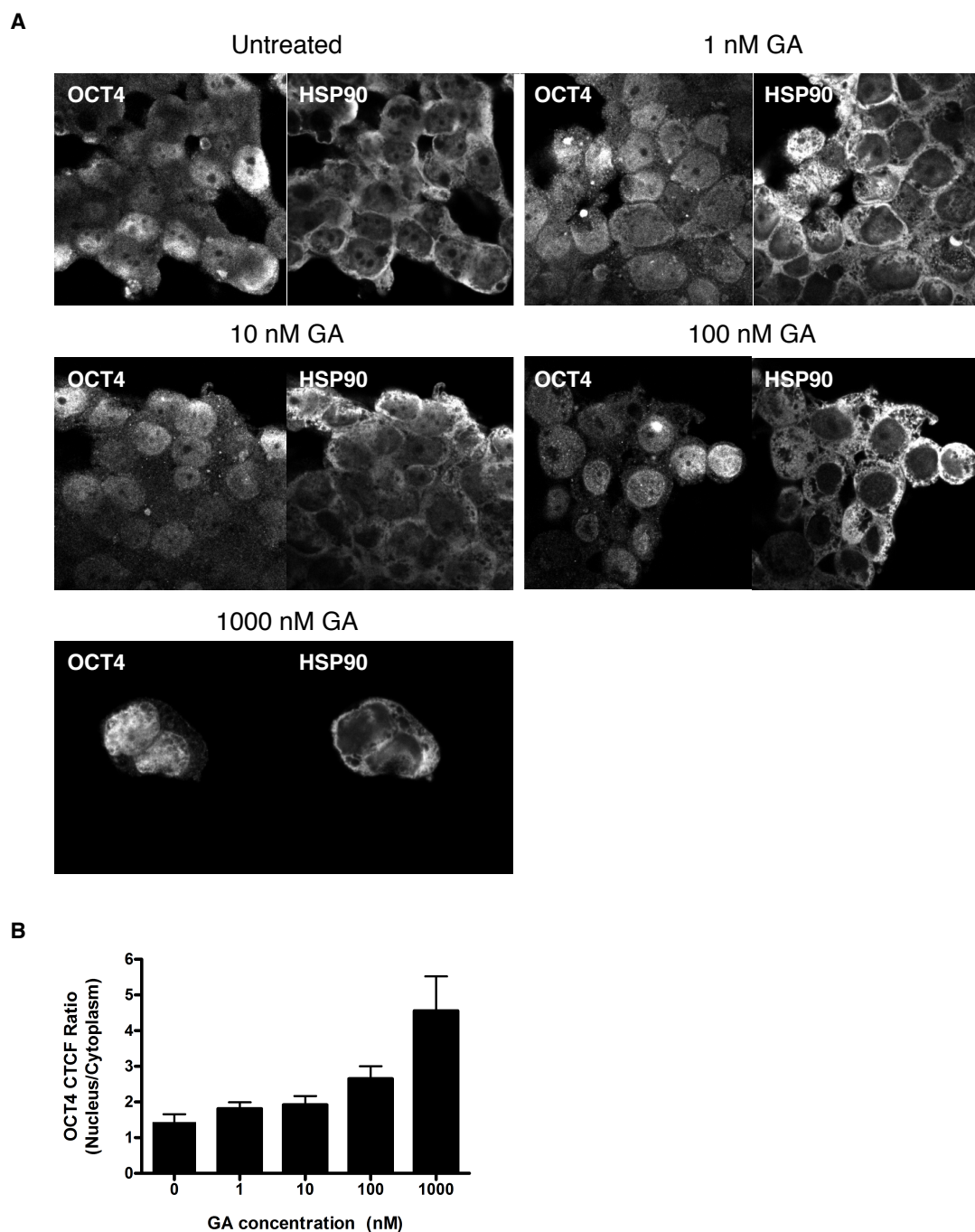


Figure 20 | HSP90 inhibition effect on human OCT4 and HSP90 colocalization using confocal microscopy. HEK-hOCT4 cells were treated with increasing concentrations of GA (0 – 1000 nM) for 24 hours. Detection of human OCT4 and HSP90 α/β was performed using confocal microscopy. (A) Images captured using a Zeiss LSM510 meta confocal microscope (Zeiss) (100x magnification). (B) Colocalization analysis was performed using Image J software to calculate CTCF for nuclear OCT4 and cytoplasmic OCT4. Cytoplasmic OCT4 CTCF calculated from the subtractions of nuclear CTCF from the OCT4 CTCF in the whole cell. Graph displays the CTCF ratio of nuclear OCT4 to cytoplasmic OCT4.

Table 2 | Effect of HSP90 inhibition on human OCT4 and HSP90 α/β colocalization.

GA (nM)	Rr	ICQ	M1	M2
0	0.405 $\pm$ 0.034	0.191 $\pm$ 0.004	0.991 $\pm$ 0.001	0.997 $\pm$ 0.001
1	0.263 $\pm$ 0.058	0.087 $\pm$ 0.023	0.985 $\pm$ 0.011	0.999 $\pm$ 0.001
10	0.381 $\pm$ 0.125	0.129 $\pm$ 0.040	0.981 $\pm$ 0.009	0.997 $\pm$ 0.002
100	0.229 $\pm$ 0.063	0.072 $\pm$ 0.049	0.982 $\pm$ 0.014	0.997 $\pm$ 0.001
1000	0.255 $\pm$ 0.363	0.098 $\pm$ 0.202	0.944 $\pm$ 0.093	0.859 $\pm$ 0.229

Rr, Pearson's coefficient (-1 to 1); ICQ, Intensity correlation quotient (-0.5 to 0.5); M1, Manders coefficient ratio channel 1 (0 to 1); M2, Manders coefficient channel 2 (0 to 1). Data recorded from triplicate y-axis slices from triplicate images.

4.2.7. Analysis of the human OCT4 and HSP90 α/β interaction

To determine whether HSP90 α/β and human OCT4 interact in the nucleus, Whole cell RIPA lysates and the nuclear fraction isolated from HEK-hOCT4 cells using REAP subcellular fractionation was used for the co-immunoprecipitation of nuclear human OCT4 (Figure 21.A and B). The presence of a band corresponding to human OCT4 was detected (using a goat anti-OCT4 antibody) in the presence of the rabbit anti-OCT4 capture antibody (Figure 21.A and B). Non-specific OCT4 binding to protein A matrix (Figure 21.A: beads alone) was noted for the whole cell lysate from HEK-hOCT4 cells however a greater concentration of human OCT4 was co-immunoprecipitated in the presence of the OCT4 capture antibody (Figure 21.A). As observed previously, HSP90 co-immunoprecipitated with human OCT4 at a low concentration relative to the OCT4 concentration (Figure 14 and Figure 21.A). A negative co-immunoprecipitation was performed using all reagents in the absence of a capture antibody to account for the interaction of proteins with the protein A matrix (Figure 21.A and B: beads alone). A band corresponding to human OCT4 was observed with co-immunoprecipitation using the OCT4 capture antibody from the nuclear fraction isolated using the REAP protocol (Figure 21.B). This suggested that nuclear human OCT4 was successfully isolated from the REAP generated nuclear fraction. We did not detect the presence of HSP90 α/β with the co-immunoprecipitation of nuclear human OCT4 (Figure 21.B), despite the fact that the amount of human OCT4 in the immunoprecipitation from the nucleus was equivalent or greater to that obtained with co-immunoprecipitations using whole cell samples (Figure 14.A).

Geldanamycin (GA) is an HSP90 α/β chemical inhibitor targeting the N-terminal ATPase domain of HSP90 α/β (Grenert *et al*, 1997; Prodromou *et al*, 1997; Stebbins *et al*, 1997; Roe *et al*, 1999). The inhibition of HSP90 has previously been shown to decrease the proportion of HSP90 α/β bound to a client protein (Whitesell and Cook, 1996). To determine whether GA was able to abrogate the interaction between human OCT4 and HSP90 α/β , HEK-hOCT cells were treated with 1000 nM GA prior to lysis in RIPA buffer. DMSO alone served as a vehicle control for the GA treatments. Human OCT4 was co-immunoprecipitated using rabbit anti-OCT4 capture antibody

with the presence of HSP90 α/β , HSP70 and human OCT4 detected using Western blot analysis (Figure 21.C). To account for non-specific interactions with the protein A matrix, a negative control co-immunoprecipitation containing beads alone was performed. A whole cell lysate of HEK-hOCT4 cells was used as a positive control for detection of HSP90 α/β , HSP70 and human OCT4. HSP90 was observed to co-immunoprecipitate with human OCT4 in the presence of GA (Figure 21.C). HSP90 was not observed for co-immunoprecipitation of DMSO vehicle control samples, however as HSP90 co-immunoprecipitates at low concentrations with high concentrations of OCT4, the proportion of OCT4 co-immunoprecipitated in this sample may not be sufficient for HSP90 detection (Figure 21.C). HSP70 was found to interact with the protein A matrix as observed by the presence of a HSP70 in the negative control sample containing beads alone (Figure 21.C). Co-immunoprecipitation using the rabbit anti-OCT4 capture antibody with DMSO vehicle control samples successfully co-immunoprecipitated human OCT4 protein. HSP70 was found to be present in very low concentrations in the DMSO vehicle control sample relative to the negative control and GA treated samples (Figure 21.C). In the case of GA treatment HSP90 α/β , HSP70 and human OCT4 were detected which may be suggestive that human OCT4 was in a complex with the molecular chaperones HSP70 and HSP90 α/β in the presence of HSP90 inhibitor GA (Figure 21.C). It was noted that the amount of human OCT4 co-immunoprecipitated in the DMSO vehicle control sample and GA treated cells differed substantially which may have influenced the resulting abundances and hence the detection of both HSP90 α/β and HSP70 in the DMSO vehicle control sample (Figure 21.C).

To determine whether GA promoted the interaction between nuclear OCT4 and nuclear HSP90 α/β we treated HEK-hOCT4 cells with increasing concentrations of GA (100 and 1000 nM GA) prior to REAP isolation of nuclear fractions and OCT4 co-immunoprecipitation. We noticed slight reductions in the amount of human OCT4 co-immunoprecipitated with increasing concentrations of GA compared to the DMSO vehicle control sample (Figure 21.B and D). Similar to the untreated nuclear co-immunoprecipitation (Figure 21.B), we did not detect the presence of HSP90 α/β with nuclear OCT4 co-immunoprecipitation from GA treated HEK-hOCT4 cells or for DMSO vehicle control samples (Figure 21.D). Although REAP subcellular

fractionation allows for co-immunoprecipitation from the isolated nuclear and cytoplasmic fractions (Suzuki *et al*, 2010), we did not have antibodies for a control protein that is a known nuclear OCT4 interacting partner. Therefore, while our data suggest that HSP90 α/β is not interacting with human OCT4 in nuclear fractions and GA does not promote the interaction between HSP90 α/β and nuclear human OCT4, we cannot discount the possibility that the nuclear isolation procedure may have disrupted the interaction between nuclear OCT4 and nuclear HSP90 α/β or that the level of HSP90 in the nuclear complex is below detectable levels.

4.2.8. Effect of HSP90 inhibition using GA on human OCT4 activity in HEK-hOCT4 cells

We had demonstrated that OCT4 interacted directly with HSP90 and that GA treatment led to its proteasomal degradation. We next determined whether the inhibition of HSP90 had an effect on OCT4 transcriptional activity. OCT4 can promote transcription of target genes in both monomer and dimer conformations (Pan *et al*, 2002). Previously we have shown that human OCT4 in HEK-hOCT4 cells is active and capable of transcriptional activation in monomeric and both PORE and MORE dimeric forms using OCT4-responsive luciferase vectors (Figures 11 and 12). To study the effect that HSP90 inhibition has on OCT4 activity, HEK-hOCT4 cells were transfected with luciferase reporter vectors containing the gene encoding for firefly luciferase under transcriptional activation of three different OCT4-responsive promoter elements (Figure 11 and Figure 22.A, C, and E). Following transfection, cells were treated with DMSO vehicle control or increasing concentrations of GA (100 nM, 500 nM and 1000 nM) for 24 hours. Post treatment, cells were lysed and fluorescence and luminescence read in triplicate. The resulting luminescence values were divided by the corresponding fluorescence values to create relative luminescence values normalized for transfection efficiency. The relative luminescence values were then normalized against the control reporter vector pTATA-37Tk-Luc (Figure 11.A), with the average for each biological triplicate and each technical triplicate treatment calculated and presented in Figure 22.

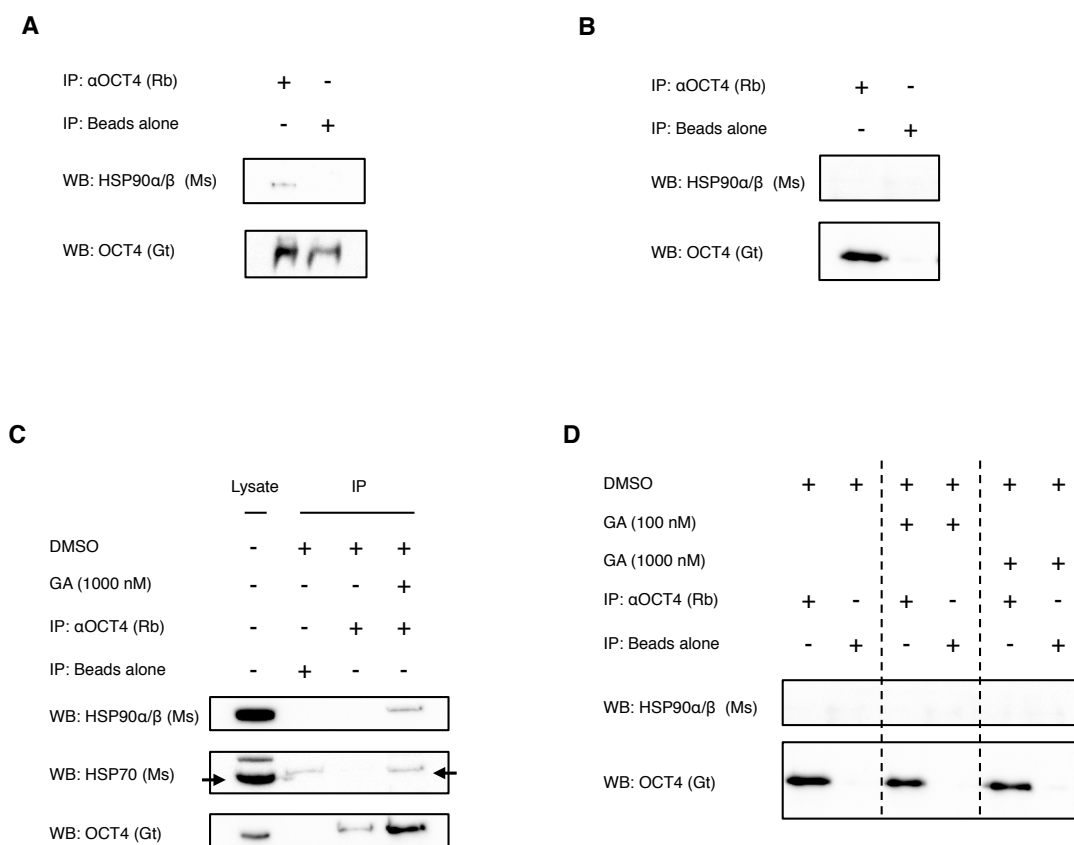


Figure 21 | Effect of GA on the interaction between human OCT4 and HSP90 α/β .

(A) Human OCT4 was co-immunoprecipitated from HEK-hOCT4 RIPA buffer lysates using rabbit anti-OCT4 capture antibody. Bound proteins were analyzed using Western blot. (B) Nuclear fractions were isolated in RIPA buffer from HEK-hOCT4 cells using the REAP subcellular fractionation protocol. Human OCT4 was co-immunoprecipitated using rabbit anti-OCT4 capture antibody. (C) HEK-hOCT4 cells were treated with DMSO or geldanamycin (GA; 1000 nM) for 24 hours prior to lysis in RIPA buffer. OCT4 co-immunoprecipitation performed using rabbit anti-OCT4 capture antibody. Bound proteins were analyzed using Western blot. (D) HEK-hOCT4 cells were treated with DMSO or GA (100 nM and 1000 nM) for 24 hours prior to subcellular fractionation using REAP subcellular fractionation protocol to isolate nuclear fractions in RIPA buffer. OCT4 co-immunoprecipitation performed using rabbit anti-OCT4 capture antibody. (A – D) Bound proteins were analyzed using Western blot analysis. Human OCT4 was probed using goat anti-OCT4 antibody, HSP90 α/β probed using mouse anti-HSP90 α/β antibody, and HSP70 was probed using mouse anti-HSP70 antibody. Negative control co-immunoprecipitations included all reagents excluding capture antibodies. ‘-’ Denotes absence of treatment or antibody, while ‘+’ denotes the presence of specified capture antibody or treatment.

Transcriptional activation by monomeric human OCT4 (6W) remained constant in the DMSO vehicle control or 100 nM GA and 500 nM GA treatments (Figure 22.B). An increase in human OCT4 monomeric activity, as illustrated by increased relative luminescence, was observed with treatment of HEK-hOCT4 cells with 1000 nM GA (Figure 22.B). Human OCT4 homodimer activity on PORE sequences increased with 100 nM GA above the levels of the DMSO vehicle control samples (Figure 22.D). However treatments with concentrations of 500 nM and 1000 nM GA greatly reduced the promoter activity to levels below that of the DMSO vehicle control samples (Figure 22.D). A similar trend, although not as substantial, was observed for MORE promoter activity. An increase in MORE promoter activity was observed with 100 nM GA (Figure 22.F). Geldanamycin concentrations of 500 nM and 1000 nM caused reduction of MORE promoter activity to below that of the DMSO vehicle control samples (Figure 22.F)

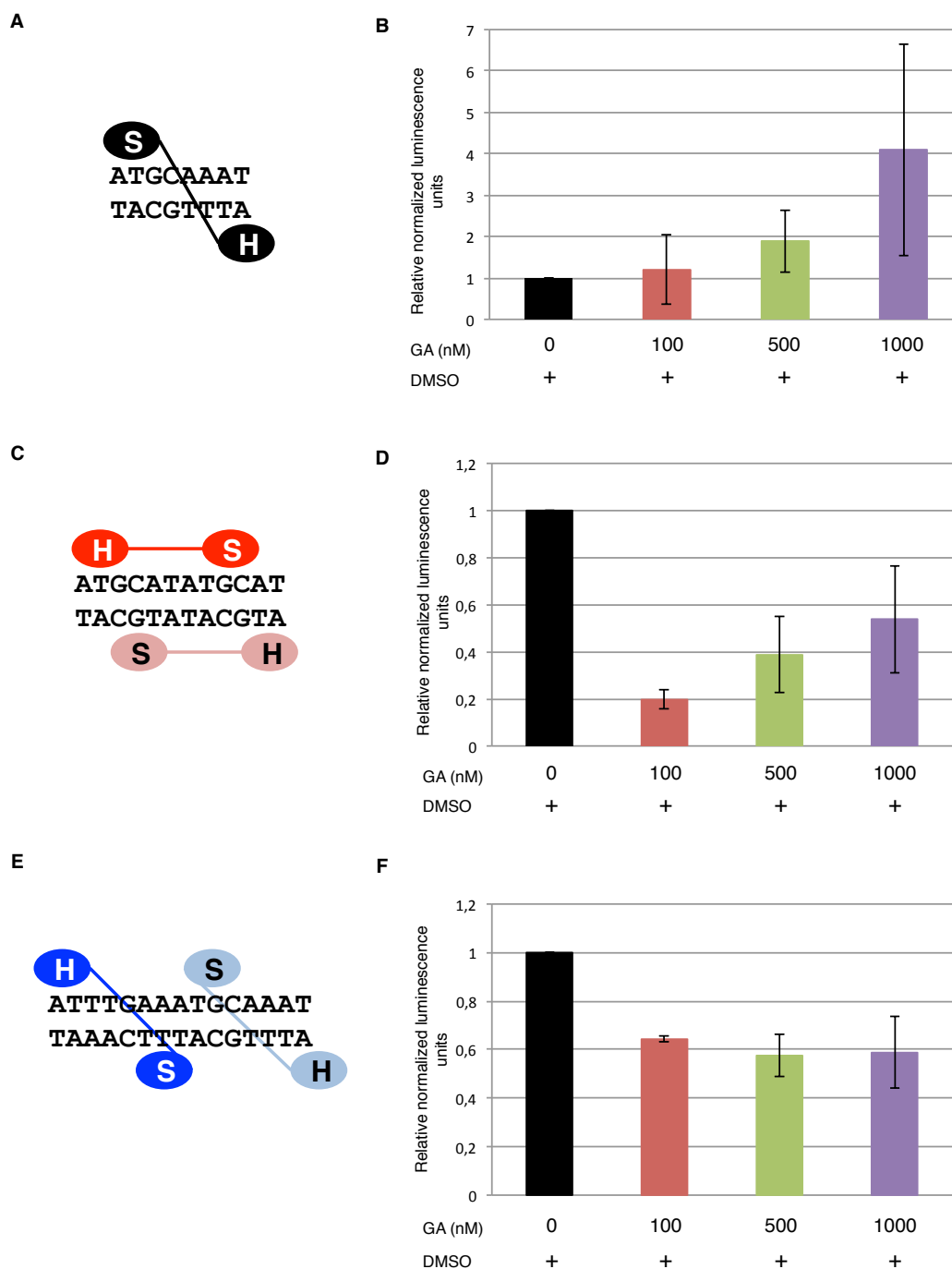


Figure 22 | Effect of HSP90 inhibition on human OCT4 activity in HEK-hOCT4 cells. HEK-hOCT4 cells were transfected with pTATA-37Tk-Luc, pTATA-6W-37Tk-Luc (6W), pTATA-PORE-37Tk-Luc (PORE) or pTATA-MORE-37Tk-Luc (MORE) together with pECFP-C1, used as a transfection control, for 24 hours. Cells were then treated with DMSO or 100 nM, 500 nM or 1000 nM GA for 24 hours. Luminescence and fluorescence readings were recorded and data analyzed as indicated. (A, C, E) Schematic diagrams of OCT4 monomer, and homodimer interactions with DNA allowing for transcriptional activation of promoter. (B, D, F) Graphs of transcriptional activation of monomer (6W), PORE and MORE promoter elements generated using triplicate technical replicates from triplicate independent transfections.

4.2.9. Phosphorylation inhibitors and interaction between human OCT4 and HSP90 α/β

Both HSP90 α/β and human OCT4 are regulated through numerous phosphorylation events (Figures 1 and 23; Mollapour and Neckers, 2012). Recently, posttranslational modifications have been demonstrated to play an important role in HSP90 client protein, co-chaperone and drug interactions (Xu *et al*, 2012; Beebe *et al*, 2013; Mollapour *et al*, 2014). There is growing evidence for the importance of phosphorylation in regulating OCT4 activity (Saxe *et al*, 2009; Brumbaugh *et al*, 2012; Lin *et al*, 2012). The phosphorylation profiles of human and murine OCT4 proteins were created using available literature and phosphorylation prediction software [NetPhos 2.0 (Blom *et al*, 1999) and PhosphoSitePlus (Hornbeck *et al*, 2012)] (Figure 23). Although most phosphorylation sites appear to be conserved between human and murine OCT4, several phosphorylation sites show species-specificity (Figure 23). We noted that the majority of phosphorylation sites occurred in the N-terminal and C-terminal transactivation domains (Figure 23). Where possible, kinases responsible for the phosphorylation of specific residues are illustrated, however in most cases the identity of the kinase responsible for phosphorylation remains uncharacterized (Figure 23). As both HSP90 and OCT4 are highly phosphorylated and phosphorylation play key roles in the regulation and fate of these proteins, we sort to characterize whether phosphorylation influences the relationship between HSP90 and human OCT4.

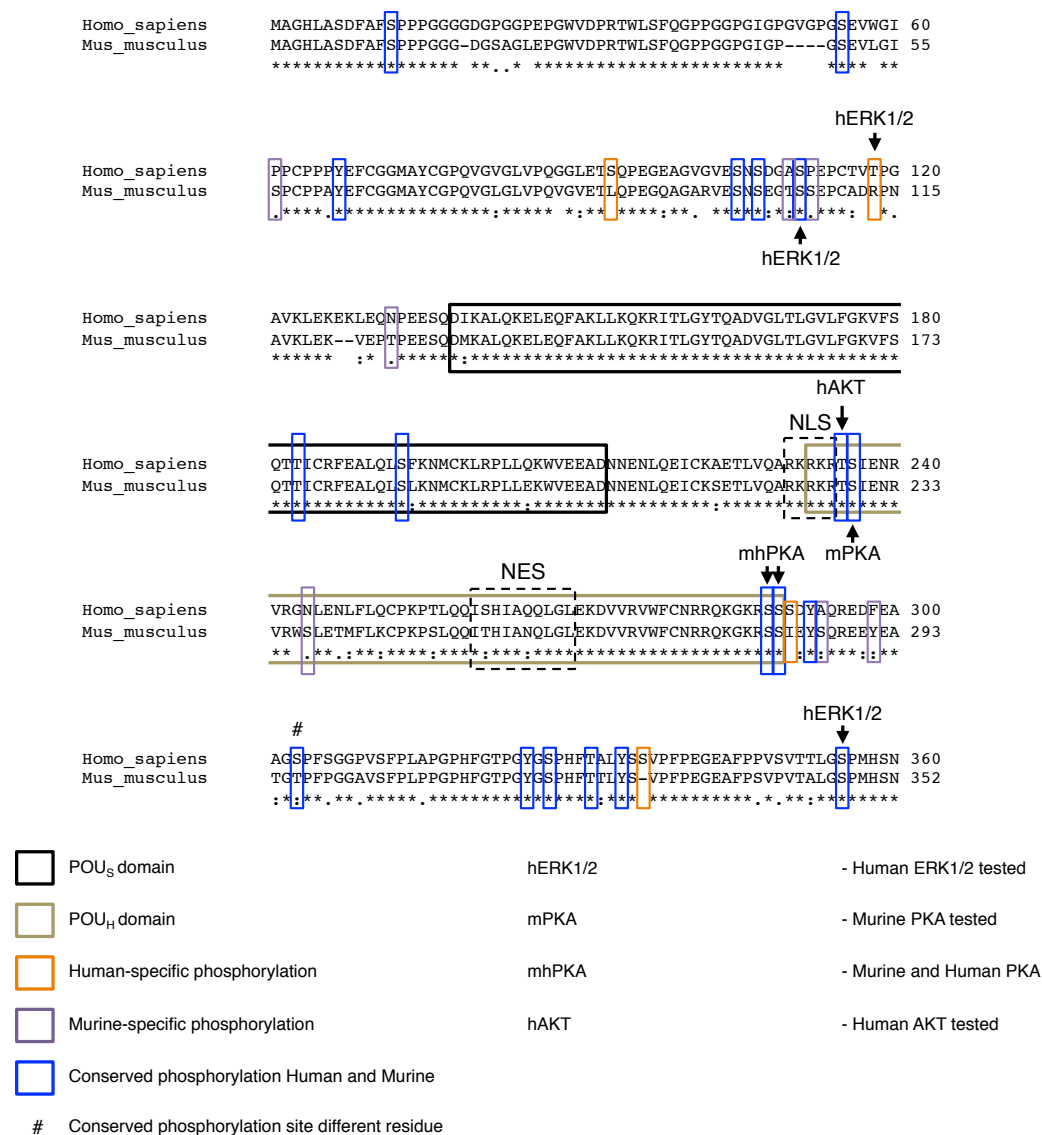


Figure 23 | Comparison of phosphorylation conservation between human and murine OCT4. Sequence alignment of human (*Homo sapiens*) and murine (*Mus musculus*) OCT4 proteins showing predicted and experimentally established phosphorylation sites. Kinases responsible for site phosphorylation are indicated where available. Solid black box, POU_S subdomain. Gold box, POU_{HD} subdomain. Orange boxes, human-specific phosphorylation sites. Purple boxes, murine-specific phosphorylation sites. Blue boxes, conserved phosphorylation site between human and murine. #, Conserved phosphorylation site between human and murine with non-conserved residue. Dashed black line, nuclear localization signal (NLS) or nuclear export signal (NES). hERK2, human residue phosphorylated by ERK2 (only analyzed with human OCT4). mPKA, murine residue phosphorylated by PKA (only analyzed with murine OCT4). mhPKA, both human and murine residues phosphorylated by PKA. hAKT, human residue phosphorylated by AKT (only analyzed with human OCT4).

To begin to characterize the effects of posttranslational modifications on the interaction between human OCT4 and HSP90 α/β , we used commercially available kinase inhibitors (H7, D4476 and ERK2i) to determine whether the interaction between human OCT4 and HSP90 α/β was phosphorylation dependent. The H7 inhibitor targets PKA and PKC kinases and inhibits enzyme activity through direct binding to the enzymes active sites (Hidaka *et al*, 1984), while the D4476 inhibitor specifically targets CK1 kinase activity (Rena *et al*, 2004). The ERK2i inhibitor, although preferentially targeting ERK2, inhibits both ERK1 and ERK2 kinase binding to target proteins but does not prevent the phosphorylation and hence activation of ERK1 and ERK2 by MEK1 and MEK2 (Hancock *et al*, 2006), HEK-hOCT4 cells were treated with kinase inhibitors or DMSO control for 24 hours prior to cell lysis in RIPA buffer. Human OCT4 was co-immunoprecipitated from RIPA lysates using rabbit anti-OCT4 capture antibody. Negative control co-immunoprecipitations were performed with beads alone to account for non-specific interactions between human OCT4 or HSP90 α/β and the protein A matrix. Human OCT4 and HSP90 α/β protein levels were analyzed using Western blot with goat anti-OCT4 and mouse anti-HSP90 α/β antibodies respectively (Figure 24). Densitometry was performed to determine the ratios of HSP90 α/β bound to human OCT4 immunoprecipitated using ImageJ software. The ratios were normalized to the DMSO control sample (Figure 24). Human OCT4 was co-immunoprecipitated in equal amounts for all treatments (Figure 24). There was little to no non-specific interactions between human OCT4 or HSP90 α/β and the protein A matrix with the exception of the ERK2i treated negative control sample (Figure 24). However, the non-specific interaction of OCT4 with the resin was below that of the OCT4 immunoprecipitation sample and therefore this was considered sufficient for interpretation. Treatments with H7 and D4476 kinase inhibitors increased the proportion of HSP90 α/β bound to human OCT4 relative to the DMSO control suggesting a promotion of the HSP90 α/β and human OCT4 interaction with the inhibition of PKA and PKC or CK1 kinases (Figure 24). Interestingly the ERK2i inhibitor decreased the proportion of HSP90 α/β bound to human OCT4 relative to the DMSO control (Figure 24) suggesting that phosphorylation of either human OCT4 and/or HSP90 α/β by ERK1 or ERK2 promotes the interaction between HSP90 α/β and human OCT4.

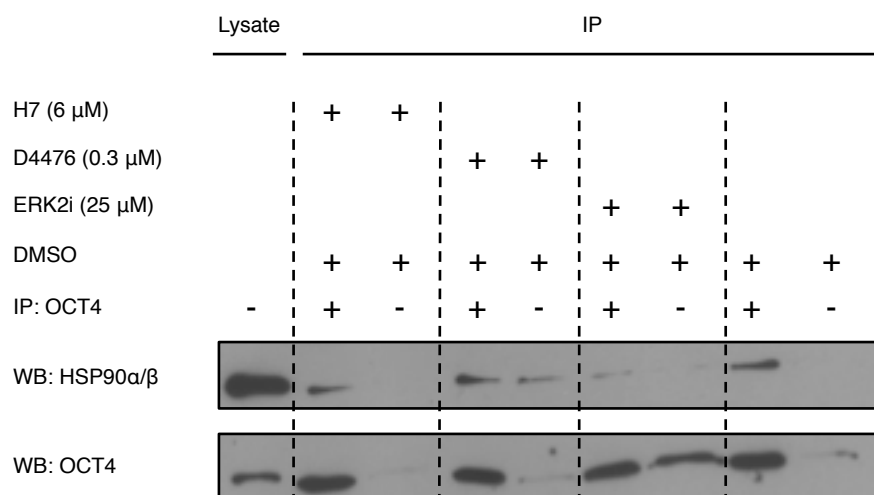


Figure 24 | Effect of kinase inhibition on the interaction between human OCT4 and HSP90 α / β .

HEK-hOCT4 cells were treated with H7 (6 μ M), D4476 (0.3 μ M), ERK2i (25 μ M) and DMSO for 24 hours prior to cell lysis in RIPA buffer. OCT4 co-immunoprecipitation was performed using rabbit anti-OCT4 capture antibody on H7, D4476, ERK2i and DMSO RIPA lysates. Bound proteins were analyzed using Western blot where human OCT4 was probed using a goat anti-OCT4 antibody while HSP90 α / β was probed using a mouse anti-HSP90 α / β antibody. ‘-’ Denotes absence of specified treatment or capture antibody while ‘+’ denotes the presence of specified treatment or capture antibody. Equal volumes from IP samples loaded in each lane.

To account for whether the changes in the interaction between human OCT4 and HSP90 α/β resulted from changes in the human OCT4 protein levels, HEK-hOCT4 cells were treated with 5-fold decreased and increased concentrations, and working concentration (concentration used for co-immunoprecipitation) of the H7, D2276 and ERK2i kinase inhibitors as well as vehicle control DMSO (Figure 25.A). Human OCT4 levels remained unchanged for 5-fold decreased or increased concentrations of H7 and D4476 (Figure 25.A). Although 5-fold decreased and working concentrations of ERK2i and DMSO had no effect on human OCT4 protein levels, in both cases the 5-fold increased concentrations caused human OCT4 degradation (Figure 25.A). In the case of the 5x ERK2i treatment there was loss of total protein (Figure 25.A). This is likely as a result of the 5x greater DMSO concentration in this sample (compared to all the kinase inhibitors used, the ERK2i inhibitor had the greatest concentration of DMSO). A time course study of human OCT4 protein levels in HEK-hOCT4 cells was performed using 25 μ M ERK2i; similar conditions that lead to a loss of interaction between Oct4 and Hsp90. The levels of human OCT4 protein were determined using Western blot. Human OCT4 protein levels initially fluctuated with 25 μ M ERK2i treatment, however increased levels of human OCT4 protein were observed for 16 and 24 hour treatment (Figure 25.B). Interestingly, inhibition of MEK1 and MEK2 kinase phosphorylation, and hence activation, of ERK1 and ERK2, using increasing concentrations of the MEK1 inhibitor U0126 (Favata *et al*, 1998), resulted in a dose-dependent decrease in human OCT4 levels (Figure 25.C). A similar decrease in HSP90 α/β protein levels observed with increasing concentrations of U0126 (Figure 25.C).

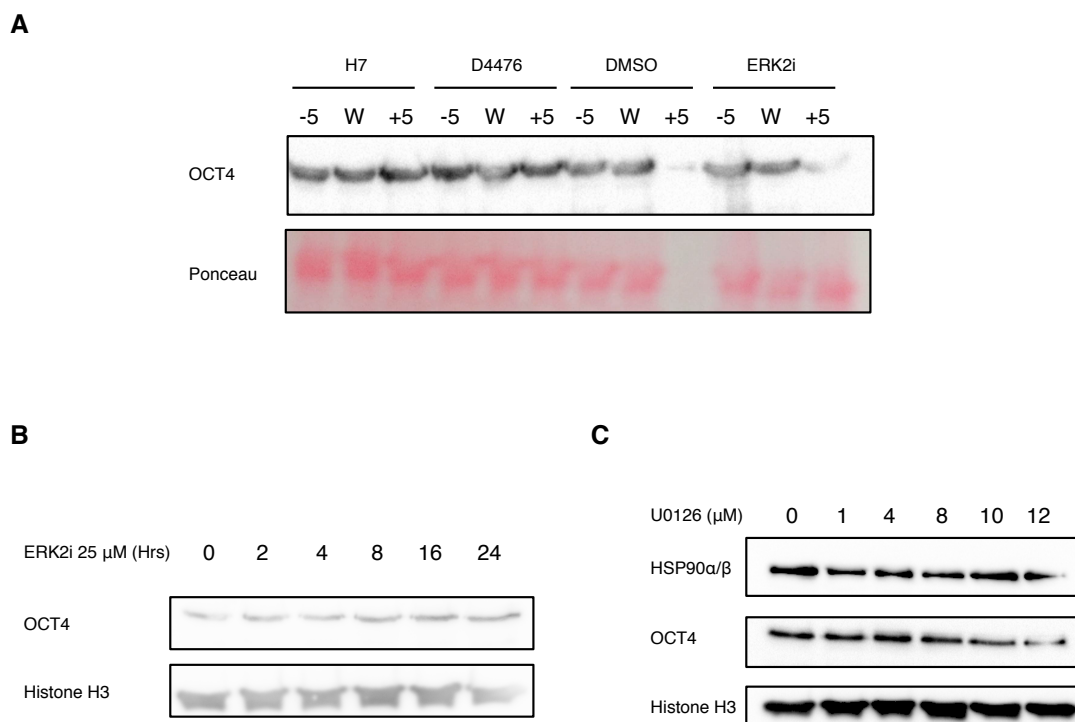


Figure 25 | Effect of kinase inhibitors on human OCT4 protein levels in HEK-hOCT4 cells. (A) HEK-hOCT4 cells were treated with H7 [1.2 μ M (-5-fold), 6 μ M and 30 μ M (+5-fold)], D4476 [0.06 μ M (-5-fold), 0.3 μ M and 1.5 μ M (+5-fold)] or ERK2i [5 μ M (-5-fold), 25 μ M and 125 μ M (+5-fold)] kinase inhibitors, or DMSO as vehicle control for 24 hours prior to cell lysis. Human OCT4 protein levels were analyzed by Western blot. (B) HEK-hOCT4 cells were treated with 25 μ M ERK2i for 0, 2, 4, 8, 16 and 24 hours prior to cell lysis in SDS-PAGE sample buffer. Human OCT4 protein levels were analyzed by Western blot. (C) HEK-hOCT4 cells were treated with increasing concentrations of MEK1 inhibitor U0126 (0 – 12 μ M) for 24 hours prior to cell lysis in SDS-PAGE sample buffer. Human OCT4 and HSP90 α/β protein levels analyzed by Western blot. Equal cell numbers were loaded with Histone H3 used as protein loading control.

Chapter 5. Discussion and Conclusion

5.1. Discussion

Understanding the transcriptional regulation of and the effects of OCT4 activity on cellular biology of stem cells has been well elucidated (Wu and Scholer, 2014). However the biochemistry behind OCT4 protein processing and regulation remains largely unknown, particularly for the human isoform. Using a lentiviral gene delivery system we were successful in the generation of a transgenic stable cell line with constitutive expression of human OCT4, HEK-hOCT4. Exogenous human OCT4 from the HEK-hOCT4 cell line was shown to have both monomeric and homodimeric transcriptional activity. We report the first interaction of human OCT4 with HSP90. As we observed both the direct interaction with HSP90, and degradation of human OCT4 upon inhibition of HSP90 with either N-domain or C-domain binding Hsp90 inhibitors, we propose that human OCT4 is an HSP90 client protein (Figure 26: 1). We show that HSP90 inhibition induced human OCT4 degradation with GA occurs via the proteome (Figure 26: 2). We suggest that GA also blocked OCT4 dimer transcriptional activity, but did not affect monomeric OCT4 transcriptional activity (Figure 26: 3). HSP90 and human OCT4 did not interact in nuclear fractions, and we therefore predict that HSP90 and Human OCT4 interact in the cytoplasm (Figure 26: 1). This is supported by the fact that only a small fraction of the highly abundant HSP90 co-immunoprecipitated with large quantities of total human OCT4. This reflects the low concentrations of human OCT4 detected in the cytoplasmic fractions, and the observation that cytosolic OCT4 is preferentially reduced relative to nuclear OCT4 in GA treated cells. We further show that this human OCT4 and HSP90 interaction is modulated by phosphorylation (Figure 26: 4), wherein kinase inhibition with ERK2i reduced the interaction of human OCT4 and HSP90 without changing the levels of OCT4 protein.

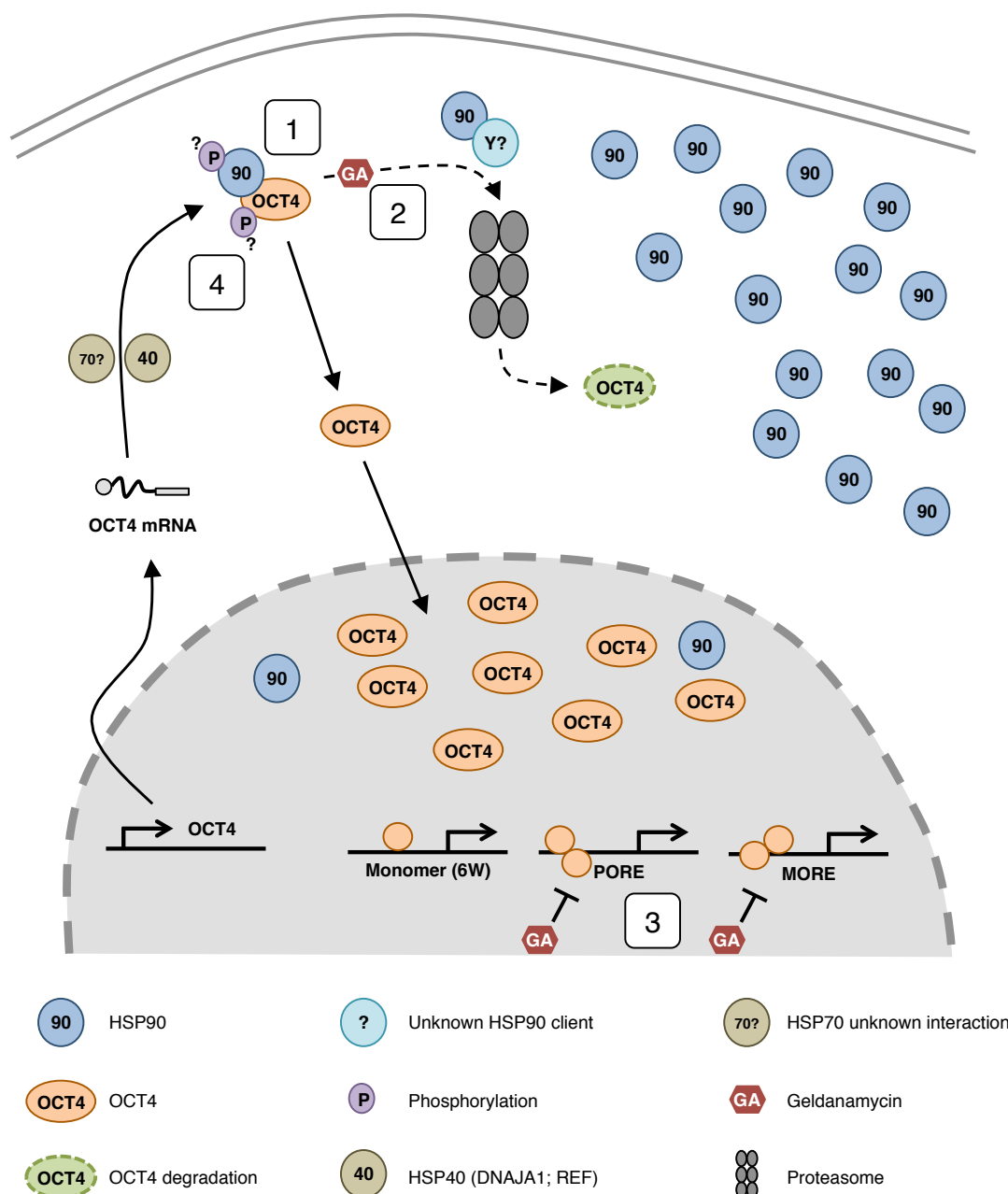


Figure 26 | Summary model for the relationship between human OCT4 and HSP90. OCT4 mRNA is translocated to the cytoplasm where it is translated. OCT4 interacts with DNAJA1, an HSP70 co-chaperone (Pardo *et al*, 2010), suggesting a possible interaction with HSP70. 1) Human OCT4 directly interacts with HSP90, with this interaction suggested to occur in the cytoplasm. 2) Inhibition of HSP90 causes the proteosomal degradation of human OCT4 suggesting that HSP90 is involved in stabilizing human OCT4. 3) Inhibition of HSP90 reduces human OCT4 dimer transactivation activation but not monomer transactivation. 4) Human OCT4 and HSP90 complex is modulated by kinase activity. ?, experimentally undetermined.

5.1.1. Alternative model to address the biochemical relationship between human OCT4 and human HSP90

Bradley and colleagues (2012) used a murine ESC model to elucidate HSP90 functioning in pluripotency, concluding that HSP90 is essential in maintaining pluripotency of murine ESCs through the regulation of OCT4 and Nanog (Bradley *et al*, 2012). As both OCT4 and Nanog were found to interact directly with HSP90 and HSP90 inhibition resulted in the degradation of both OCT4 and Nanog proteins, they suggested that OCT4 and Nanog are potential novel client proteins of HSP90 (Bradley *et al*, 2012). However, the use of stem cells as a model for testing the biochemical relationship between HSP90, a protein responsible for global cellular homeostasis, and OCT4 and Nanog, proteins responsible for the master regulation of stem cell biology, is potentially flawed. There are possible complications due to off-target responses resulting from HSP90 activity modulation, either by chemical inhibitors or siRNA, which challenge the interpretation of these data. Specifically, it is not possible, using this model system, to determine whether HSP90 inhibition induced degradation of OCT4, therefore leading to differentiation of murine embryonic stem cells; or whether HSP90 inhibition induced differentiation through deregulation of stem cell signaling pathways which resulted in the concomitant loss of OCT4.

HSP90 regulates several intermediates of stem cell-related signaling pathways that are involved in maintaining and regulating stem cell biology (Figure 3). These HSP90-regulated stem cell-related signal transduction pathways include, but are not limited to, the MAPK/ERK signaling pathway (Bauweester *et al* 2004; Piper *et al*, 2006; Shinozaki *et al* 2006; Truman *et al* 2008; Fang *et al*, 2009; Haupt *et al*, 2012), GSK3 β signaling pathway (Lochhead *et al*, 2006; Banz *et al*, 2009), Wnt/ β -catenin signaling pathway (Cooper *et al*, 2011), TGF β signaling pathway (Wrighton *et al*, 2008; Shang *et al*, 2014), and the LIF/JAK/STAT3 signaling pathway (Sato *et al*, 2003; Setati *et al*, 2010; Prinsloo *et al* 2012).

The LIF/JAK/STAT3 signaling pathway is critical for murine ESC self-renewal (Smith *et al*, 1988; Yoshida *et al*, 1994; Takeda *et al*, 1997; Niwa *et al*, 1998; Raz *et al*, 1999; Matsuda *et al*, 1999) however it is not required for human embryonic stem

cell self-renewal (Deheron *et al*, 2004). The presence of LIF induces JAK mediated phosphorylation of STAT3 resulting in the dimerization, activation and nuclear translocation of phosphorylated STAT3 (Zhong *et al*, 1994; Kretzschener *et al* 2004; Ushijima *et al*, 2005). HSP90 interaction with STAT3 is required for both phosphorylation-mediated dimerization and activation, and subsequent translocation of phosphorylated dimerized STAT3 into the nucleus (Setati *et al*, 2010; Prinsloo *et al*, 2012). HSP90 inhibition prevents the phosphorylation of STAT3 thereby reducing the levels of phosphorylated STAT3 and nuclear translocation in the presence of LIF (Sato *et al*, 2003, Setati *et al*, 2010). Abrogation of this pathway results in murine ESC differentiation (Niwa *et al*, 1998; Raz *et al*, 1999; Matsuda *et al*, 1999). Bradley and colleagues (2012) observed a reduction of phosphorylated STAT3 with HSP90 inhibition in murine ESCs (Bradley *et al*, 2012), as has been previously demonstrated with HSP90 inhibition (Sato *et al*, 2003, Setati *et al*, 2010). Interestingly, in the Bradley study the loss of phospho-STAT3 occurred at a lower concentration of the HSP90 inhibitor than that required to reduce OCT4 levels. Therefore an alternative conclusion to the observed reductions in murine OCT4 and Nanog proteins with HSP90 inhibition by Bradley and colleagues (2012), may rather be as a result of induction of differentiation due to HSP90 inhibition abrogating, for one, the LIF/JAK/STAT3 signaling pathway. Although induction of differentiation would explain the reduced Nanog protein and OCT4 protein and mRNA levels, the authors did not observe a reduction in Nanog mRNA. Nanog is one of many target genes of activated STAT3 (Okumura *et al*, 2011). HSP90 additionally is involved in stabilization of Trithorax protein complexes (Tariq *et al*, 2009) that regulate gene expression through providing gene accessibility and therefore activation of genes (Swartz and Pirrotta, 2007). Although not proven, HSP90 inhibition may prevent pluripotent effector molecules such as OCT4 and Nanog from accessing necessary target genes through the abrogation of Trithorax complexes.

Using the HEK-hOCT4 model we are able to propose that human OCT4 is an HSP90 client protein based on our observations that human OCT4 directly interacts with HSP90, and inhibition of HSP90 activity results human OCT4 degradation. Importantly, the reduction of OCT4 protein levels observed in our study are independent of differentiation associated reductions to OCT4 gene expression, as may be the case in the study performed by Bradley and colleagues (2012). However,

although the model generated in our study allows for the biochemical analysis of the relationship between OCT4 and HSP90 it does not address the biological significance of this relationship. In this context, the murine ESC model used by Bradley and colleagues (2012) is superior to ours since it provides the biological context supporting the functional relevance of the relationship between OCT4 and HSP90. However, one concern is that the Bradley model is murine ESC and there are well characterized differences between the biology of human and murine ESC (Schnerch *et al*, 2010). Therefore our future work is to proceed into analysis of the human OCT4 and HSP90 complex within the biological context of pluripotent stem cells using a human iPSC model (Figure 27). Several studies analyzing gene expression and DNA methylation in iPSC and ESC (both human and murine) have illustrated that while some iPSC clones show inherent differences compared to ESC clones, many display indistinguishable differences to ESC counterparts (Chin *et al*, 2009; Deng *et al*, 2009; Guenther *et al*, 2010; Newman and Cooper, 2010; Bock *et al*, 2011). However even amongst ESC clones differences and variations have been noted (Ward *et al*, 2004; Osafune *et al*, 2008). We have started initial work in setting up human iPSC culture protocols in order to determine the biological relevance of the human OCT4 and HSP90 interaction. The human iPSC cell line Nelson1 was a kind gift from Dr Janine Scholefield and Dr Musa Mhlanga (CSIR, Pretoria). This cell line was developed in South Africa and has been validated through pluripotency marker expression, karyotype analysis, tri-germ layer differentiation assays and inactivation of exogenous genes used to reprogram the cells. Although both feeder and feeder-free culture systems are currently being developed (Figure 27), we propose to use feeder-free iPSC culture to negate the effect of feeder cells in biochemical assays. Our initial focus will be on confirming our biochemical analyses in the biological context of iPSCs. First we will determine whether the interaction between human OCT4 and HSP90 occurs in iPSCs, after which we will analyze the effect of HSP90 inhibition on human OCT4 and cellular differentiation.

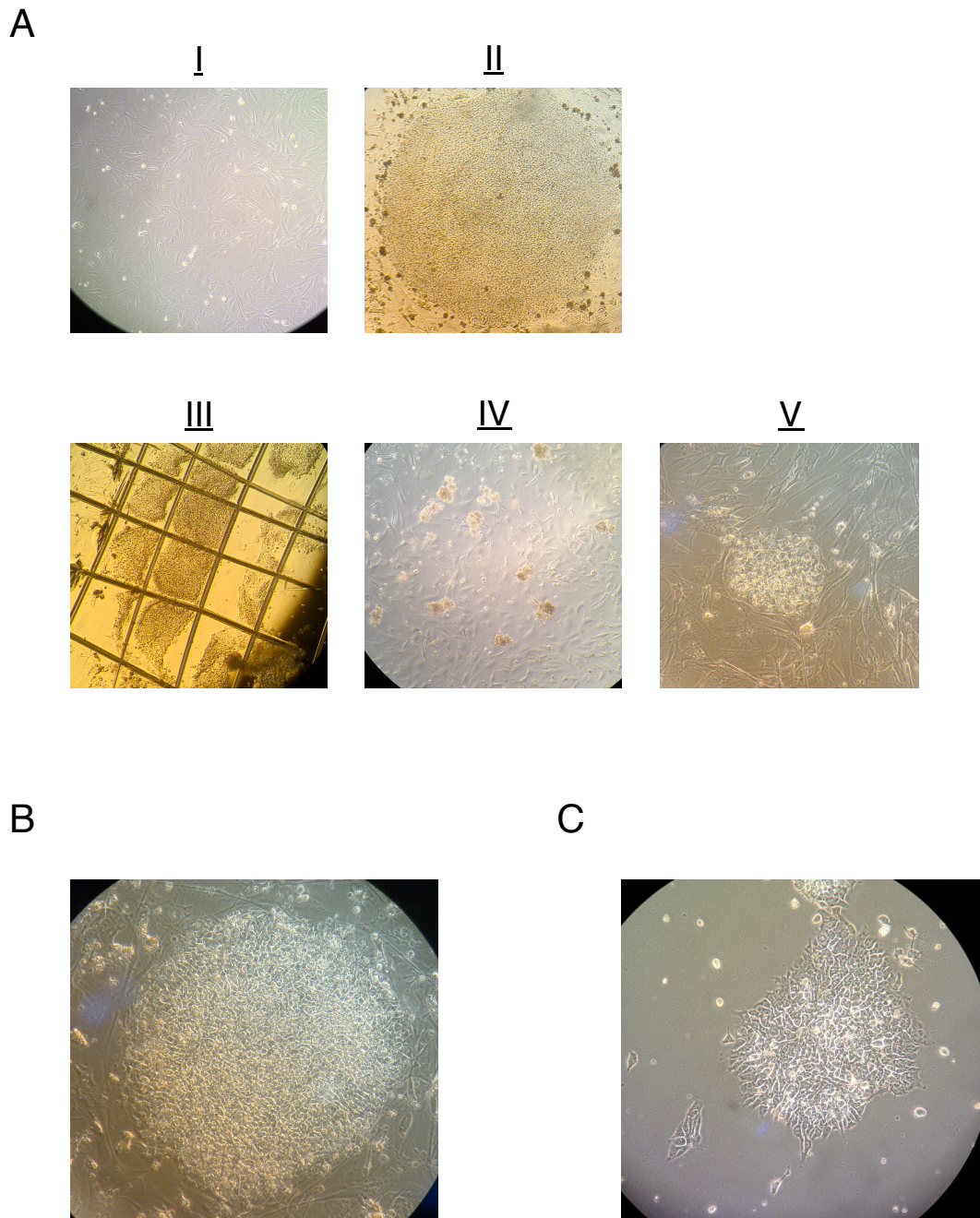


Figure 27 | Optimization of human iPSC culture for HSP90-OCT4 analysis in context of pluripotency and differentiation.

Human iPSCs (Cell line Nelson1) will be used to determine the biological relevance of the HSP90-OCT4 relationship. (A) Initial protocols for the maintenance of human iPSCs on MEF feeder layers. I, inactivated MEF feeder-layer; II, iPSC feeder-dependent colony; III, mechanical passage of feeder-dependent iPSC colonies; IV, seeding of feeder-dependent iPSC cut colonies onto MEF feeder-layer; V, iPSC feeder-dependent colony re-establishment after mechanical passage. (B) Feeder-dependent human iPSC colony morphology. (C) Feeder-free human iPSC colonies grown on Geltrex.

5.1.2. Human OCT4 is an HSP90 client

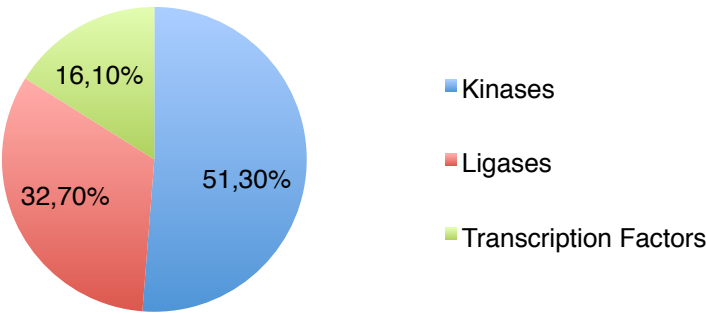
HSP90 interacts with and stabilizes a large cohort of cellular proteins (over 300 are presently described), known as client proteins. These client proteins include a wide range of protein types and may include both wild type and mutant proteins (Taipale *et al*, 2012). The accepted description of an HSP90 client protein is a protein that directly interacts with HSP90 and requires HSP90 for its stability and/or activity (Taipale *et al*, 2010). Importantly, a protein is given the ‘HSP90 client’ status only if it displays both of these characteristics. HSP90 does not function in the folding of native polypeptides, but rather interacts with folded or partially folded client proteins. Recognition of a client protein by HSP90 appears to rely on conformational determinants, rather than a linear motif, which explains why HSP90 can interact with such a wide variety of proteins and makes prediction of HSP90 client proteins difficult. The HSP90-client protein interaction results in the maturation and/or stabilization and hence activity of the client protein. However, at present there does not appear to be any correlation between the affinity of the HSP90-client interaction and the dependence of that client on HSP90 for stability. We propose that human OCT4 is an HSP90 client based on our observations that OCT4 interacts directly with HSP90, and that inhibition of HSP90 induces OCT4 degradation and loss of homodimer activity. The fact that the HSP90-OCT4 interaction is affected by posttranslational modifications supports this conclusion, as differential modification has been shown to be one mechanism by which HSP90 regulates client protein binding (Mollapour *et al*, 2010).

5.1.2.1. Human OCT4 directly interacts with HSP90 α/β

Our co-immunoprecipitation assays revealed for the first time that human OCT4 interacts directly with HSP90 α/β . An interaction between murine OCT4 and HSP90 has previously been described in the murine system (Bradley *et al*, 2012). We are as yet to discern whether this interaction occurs with both HSP90 cytosolic isoforms (HSP90 α and HSP90 β), or whether the interaction between human OCT4 and HSP90 is isoform-specific. In the study, Bradley and colleagues (2012) identified that murine OCT4 interacted with both murine HSP90 α and HSP90 β isoforms.

Although HSP90 interacts with over 300 client proteins (Taipale *et al*, 2012), the interaction between HSP90 and transcription factors, like OCT4, represents a minor proportion of HSP90 interactions. A large-scale HSP90 interaction analyses by Taipale and colleagues (2012) identified that of the over 300 HSP90 client proteins identified in there study, 51% of these were kinase proteins while only 16% were transcription factors (Figure 28.A; Taipale *et al*, 2012). Furthermore, approximately 60% of the total of 315 kinases analyzed were identified as being HSP90 client proteins (Figure 28.B; Taipale *et al*, 2012). In contrast, only 6% out of a total of 978 transcription factors analyzed were determined as being HSP90 client proteins (Figure 28.B; Taipale *et al*, 2012). Although human OCT4 was among the transcription factors screened, Taipale and colleagues (2012) did not identify human OCT4 as a HSP90 client protein. However in this study, the client proteins were only transiently expressed and only the protein level and not the activity of the proteins was screened. In contrast to the study by Taipale and colleagues (2012), human OCT4 in our model is constitutively expressed and transcriptionally active. Additionally observations from the morphological changes exhibited in HEK-hOCT4 cells versus HEK293FT cells are suggestive that the constitutive expression of transcriptionally active human OCT4 has resulted in some form of cellular programming. This together with the identification of a murine OCT4 and HSP90 complex in murine ESCs (Bradley *et al*, 2012) implies that a stabilized OCT4 transcriptional network may be required for the detection of the relationship between HSP90 and human OCT4. Transient exogenous expression of OCT4 was not sufficient for the interactions between OCT4 and HSP90 to be observed (Taipale *et al*, 2012).

A



B

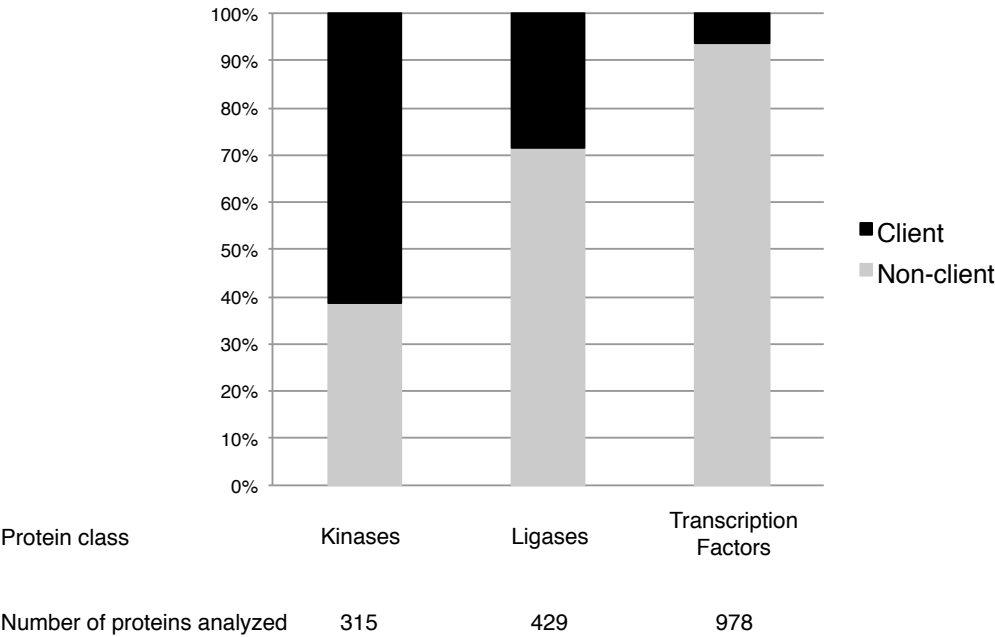


Figure 28 | Proportional analysis of kinase, ligase and transcription factor HSP90 client proteins.
(A) Proportional of HSP90 kinase (blue), ligase (red) and transcription factor (green) client protein classes identified by Taipale and colleagues (2012). (B) Proportion of total kinases, ligases and transcription factors tested that were identified as HSP90 client proteins. Black bars; HSP90 client proteins. Grey bars; non-client protein.

The cytosolic isoforms of HSP90 are one of the most highly abundant proteins in a cell, constituting approximately 3% of total cellular protein (Mahalingam *et al*, 2009). Although predominantly localized in the cytoplasm of cells, and not containing a nuclear localization signal (NLS), the presence of HSP90 in the nucleus of cells is well accepted (Sawarkar and Paro, 2012). Using subcellular fractionation techniques we found that human OCT4 in HEK-hOCT4 cells displayed a predominant nuclear localization with some cytoplasmic localized human OCT4. Additionally we found the concentration of HSP90 within the nucleus substantially lower than that of the cytoplasmic fractions in HEK-hOCT4 cells. We observed that nuclear human OCT4 and nuclear HSP90 α/β do not interact in REAP-isolated nuclear fractions from HEK-hOCT4 cells, suggesting that the interaction between human OCT4 and HSP90 is occurring in the cytoplasm. This is supported by the fact that low levels of HSP90 relative to human OCT4 were detected in OCT4 co-immunoprecipitations from whole cell lysates. This may reflect the fact that cytoplasmic OCT4, which is less abundant than nuclear OCT4, is interacting with HSP90 in the cytoplasm. Since nuclear OCT4 will need to be translated in the cytoplasm, it is plausible that the interaction between OCT4 and HSP90 in the cytoplasm is required for stabilization of nascent OCT4 prior to nuclear transport. This is supported by the fact that OCT4 interacts with the HSP70 co-chaperone DNAJA1 (Pardo *et al*, 2010). This is the initial stage of HSP70-HSP40 mediated *de novo* protein folding prior to association with HSP90 chaperone machine; a process that is conserved for many HSP90 client proteins (Li *et al*, 2012)

However, it is also possible that HSP90 levels in the nuclear complex are too low as to be undetectable by Western blot analysis, despite the fact that we isolated high levels of OCT4 in the nuclear co-immunoprecipitations. Indeed, it is known that nuclear HSP90 represents only 2 to 3% of total HSP90 protein levels (Sawarkar and Paro, 2012). Additionally, although the REAP subcellular fractionation technique allows for the isolation of intact protein complexes from both cytoplasmic and nuclear fractions (Suzuki *et al*, 2010), we cannot rule out the possibility that the REAP technique in our particular study did not allow for isolation of intact protein complexes. This needs to be verified by the detection of known human OCT4 interacting partners with the isolation of nuclear OCT4 using the REAP fractionation technique. Esrrb or CDK1 are two examples of proteins that interact with nuclear

OCT4 in a DNA-independent manner (van den Berg *et al*, 2008; Li *et al*, 2012). Detection of either Essrb or CDK1 with nuclear OCT4 isolated using the REAP technique would confirm that intact nuclear OCT4 multi-protein complexes are being isolated. This would confirm that HSP90 α/β does not interact with human OCT4 in the nucleus. Additionally, we should demonstrate the specific interaction between human OCT4 and HSP90 α/β in cytoplasmic extracts.

5.1.2.2. *Inhibition of HSP90 causes loss of OCT4 protein levels*

HSP90 client proteins are reliant on HSP90 activity for stability, with the inhibition of HSP90 activity resulting in degradation of HSP90 client proteins (Karogaz *et al*, 2015). We have shown for the first time that inhibition of HSP90 using both GA (N-terminal HSP90 inhibitor) and NOV (C-terminal HSP90 inhibitor) results in human OCT4 degradation. The degradation of murine OCT4 has previously been described with HSP90 inhibition in murine ESCs (Bradley *et al*, 2012). However HSP90 inhibition in murine ESCs results in the abrogation of the LIF/JAK/STAT3 signaling pathway by reducing phosphorylated STAT3 (active STAT3) (Sato *et al*, 2003; Setati *et al*, 2010), a signaling pathway essential for maintaining the undifferentiated stem cell state in the murine system (Niwa *et al*, 2000). This may suggest that loss of murine OCT4 in this study was due to the induction of differentiation (Bradley *et al*, 2012). This notion is supported by the authors' observations that HSP90 inhibition reduced the levels of murine OCT4 mRNA in the murine ESC model (Bradley *et al*, 2012). As the effects of HSP90 inhibition on HSP90 client proteins are observed post-translationally (Chen *et al*, 2012), a decrease to OCT4 mRNA levels is most likely due to the resulting effect of HSP90 inhibition to stem cell-related signaling pathways. However it must be noted that OCT4 does to some extent mediate its own transcriptional activation with the aid of a transcription factor binding partner SOX2 (Okumura-Nakanishi *et al*, 2005) and therefore a reduction in OCT4 mRNA levels would be expected with the reduction in OCT4 protein levels. Importantly, human OCT4 degradation reported in our study is independent of both transcriptional deregulation due to perturbations of stem cell-related signaling pathways and HSP90 inhibition induced cellular differentiation. Our data suggest that HSP90 plays a role in the stabilization of human OCT4. However, as we have not assessed the effect of GA

treatment on human OCT4 mRNA levels, we cannot with absolute certainty state that the reductions of human OCT4 with GA treatment is solely due to degradation alone.

We observed that human OCT4 was less sensitive to HSP90 inhibition compared to HSP90 model client proteins CDK4 and AKT, with human OCT4 degradation occurring at concentrations above 250 nM GA. Our data, as well as data from Bradley and colleagues (2012), suggests that other clients of HSP90 in murine ESCs may have been affected before OCT4 levels were perturbed. HSP90 client proteins have been shown to display varied degradation sensitivities to HSP90 inhibition and not all respond in the same manner (Taipale *et al*, 2012). From the study by Taipale and colleagues (2012) it is evident that some kinase proteins, although identified as showing physical interactions with HSP90, showed no protein degradation upon HSP90 inhibition irrespective of whether they displayed strong or weak associations with HSP90 and therefore were not classified as HSP90 client proteins (Taipale *et al*, 2012). Indeed some kinases that were identified as client proteins in the study, and further classified as showing ‘strong’ client status by the authors, displayed only slight or minimal reductions in protein levels (Taipale *et al*, 2012). Both of the HSP90 model client proteins used in our study are kinases. The relationship between HSP90 and kinase client proteins is well established, with over half of kinase proteins classified as HSP90 clients (Figure 28.B; Taipale *et al*, 2012). However the interaction and stabilizing effect of HSP90 on transcription factor client proteins is more novel with only an estimated 6% of transcription factors described as HSP90 clients (Figure 28.B; Taipale *et al*, 2012).

As we have provided evidence suggesting that the interaction between HSP90 and human OCT4 occurs in the cytoplasm, the observed sensitivity of human OCT4 to HSP90 inhibition may be proportional to cytoplasmic versus nuclear human OCT4 concentrations in these subcellular localizations and/or may be reflective of the function of HSP90 on human OCT4. Hence HSP90 inhibition preferentially degrades the less concentrated cytoplasmic proportion of human OCT4, while leaving the nuclear fraction unaffected under the conditions of our assay. This is supported by our observations that along with HSP90 inhibition reducing human OCT4 protein levels, HSP90 inhibition causes a dose-dependent increase in the ratio of nuclear and cytoplasmic human OCT4 (although at the highest concentration of GA treatment the

number of viable cells present were very low), as well as decreasing co-localization between human OCT4 and HSP90 α/β . For both subcellular fractionation methods, lower molecular weight bands were detected with the human OCT4 antibody. These bands were present in both nuclear and cytoplasmic fractions although displayed a predominantly cytoplasmic localization. With GA treatments, the proportion of these bands within the cytoplasmic fraction increased substantially. It is tempting to speculate that these lower molecular weight bands represent OCT4 degradation products with the increased cytoplasmic proportions accumulating with GA treatment as a result of degradation of cytosolic human OCT4 due to HSP90 inhibition. Cytosolic human OCT4-derived lower molecular weight bands have been observed in cytosolic fractionations from subcellular fractionations of seminomas and embryonal carcinomas (Cheng *et al*, 2007). This is an interesting proposition in light of our data inferring that the HSP90 and human OCT4 interaction occurs in the cytoplasm.

Additional experiments preventing the nuclear import or export of human OCT4 will provide insight into determining whether HSP90 interacts only with cytoplasmic OCT4 and whether inhibition induces preferential degradation of cytoplasmic human OCT4 as opposed to nuclear human OCT4. Although inhibition of nuclear importation of human OCT4 could be achieved using NLS mutants of human OCT4 (Pan *et al*, 2004), inhibition of human OCT4 nuclear exportation would prove difficult, as nuclear export of OCT4 has been suggested to occur through an unknown mechanism with alterations to a putative NES not affecting nuclear export of OCT4 (Oka *et al*, 2013). As the studies on the NLS and NES sequences of OCT4 to date have been conducted on the murine protein, these experiments will further serve to validate whether the equivalent sequences in human OCT4 have an analogous function.

In HEK-hOCT4 cells, human OCT4 displayed a natural protein turnover rate together with HSP90 α/β as illustrated by accumulation of human OCT4 and HSP90 α/β proteins upon treatment with proteasome inhibitor MG132. This is suggestive that human OCT4 degradation is mediated through the proteasome; a result in line with murine OCT4, as proteasome inhibition rescued murine OCT4 degradation (Xu *et al*, 2006; Saxe *et al*, 2009). Additionally our data suggest that HSP90 inhibition induced

degradation of human OCT4 through the proteasome, a similar observation made by Bradley and colleagues for HSP90 inhibition induced degradation of murine OCT4 with 17-AAG (Bradley *et al* 2012). OCT4 translation occurs in the cytoplasm after which OCT4 is translocated to the nucleus. This may explain why we observed preferential loss of cytosolic OCT4 in our confocal experiments. The destabilization of the human OCT4 and HSP90 interaction in the cytoplasm and resulting degradation of cytoplasmic human OCT4 will ultimately lead to reductions in nuclear human OCT4 due to OCT4 natural protein turnover. The time frames of our HSP90 inhibition studies were likely not long enough to observe the effect on nuclear OCT4. OCT4 protein half-life varies between 5 to 8 hours in murine ESCs (Plachta *et al*, 2011), while OCT4 in human embryonic carcinomas display a slightly elevated half-life of between 6 to 12 hours (Lin *et al*, 2012). Interestingly, sumoylation and phosphorylation post-translational modifications substantially increase the half-life of OCT4 in murine and human systems (Wei *et al*, 2007; Lin *et al*, 2012). Additionally, Lin and colleagues (2012) demonstrated, using an OCT4 phospho-mimetic mutant, mimicking AKT phosphorylation of Threonine 235 on human OCT4, that phosphorylation stabilizes human OCT4 and facilitates nuclear translocation in human embryonic carcinomas (Lin *et al*, 2012). It must be noted that AKT is a client protein of HSP90 and, as illustrated in our study, inhibition of HSP90 induces degradation of AKT.

Bradley and colleagues (2012) used the GA analog 17-Allylamino-17-demethoxygeldanamycin (17-AAG) at a concentration of 250 nM for HSP90 inhibition when assessing the relationship between HSP90 and murine OCT4 (Bradley *et al*, 2012). Both GA and 17-AAG have been described as having equivalent HSP90 inhibition at similar doses even though GA binding to HSP90 is stronger than that of 17-AAG (Schulte and Neckers, 1998). Additionally 17-AAG exhibits reduced *in vivo* toxicity in comparison to GA (Page *et al*, 1997). It is not known whether the action of HSP90 inhibitors is equivalent throughout the cytoplasm and nucleus, although both GA and 17-AAG are unable to enter the mitochondria of cells (Kang *et al*, 2007). The vast proportion of HSP90 within the cytoplasm may additionally sequester HSP90 inhibitors thereby preventing these inhibitors from entering the nucleus or substantially reducing the nuclear concentration of the inhibitor. As we present evidence suggesting that HSP90 interaction with human

OCT4 does not occur in the nucleus of HEK-hOCT4 cells, HSP90 relationship with OCT4 is likely to be involved in cytoplasmic stabilization of human OCT4 and therefore inhibition of nuclear HSP90 would be predicted to be of little consequence.

5.1.2.3. *Inhibition of HSP90 reduces human OCT4 activity*

OCT4 and POU transcription factors are able to activate transcription as monomers recognizing the octamer motif (5'-ATGCAAT-3'), or as homodimer complexes recognizing different octamer-related motifs, and interacting with DNA in different homodimer configurations (PORE, 5'-ATTTGAAAGGCAAAT-3'; MORE, 5'-ATGCATATGCAT-3') (Remenyi *et al*, 2001). We assessed the effect of HSP90 inhibition on human OCT4 activity and transactivation using luciferase reporter assays for monomer activation (6W), and homodimer activation (PORE and MORE). In the case of homodimer transactivation, we observed a subtle dose-dependent decrease in homodimer transactivation of both PORE and MORE elements. This response was expected due to the fact that GA concentrations above 250 nM induced degradation of human OCT4. Geldanamycin treatment had no substantial effect on monomeric activity at lower concentrations (100 nM and 500 nM), however monomer transactivation was increased at 1000 nM GA. This suggested that, conversely to our previous evidence that HSP90 inhibition leads to human OCT4 protein degradation, HSP90 inhibition increases human OCT4 monomer activity. Monomeric OCT4 is able to bind to PORE elements however this binding does not elicit transcriptional activation, but rather results in transcriptional suppression of the element (Remenyi *et al*, 2001). The promotion of monomeric OCT4 activity over homodimeric OCT4 activity would suggest that HSP90 functions in the dimerization of human OCT4 either directly or indirectly. If HSP90 does function directly in the stability of human OCT4 dimerization, then this interaction should occur in the nucleus, as OCT4 dimerization is DNA dependent (Remenyi *et al*, 2001). However because HSP90 α/β did not co-immunoprecipitate with nuclear human OCT4, we believe that this is unlikely.

It is also possible that HSP90 inhibition induced changes in OCT4 post-translational modifications that culminated in altered activity. Transcriptional activation of PORE

and MORE elements by OCT4 are regulated by phosphorylation of numerous specific residues on OCT4 (Brehm *et al*, 1997). Phosphorylation of Serine 159 or Serine 335 prevents MORE activation without affecting PORE activation (Remenyi *et al*, 2001; Kang *et al*, 2009). Phosphorylation of Serine 105 prevents DNA interaction by abrogating both monomer and homodimer transcriptional activity by creating a steric hindrance between the POU_S DNA binding domain and DNA (Remenyi *et al*, 2001). Therefore neither of these changes would be consistent with our observations. However, phosphorylation of Serine 229 by PKA, abolishes PORE but not MORE activity by steric hindrance within the homodimer interface (Saxe *et al*, 2009) and therefore might explain our observations.

Our data suggesting that the interaction between HSP90 and human OCT4 does not occur in the nucleus does not appear consistent with the observations that HSP90 inhibition may result in decreased homodimer transactivation (which is DNA dependent) but not monomer transactivation. However, two theories may account for these observations: 1) HSP90 inhibition modulates a client protein or client proteins that are responsible for catalyzing post-translational modifications of human OCT4, thereby HSP90 inhibition indirectly affects the homodimer transactivation of human OCT4; or 2) HSP90 inhibition reduces human OCT4 protein levels thereby abrogating homodimer transactivation and promoting monomeric human OCT4 activity due to a stoichiometric shift. As numerous kinases are HSP90 client proteins (Taipale *et al*, 2012), with some of these clients implicated in OCT4 phosphorylation (PKA and AKT, or the ERK pathway-mediating MAPKs), the possibility that HSP90 inhibition results in degradation of a kinase or kinases responsible for OCT4 phosphorylation thereby indirectly affecting transactivation of OCT4 homodimers is a likely scenario. Indeed we observed this scenario with HSP90 inhibition causing the degradation the HSP90 model client protein and OCT4 phosphorylating kinase AKT. We have also demonstrated that HSP90 inhibition reduced the levels of human OCT4 which may favour the formation of monomers over dimers, although we currently have no direct evidence of an effect on OCT4 dimer formation. We are therefore as yet unable to discern between the two hypotheses, or in fact the possibility that both may be the case. Although firefly luciferase is a known client protein of HSP90 (Thulasiraman and Matts, 1996; Ahner *et al*, 2005; Boschelli *et al*, 2010), the fact that the monomer activity increased while homodimer activity decreased suggests that loss

of activity was not due to the effects of HSP90 inhibition on firefly luciferase protein, but rather due to modulation of firefly luciferase expression from the reporter constructs.

5.1.3. Human OCT4 and HSP90 interaction is affected by phosphorylation

Both HSP90 and OCT4 are heavily regulated by post-translational modifications, most significantly phosphorylation. Although numerous phosphorylation sites have been identified on OCT4, the identity of kinases responsible for the majority of these phosphorylations remains relatively unknown (Brumbaugh *et al*, 2012). We have begun to assess the effect of kinase inhibitors on the interaction between HSP90 and human OCT4 to determine if the interaction between HSP90 and human OCT4 is dependent on phosphorylation events, as is the case with HSP90 and stem cell-related transcription factor STAT3 (Setati *et al*, 2010; Prinloo *et al*, 2012) and other HSP90 clients (Mollapour *et al*, 2010). Although displaying a relatively high protein sequence identity together with conserved phosphorylation sites (Figure 23), murine and human OCT4 show species-specific phosphorylation motifs (Figure 23). Three commercially available kinase inhibitors were used, namely H7 (targeting PKA and PKC; Hidaka *et al*, 1984), D4476 (targeting CK1; Rena *et al*, 2004) and ERK2i (preferentially targeting ERK2-mediated phosphorylation of protein substrates but also inhibits ERK1-mediated phosphorylation of protein substrates; Favata *et al*, 1998). In all cases, kinase inhibition modulated the interaction between HSP90 α/β and human OCT4. Although these results need to be validated and verified, they suggest that phosphorylation of either or both proteins modulate the interaction between HSP90 α/β and human OCT4.

Treatment with the PKA and PKC chemical inhibitor H7 and the CK1 chemical inhibitor D4476 showed increased interaction between HSP90 and human OCT4. Both PKA and PKC have been illustrated to phosphorylate HSP90 (Lei *et al*, 2007; Wang *et al*, 2009; Lu *et al*, 2014) however the functional significance of these phosphorylation events on the wide range of HSP90 clients is unknown. PKA phosphorylation of HSP90 has been shown to target HSP90 to the plasma membrane and for extracellular secretion (Lei *et al*, 2007; Wang *et al*, 2009). Therefore the

inhibition of PKA phosphorylation of HSP90 using the H7 chemical inhibitor may increase the co-localization between cytoplasmic HSP90 and cytoplasmic human OCT4 resulting in increased interaction. PKA has additionally been shown to phosphorylate murine OCT4, resulting in increased murine OCT4 protein levels without increasing murine OCT4 mRNA levels. This suggests that PKA phosphorylation of murine OCT4 may promote protein stability as well as increased PORE-specific homodimer activation (Saxe *et al*, 2009). This PKA phosphorylation site is conserved in human OCT4 (Figure 23) however the phosphorylation of human OCT4 by PKA is yet to be confirmed experimentally.

Of particular interest was the reduction of the HSP90 and human OCT4 complex with inhibition of ERK1/2, because of the biological significance of the MAPK/ERK pathway in regulating stem cell nature. Previously ERK2 has been shown to phosphorylate human OCT4 at three sites *in vitro*: Serine 111, Threonine 118 and Serine 355 (Brumbaugh *et al*, 2012). Serine 111 is the only conserved ERK phosphorylation site in murine OCT4 (Figure 23; murine OCT4 Serine 106). The MAPK/ERK pathway is stimulated by FGF (Figure 2 and 3) and is essential in promoting self-renewal in human ESCs, but not in murine ESCs (Yoshida-Koide *et al*, 2004; Xu *et al*, 2005; Yu *et al*, 2011). All three of these ERK2 phosphorylation residues are located outside of the DNA binding domains of OCT4 (Brumbaugh *et al*, 2012), which suggests that ERK2 phosphorylation of human OCT4 positively regulates human OCT4 transactivation or alters the activation of specific gene targets through homodimer regulation. Additionally, although reducing the amount of OCT4 in a complex with HSP90, ERK1 and ERK2 inhibition using the ERK2i did not cause human OCT4 degradation; which suggests that the effect of ERK1 and ERK2 inhibition did not destabilize the HSP90 and human OCT4 complex.

It was noted that the inhibition of MEK1 and MEK2, the kinases responsible for phosphorylation and hence activation of ERK1 and ERK2, using high concentrations of the MEK1/2 inhibitor U0126 caused human OCT4 degradation. This is interesting to note as evidence suggests that MEK1 is a HSP90 client protein (Stancato *et al*, 1997; Shinozaki *et al*, 2006), therefore human OCT4 degradation resulting from HSP90 inhibition could be mediated through modulation of the MAPK/ERK pathway. The ability of ERK1 and ERK2 to phosphorylate HSP90 has not been experimentally

described. Bioinformatic analyses reveal no conclusive ERK phosphorylation motifs on HSP90, however we cannot discount the possibility that the reduction in the interaction between HSP90 and human OCT4 with the inhibition of ERK1 and ERK2 also results from loss of ERK1 and ERK2 phosphorylation of HSP90. Although our data suggest that the HSP90 and human OCT4 complex is influenced dynamically by specific phosphorylation modifications to human OCT4 and/or HSP90, further analysis of the effect of phosphorylation on the HSP90 and human OCT4 complex is required with the use of phospho-mimetic and phospho-inhibiting human OCT4 mutations. Future research should validate the inhibition of the specific kinases in question with treatment using the kinase inhibitors. The kinase inhibitor concentrations used in our experiments were based on concentrations used in literature. For future analyses the concentrations of each kinase inhibitor should be titrated to establish mechanistically relevant concentrations. Additionally, future experiments should include the establishment of mechanistically relevant treatment incubation periods for the kinase inhibitors.

5.1.4. Evidence supporting an additional indirect relationship between human OCT4 and HSP90

Interestingly, co-treatment of HEK-hOCT4 cells with GA and low concentrations of MG132 (0.1 – 1 μ M) resulted in greater reductions in human OCT4 protein levels than either treatment alone. If the relationship between HSP90 inhibition and human OCT4 degradation is additionally mediated via a third protein responsible for OCT4 degradation, the fact that human OCT4 degradation was observed with co-treatments with GA and low concentrations of MG132 (0.1 – 1 μ M) would suggest that low MG132 concentrations are sufficient to rescue this OCT4-degrading protein from GA-induced proteasomal degradation, or that HSP90 inhibition may activate the protein in question. Under these conditions, OCT4 degradation through the proteasome is less sensitive to proteasomal inhibition and therefore low concentrations of MG132 are unable to rescue the combined effects of OCT4 degradation due to HSP90 inhibition, as well as the possible accumulation of an OCT4 degrading protein.

The ubiquitin ligase family of proteins presents one possibility. Murine OCT4 degradation has been found to be mediated by Wwp2 and Itch E3 ubiquitin ligases, while human OCT4 degradation in human ESCs is mediated through WWP2 (Xu *et al*, 2006; Xu *et al*, 2009; Liao and Jin, 2010; Liao *et al* 2012). Of interest is that murine Wwp2 and Itch E3 ubiquitin ligases catalyze the ubiquitination of murine OCT4 in context-dependent manners (Liao *et al*, 2012). While Itch catalyzes ubiquitination of murine OCT4 in pluripotent murine ESCs, Wwp2 catalyzes ubiquitination of murine OCT4 only upon induction of differentiation and has no effect on murine OCT4 in pluripotent murine ESCs (Xu *et al*, 2006; Liao and Jin, 2010; Liao *et al*, 2012). Itch activity additionally, and contradictorily, was shown to enhance PORE promoter activity by murine OCT4 (Liao *et al*, 2012). Human WWP2 has been shown to mediate human OCT4 degradation in human ESCs under physiological conditions, context-independently (Xu *et al*, 2009).

Numerous ubiquitin ligases are HSP90 client proteins (Figure 28.A and B; Taipale *et al*, 2012) including WWP1 (Taipale *et al*, 2012), a ligase belonging to the C2-WW-HECT E3 subgroup of HECT ligase subfamily that includes Wwp2 and Itch (Bernassola *et al*, 2008). However WWP2, the human homolog of murine Wwp2, has not been identified as being an HSP90 client protein (Taipale *et al*, 2012). However C2-WW-HECT E3 ligases do show redundancy in functions owing to the conservation of domain structure in this sub-family (Ingham *et al*, 2004). In *C.elegans*, knockdown of Wwp1 prevents embryogenesis, a biological process requiring OCT4 (Huang *et al*, 2000). Although WWP1 may be a candidate due to its E3 ubiquitin ligase functioning, as an HSP90 client protein the inhibition of HSP90 would be expected to result in degradation of WWP1.

5.1.5. Functional relevance of the human OCT4 and HSP90 complex

We provide data suggesting that human OCT4 is an HSP90 client protein based on our observations that human OCT4 and HSP90 directly interact and inhibition of HSP90 results in the degradation of human OCT4 and a loss in homodimer activity. Although we suggest the biochemical relevance of the human OCT4 and HSP90 interaction, in our model we cannot address the biological relevance of this interaction. OCT4 activity is finely tuned within the biological context of pluripotent

stem cells with growing evidence suggesting that this fine-tuning is mediated, amongst other things, by post-translational modifications. Our data, too, suggests that post-translational modifications modulate the interaction between human OCT4 and HSP90. Therefore to provide a biological relevance for the relationship between human OCT4 and HSP90 we need to study this complex within the biological context and environment of pluripotent stem cells. The use of human iPSCs (Figure 27) will provide us with the biological context and framework within which human OCT4 functions thereby providing the biological relevance of the human OCT4 and HSP90 complex.

Our data suggests that HSP90 is involved in the cytoplasmic stabilization of human OCT4. In our HEK-hOCT4 model, human OCT4 displayed both cytoplasmic and nuclear localization although the predominant localization was nuclear. The inverse was observed with HSP90, with the vast majority of HSP90 α/β was localized to the cytoplasmic compartment with only a low proportion showing nuclear localization. Both murine ESCs and human ESCs have a characteristically high nuclear versus cytoplasmic ratio (Sato *et al*, 2004). The dispersion of HSP90 within murine ESCs or human ESCs is yet to be determined and it is unknown as to whether the proportional localization of HSP90 differs in ESCs due to the differences in cytoplasmic-to-nucleus spatial ratio. In murine and human ESCs, OCT4 displays a punctate nuclear staining with no detectable cytoplasmic staining (Sato *et al*, 2004). However, OCT4 will still need to be translated in the cytoplasm prior to nuclear translocation and therefore it is likely that during this stage that HSP90 may play the stabilization role. Studies have shown that OCT4 is a nucleocytoplasmic shuttling protein (Plachta *et al*, 2011; Oka *et al*, 2013). Kinetic studies on murine OCT4 in murine embryos have illustrated that in murine ESCs, while OCT4 is a nucleocytoplasmic shuttling protein, the rate of nuclear import exceeds nuclear export and the near entire proportion of OCT4 remains in the nucleus (Plachta *et al*, 2011). Interestingly, the authors noted that with early-stage lineage commitment and initiation of differentiation, OCT4 kinetics change with the rate of nuclear export increases with the proportion of OCT4 retained in the nucleus decreasing (Plachta *et al*, 2011). This suggests that the OCT4 in pluripotent stem cells only presides in the cytoplasm transiently after cytoplasmic translation prior to nuclear import and (presumably) post nuclear export prior to

OCT4 degradation. These observations allude to the possibility that a cytoplasmic relationship, as we suggest between human OCT4 and HSP90, may only occur transiently within a self-renewing stem cell environment. However, upon the induction of differentiation the changes in OCT4 kinetics may enrich for cytoplasmic interactions and may suggest that HSP90 will be important during this stage.

Interestingly a splice variant of OCT4, OCT4B, resulting in a truncated N-terminal transactivation domain, while still containing an NLS, displays a predominantly cytoplasmic localization (Cauffman *et al*, 2006). Although this isoform is unable to maintain the transcriptional profile of full length OCT4 (Wang *et al*, 2009), evidence suggests a role for this isoform in anti-apoptosis responses to stress (Wang *et al*, 2009; Gao *et al*, 2012). This is unlikely to occur in our model since exogenous OCT4 is encoded by an intron-less cDNA sequence therefore negating the possibility of different isoforms arising from mRNA splice variations.

Cytoplasmic human OCT4 has been described as a diagnostic marker for embryonal carcinomas (de Jong *et al*, 2005; Cheng *et al*, 2007), seminomas (Cheng *et al*, 2007), adrenal medulla and related neoplastic tissue (Alexander *et al*, 2012), as well as Merkel cell carcinoma (Kao *et al*, 2014). This is interesting in the light of the link between ‘stemness’ and cancer, as well as the well-characterised role played by HSP90 in stabilizing oncogenic proteins. However the function and relevance of cytoplasmic human OCT4 in these instances, if any, is presently unknown. Overexpression of OCT4 has been observed to cause neoplasia in tissue (Hochedlinger *et al*, 2005), therefore the presence of cytoplasmic OCT4 may suggest a tumorigenic transformation of these cells. Cytoplasmic OCT4 is a diagnostic marker of numerous embryonic cancers (de Jong *et al*, 2005; Alexander *et al*, 2012; Kao *et al*, 2014). A tumourigenic transformation may explain the morphological changes evident between the parent HEK293FT cell line and the HEK-hOCT4 cell line; a transformation that is supported by the fact that OCT4 in our model system was shown to be transcriptionally active. Indeed the change in morphology observed between HEK293FT and HEK-hOCT4 is similar to that of cells having undergone epithelial-to-mesenchymal transition (EMT), a phenotype associated with malignancy (Dai *et al*, 2013). We have not however verified this using EMT marker analyses or functional analyses for EMT and this is therefore only speculation.

The effect of HSP90 inhibition on the transcriptional activity of OCT4 may also have functional relevance. If the loss of homodimer activity upon HSP90 inhibition can be validated in the iPSC model, then this may provide insight into the mechanism by which reductions of OCT4 associated with HSP90 inhibition leads to stem cell differentiation. Different subsets of OCT4 regulated genes will be controlled by upstream elements containing different combinations of the octamer binding motifs (Jung *et al*, 2010). The loss of PORE and MORE activity, but not monomer activity, upon HSP90 inhibition, would therefore likely lead to a changes in the OCT4 transcriptional program which could culminate in loss of pluripotency and induce differentiation. It is interesting to note that ESC in which HSP90 was inhibited did not undergo random, non-specific differentiation, but rather demonstrated defects in differentiation of specific lineages (Bradley *et al*, 2012). HSP90 inhibition in murine ESCs resulted in preferential mesoderm lineage differentiation (Bradley *et al*, 2012).

5.2. Concluding remarks and Future Prospects

We provide data suggesting that human OCT4 is a HSP90 client protein owing to the direct interaction observed between human OCT4 and HSP90 and the observation of human OCT4 protein degradation resulting from the inhibition of HSP90. We provide evidence suggesting that the interaction between human OCT4 and HSP90 does not occur in the nucleus. To further analyze the subcellular localization and resulting effect of human OCT4 interaction with HSP90 we have begun to generate FRET compatible fluorescently-tagged constructs encoding for human OCT4 and HSP90 (Appendix Figures A1-A4). These vectors will allow us to determine the subcellular localization of the HSP90 and human OCT4 complex in live cells, as well as to further assess the effects of kinase or HSP90 inhibitors to either promote or abrogate the interaction between HSP90 and human OCT4. Our data additionally cannot discount the possibility that HSP90 additionally mediates the nuclear import of human OCT4. The use of FRET compatible fluorescently tagged human OCT4 and HSP90 together with HSP90 inhibitors, nuclear import inhibitors and nuclear export inhibitors will allow us to begin to question the two theories.

In order to verify the role of HSP90 in the cytoplasmic stabilization of human OCT4, it would be necessary to both promote nuclear OCT4 retention, and negate nuclear translocation of human OCT4 in our model. As a NLS has been identified in OCT4 (Pan *et al*, 2004) and is completely conserved among mammalian species (Figure 12), OCT4-NLS mutants blocking nuclear import could be used. If our theory is correct, then OCT4-NLS mutants should enrich for the OCT4 and HSP90 complex. Recently, a putative Crm1-like NES has been identified for OCT4 showing high conservation between mammalian species (Figure 12; Oka *et al*, 2013). Inhibition of Crm1/Exportin-1 mediated nuclear export was however unable to completely prevent nucleocytoplasmic shuffling of OCT4 suggesting multiple possible mechanisms for nuclear export (Oka *et al*, 2013). Additionally OCT4 nuclear export kinetics support the notion that nuclear export is a mechanistic facilitated process, rather than resulting from passive diffusion (Plachta *et al*, 2011). Therefore preventing nuclear export using chemical inhibitors like leptomycin B, may or may not be useful.

In conclusion, our data supports our original hypothesis that OCT4 is an HSP90 client protein. We have achieved the aim of producing a model cell line that is not dependent on OCT4 signaling to facilitate the biochemical analysis of this interaction. We achieved our second aim by using our model to define human OCT4 as an HSP90 client and to begin to understand the role of HSP90 in the biochemistry of human OCT4. Our data extend the biochemical understanding of human OCT4 and demonstrate that OCT4 is dependent on HSP90 for stability (Figure 26). HSP90 inhibition affects both OCT4 levels and activity (Figure 26). The priority for future study is therefore to validate the biological relevance of these findings in a suitable model, like human iPSC. If this can be determined, then the modulation of HSP90 activity may provide another mechanism by which to regulate stem cell pluripotency and/or differentiation. There is a strong emphasis on the use of iPSC as ‘disease in a dish’ models for various disorders (Beltrao-Braga *et al*, 2013; Svendsen, 2013). However, these studies are often limited by the ability to prevent or induce differentiation in a lineage specific manner. The understanding of the role of HSP90 in OCT4 biology, together with the availability of an ever growing number of HSP90 inhibitors may find application in this area in the future.

Chapter 6. References

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Chapter 7. Appendix: Development of FRET vectors to study HSP90-OCT4 interactions.

7.1.Methodology

Table A1 | Chemical reagents and equipment suppliers

Chemical/Equipment	Supplier	Country
Chemidoc	Bio-Rad	USA
ImageJ	National Institute of Health	USA
FBS	BioWest	France
NEAA	Gibco, Life Technologies/Thermo Fisher Scientific	USA
Sodium Pyruvate	Sigma-Aldrich	USA
PSA	Sigma Aldrich	USA
G418	Santa Cruz Biotechnology	USA
Puromycin	Sigma Aldrich	USA
Protease inhibitor cocktail	Sigma Aldrich	USA
Triton X-100	Sigma Aldrich	USA
Heochst	Santa Cruz Biotechnology	USA
Dako Fluorescent mounting media	Dakota	USA
CKX41 microscope	Olympus	USA
Attachment factor	Gibco, Life Technologies/Thermo Fisher Scientific	USA
Protein A/G MagReSyn	ReSyn Biosciences	RSA
Restriction ending leases	NEB	USA
Restriction ending leases	Promega	USA
KAPA Universal Ladder	KAPA Biosystems	RSA

Table A2 | Primary antibodies and suppliers.

Primary Antibody	Species	Supplier	Country
α HSP90 α/β	Mouse	Santa Cruz	USA
α HSP90 α/β	Goat	Santa Cruz	USA
α OCT4	Rabbit	AbCAM	USA
α OCT4	Goat	AbCAM	USA
α Histone H3	Rabbit	Cell Signaling Technologies	USA
α GAPDH-HRP	Rabbit	AbCAM	USA
α AKT(pan)	Mouse	Cell Signaling Technologies	USA
α CDK4	Rabbit	AbCAM	USA
α HSP70	Mouse	Santa Cruz	USA
α P23	Mouse	AbCAM	USA
α GFP	Rabbit	Santa Cruz	USA

Table A3 | Secondary antibodies and suppliers.

Secondary Antibody	Species	Supplier	Country
α Rabbit (IgG) - HRP	Donkey	AbCAM	USA
α Mouse (IgG) - HRP	Mouse	AbCAM	USA
α Goat (IgG) - HRP	Donkey	AbCAM	USA
α Rabbit (IgG) - 488	Donkey	AbCAM	USA
α Mouse (IgG) - 546	Donkey	Invitrogen	USA
α Goat (IgG) - 660	Donkey	AbCAM	USA

7.2.Generation of pECFP-C1-HSP90AB1 and pEYFP-C1-hOCT4 fluorescent fusion protein vectors and FRET control vector pEYFP-C1-ECFP

In order to further analyze the interaction between human OCT4 and HSP90 we have started to produce fluorescently tagged fusion constructs of both human OCT4 and HSP90. Vectors pEYFP-C1 (Figure A1.A) and pSIN-hOCT4-Puro (Figure A1.B) were used to generate pEYFP-C1-hOCT4 (Figure A1.C), a fluorescent protein fusion vector encoding for hOCT4 with an EYFP fluorescent tag. pEYFP-C1 backbone was isolated using *HindIII* and *BamHI* restriction endonuclease digestion (restriction endonuclease recognition sites highlighted in Figure A1.A). The hOCT4 coding region containing a single complimentary overhang to the pEYFP-C1 backbone was isolated from pSIN-hOCT4-Puro using *SpeI* and *BamHI* restriction endonuclease digestion (restriction endonuclease recognition sites highlighted in Figure A1.B). The complimentary *BamHI* 3' overhangs of pEYFP-C1 backbone and hOCT4 coding region were ligated together using T4 DNA Ligase. This ligation ensured the correct orientation of the hOCT4 coding region in the pEYFP-C1 vector. DNA polymerase large Klenow fragment was then used to generate blunt-ends of the non-complimentary *HindIII* and *SpeI* 3' overhangs. The subsequent linear DNA was blunt-end ligated using T4 DNA Ligase. *HindIII* and *SpeI* restriction endonucleases together with the 5' to 3' polymerase activity of DNA polymerase large Klenow fragment ensured the hOCT4 coding region was ligated in-frame of the EYFP coding region therefore creating the EYFP tagged hOCT4 fusion protein.

The resulting pEYFP-C1-hOCT4 ligation was transformed into chemically competent JM109 *E.coli* cells, with transformants verified using *HindIII* restriction endonuclease digestion (Figure A1.D). pEYFP-C1 vector contains a single *HindIII* recognition site (highlighted in Figure 5X.A) while the pEYFP-C1-hOCT4 vector contains two *HindIII* restriction endonuclease recognition sites (highlighted in Figure A1.C) with an additional *HindIII* recognition site incorporated with the ligation of hOCT4 coding region into the pEYFP-C1 backbone. The presence of a single band of predicted approximate size 4731 bp was observed when pEYFP-C1 vector was digested by *HindIII* restriction endonuclease (Figure A1.D: lane 3). Uncut pEYFP-C1-hOCT4

transformant A showed the presence of two bands predicted to be supercoiled and open circular DNA conformations (Figure A1.D: lane 4 lower and upper band respectively). pEYFP-C1-hOCT4 transformant A was not digested by *HindIII* as illustrated by the presence of two bands corresponding to the bands present in the uncut sample (Figure A1.D: lanes 4 and 5). Digestion of pEYFP-C1-hOCT4 transformants B and C using *HindIII* produced two bands of expected approximate sizes 1003 bp and 4856 bp (Figure A1.D: lanes 7 and 9 lower and upper bands respectively).

The pECFP-C1 vector (Figure A2.A) was used as the backbone vector into which the HSP90AB1 coding region [excised from pHSP90AB1-EGFP vector (Figure A2.B)] was inserted to generate pECFP-C1-HSP90AB1 (Figure A2.C), a vector encoding for HSP90AB1-ECFP fluorescent fusion protein. The HSP90AB1 coding region was excised and isolated from pHSP90AB1-EGFP using *SalI* and *SacII* restriction endonuclease digestion (*SalI* and *SacII* restriction endonuclease recognition sites highlighted in Figure A2.B). A pECFP-C1 backbone containing complimentary *SalI* and *SacII* 3' overhangs was generated and isolated by digestion of the pECFP-C1 vector using *SalI* and *SacII* restriction endonuclease digestion (*SalI* and *SacII* restriction endonuclease recognition sites highlighted in Figure A2.B). The HSP90AB1 coding region was then ligated into pECFP-C1 backbone through *SalI* and *SacII* complimentary 3' overhangs using T4 DNA Ligase.

Constructs resulting from the ligation procedure were transformed into chemically competent JM109 *E.coli* cells with subsequent transformants screened for the presence of the ECFP-HSP90AB1 DNA fragment using *NheI* and *BamHI* restriction endonuclease digestion (Figure A2.D). For uncut samples of the pECFP-C1 control vector and the pECFP-C1-HSP90AB1 transformants two bands were observed, the lower band representing supercoiled DNA conformations and the upper band representing open circular DNA conformations (Figure A2.D: lanes 2, 6,10, 14, 18). Digestion of pECFP-C1 using *BamHI* and *NheI* restriction endonucleases alone linearized the vector producing a band corresponding to the expected size of the vector (4731 bp; Figure 6X.D: lanes 2 and 5 respectively). Additional bands of lower concentration were observed for the *BamHI* restriction endonuclease digestion of pECFP-C1 (Figure A2.D: lane 3). These bands corresponded to the bands observed in

the pECFP-C1 uncut sample (Figure A2.D: lane 2) and were therefore predicted to be uncut pECFP-C1 resulting from inefficient digestion by *Bam*HI restriction endonuclease. Inefficient digestion by *Bam*HI was additionally observed when the pECFP-C1-HSP90AB1 transformants A, B and C were digested with *Bam*HI (Figure 6X.D: lanes 7, 11 and 15). These digestions generated incomplete vector digestion with the presence of supercoiled and open circular DNA conformations visible in each lane (Figure A2.D: lanes 7, 11 and 15 lower and upper bands respectively). However *Bam*HI inefficient digestion did produce a low concentration (concentration based on band intensity) of linearized vector (Figure A2.D: lanes 7, 11 and 15 middle band) the approximate size of the pECFP-C1-HSP90AB1, 6899 bp. *Bam*HI restriction endonuclease digestion of pECFP-C1-HSP90AB1 transformant D generated a single band corresponding to the predicted size of pECFP-C1-HSP90AB1, 6899 bp (Figure A2.D: lane 19). For all transformants, *Nhe*I restriction endonuclease digestion resulted in the generation of a single band of approximate size 6899 bp corresponding to the size of pECFP-C1-HSP90AB1 (Figure A2.D: lanes 8, 12, 16 and 20). Restriction endonuclease digestion of pECFP-C1-HSP90AB1 transformants using both *Nhe*I and *Bam*HI restriction endonucleases generated 2 bands of sizes 2967 bp and 3932 bp corresponding to the predicted sizes of pECFP-C1-HSP90AB1 restriction endonuclease digestion using *Nhe*I and *Bam*HI (Figures A2.D: lanes 9, 13, 17 and 21 lower and middle bands). In all cases the restriction digestion of pECFP-C1-HSP90AB1 transformants using *Nhe*I and *Bam*HI showed a band corresponding to the linear size of pECFP-C1-HSP90AB1 (Figure A2.D: lanes 9, 13, 17 and 21 upper band). This was predicted to be as a result of inefficient digestion by the *Bam*HI restriction endonuclease as inefficient *Bam*HI restriction endonuclease digestion had previously been observed (Figure A2.D: lanes 7, 11, 15 and 19).

The pEYFP-C1-ECFP vector was developed as a control vector for FRET-co-localization and encodes for the EYFP-ECFP fluorescent fusion protein. The control vector was created using the pEYFP-C1 backbone (Figure A3.A) into which the ECFP coding region, isolated from the pECFP-C1 vector (Figure A3.B), was ligated generating pEYFP-C1-ECFP (Figure 7X.C). pEYFP-C1 backbone was generated and isolated using *Hind*III and *Bam*HI restriction endonuclease digestion of the pEYFP-C1 plasmid (restriction endonuclease recognition sites highlighted in Figure A3.A). The ECFP coding region was excised and isolated from pECFP-C1 using *Nhe*I and

*Bam*HI restriction endonuclease digestion (restriction endonuclease recognition sites highlighted in Figure A3.B) thereby creating a single *Bam*HI 3' complimentary overhang with the pEYFP-C1 backbone ensuring correct gene orientation for ligation into the pEYFP-C1 backbone. The *Bam*HI 3' overhang of the ECFP coding region was ligated to the complimentary *Bam*HI 3' overhang of the pEYFP-C1 backbone. DNA polymerase large Klenow fragment was used to generate blunt-ends of the non-complimentary *Hind*III and *Nhe*I 3' overhangs of the linear pEYFP-C1-ECFP construct and subsequently blunt-end ligated to form pEYFP-C1-ECFP using T4 DNA ligase. pEYFP-C1-ECFP ligations were transformed into chemically competent JM109 *E.coli* cells with the resulting transformants screened for the presence of the pEYFP-C1-ECFP vector using *Bgl*II restriction endonuclease digestion (Figure A3.D).

pEYFP-C1 contains a single *Bgl*II restriction endonuclease recognition site (restriction recognition site highlighted in Figure A3.A). The insertion of the ECFP coding region, generated by *Nhe*I and *Bam*HI restriction digestion excision of ECFP from pECFP-C1 and subsequent liagtion of this coding region into pEYFP-C1 backbone, incorporates an additional *Bgl*II restriction endonuclease recognition site into the pEYFP-C1 ECFP vector (restriction endonuclease recognition sites highlighted in Figure A3.C). For all vectors two DNA bands were observed in the uncut samples representing supercoiled (Figure A3.D: lanes 2, 4 and 6 lower bands) and open circular (Figure A3.D: lanes 2, 4 and 6 upper bands) DNA conformations. However the 2 bands from the uncut sample of pEYFP-C1-ECFP transformant A (Figure A3.D: lane 4) had a slower migration rate than the corresponding bands for the pEYFP-C1 and pEYFP-C1-ECFP transformant B uncut DNA samples (Figure A3.D: lanes 2 and 6 respectively). *Bgl*II restriction endonuclease digestion of pEYFP-C1 generated a single band of approximate expected size 4731 bp (Figure A3.D: lane 3). The digestion of pEYFP-C1-ECFP transformant A using *Bgl*II restriction endonuclease produced two DNA bands of approximate sizes 765 bp and 4731bp (Figure A3.D: lane 5 lower and upper bands respectively). The number of bands and sizes of the bands corospond to the expected number of bands and sizes of bands generated from pEYFP-C1-ECFP digestion using *Bgl*II restriction endonuclease. Digestion of pEYFP-C1-ECFP transformant B with *Bgl*II restriction endonuclease produced a single band of approximate size 4731 bp (Figure A3.D: lane 7)

corresponding to the single band produced when pEYFP-C1 was digested with *Bgl*/II restriction endonuclease (Figure A3.D: lane 3). Suggesting that pEYFP-C1-ECFP transformant B did not contain the ECFP coding region but rather resulted from the re-circularization of the pEYFP backbone.

Transformants showing successful ligations were then transfected into HEK293FT cells for 48 hours. The expression of fluorescently tagged HSP90AB1-ECFP, human OCT4-EYFP and EYFP-ECFP was analyzed by fluorescence microscopy and Western blot (Figure A.4). For all proteins fluorescence was observed (Figure A.4.A, B and C). For human pEYFP-C1-hOCT4 and pEYFP-C1-ECFP both nuclear and cytoplasmic staining was observed (Figure A.4.A and C), while for pECFP-C1-HSP90AB1 predominant cytoplasmic staining was observed (Figure A.4.B). In order to confirm that the observed fluorescence was due to fluorescently tagged protein constructs and not resulting from expression of only the fluorescent tag, transfectants were lysed in SDS-PAGE sample buffer and used for Western analysis probing for GFP. ECFP (26 kDa) and EYFP (35 kDa) are mutants of GFP and are recognized by GFP antibodies. If the fluorescently tagged fusion constructs are expressed then probing for GFP should detect a band corresponding to the expected additive size of the fusion construct, with HSP90AB1-ECFP producing a band of approximately 120 kDa, OCT4-EYFP producing a band of approximately 70-75 kDa and EYFP-ECFP producing a band of approximately 55 kDa. For the pECFP-C1-HSP90AB1 transfection a band of approximately 120 kDa was observed when anti-GFP antibody was used (Figure A.4.D) suggesting that pECFP-C1-HSP90AB1 successfully expressed HSP90AB1 tagged with ECFP. For both the pEYFP-C1-hOCT4 and pEYFP-C1-ECFP transfections a single band of approximately 35 kDa corresponding to EYFP was observed (Figure A.4.D). Additionally human OCT4 was not detected in pEYFP-C1-hOCT4 transfections (data not shown). Restriction endonuclease diagnostic analysis confirmed the presence of human OCT4 and ECFP coding regions in the pEYFP-C1-hOCT4 and pEYFP-C1-ECFP vectors, however Western blot analysis revealed only the presence of EYFP, indicating that a possible stop codon is present between EYFP and human OCT4 or ECFP.

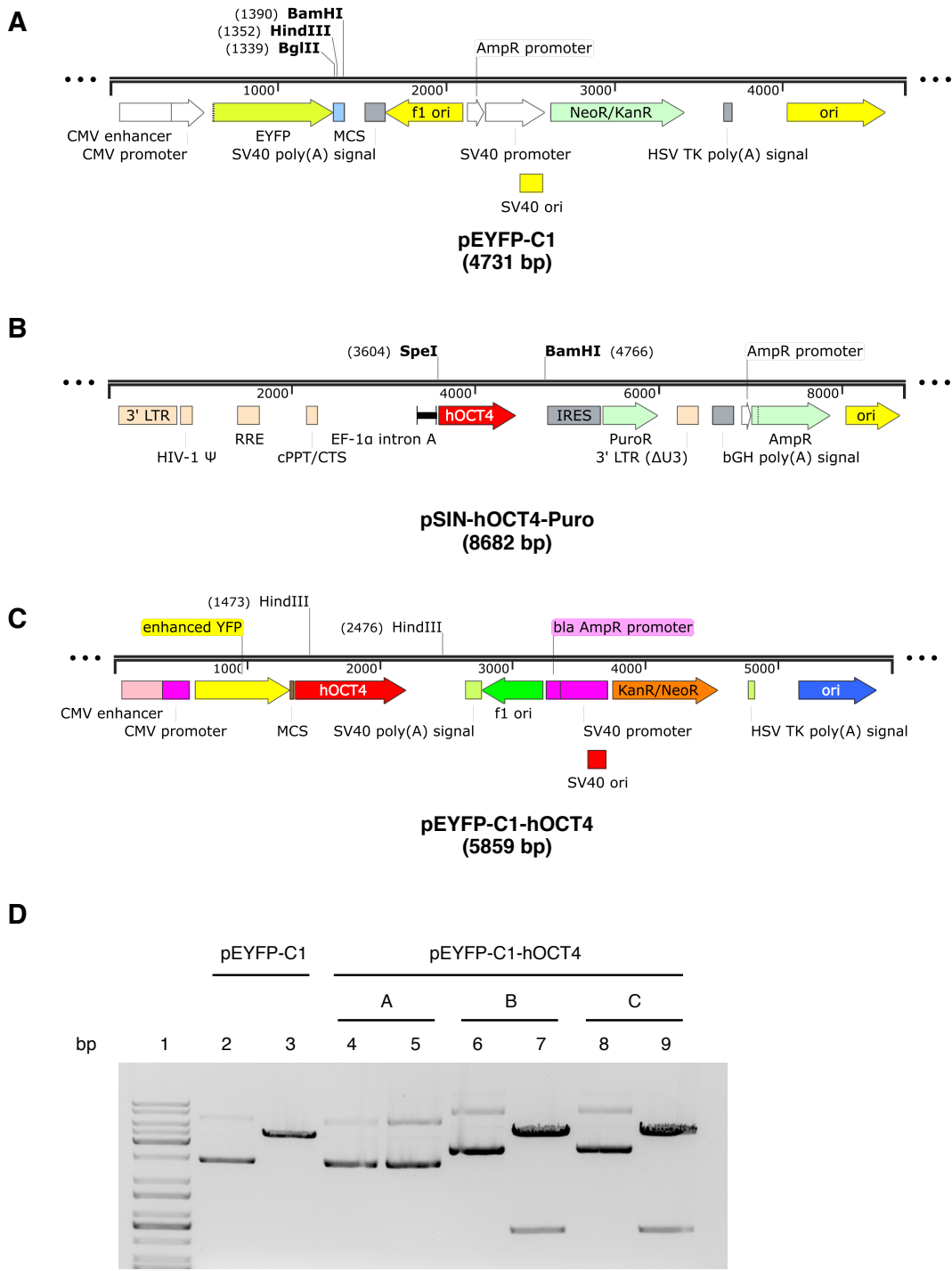


Figure A1 | Generation of pEYFP-C1-hOCT4 vector.
Linear map representations of pEYFP-C1 (A) and pSIN-hOCT4-Puro (B) vectors used to generate fluorescent fusion protein construct pEYFP-C1-hOCT4 (C). (D) Screening of pEYFP-C1-hOCT4 transformants using *Hind*III restriction endonuclease to identify transformants containing pEYFP-C1-hOCT4. KAPA universal DNA ladder: lane 1. Uncut DNA: lanes 2, 4, 6, 8. *Hind*III restriction endonuclease digestion: lanes 3, 5, 7, 9.

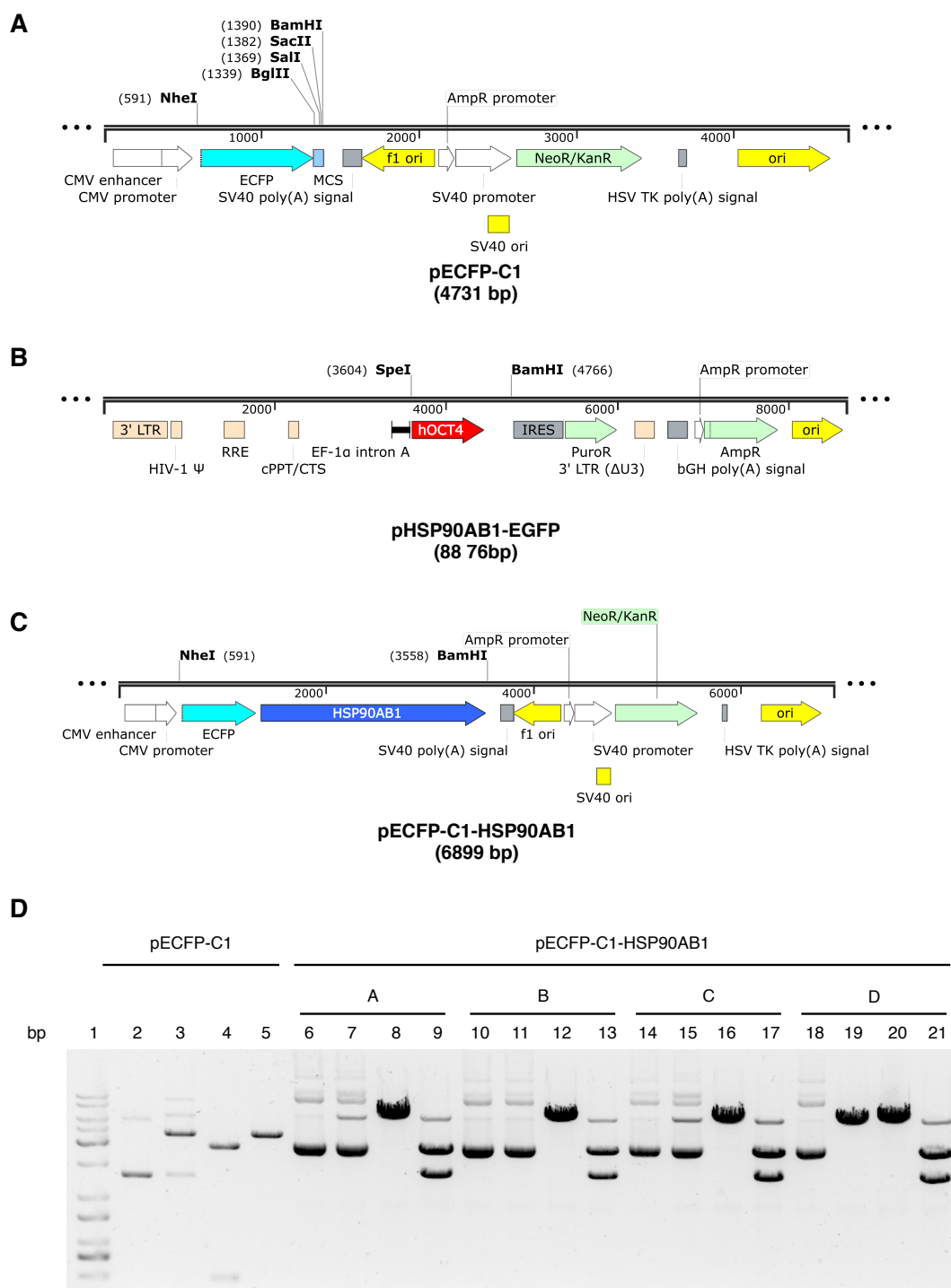


Figure A.2 | Generation of pECFP-C1-HSP90AB1 vector.

Linear map representations of pECFP-C1 (A) and pHSP90AB1-EGFP (B) vectors used to generate fluorescent fusion protein construct pECFP-C1-HSP90AB1 (C). (D) Screening of pECFP-C1-HSP90AB1 transformants using *Bam*HI and *Nhe*I restriction endonucleases to identify transformants containing pECFP-C1-HSP90AB1. KAPA universal DNA ladder: lane 1. Uncut DNA: lanes 2, 6, 10, 14, 18. *Bam*HI digestion: lanes 3, 7, 11, 15, 19. *Nhe*I digestion: lanes 5, 8, 12, 16, 20. *Bam*HI and *Nhe*I digestions: lanes 4, 9, 13, 17, 21.

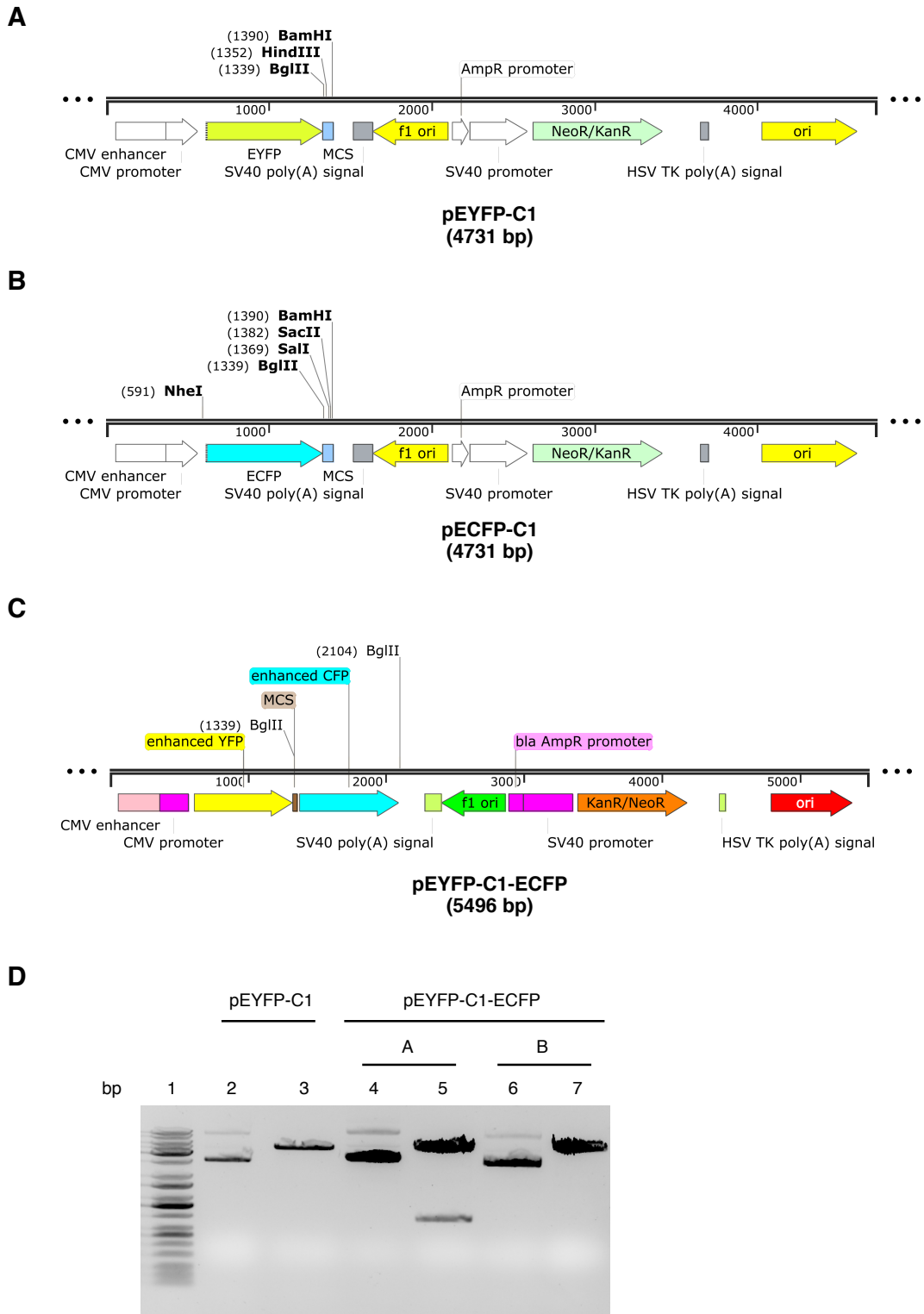


Figure A.3 | Generation of pEYFP-C1-ECFP FRET control vector. Linear map representations of pEYFP-C1 (A) and pECFP-C1 (B) vectors used to generate FRET control vector pEYFP-C1-ECFP (C). (D) Screening of pEYFP-C1-ECFP transformants using *Bgl*II restriction endonuclease to identify transformants containing pEYFP-C1-ECFP. KAPA universal DNA ladder: lane 1. Uncut DNA: lanes 2, 4, 6. *Bgl*II restriction endonuclease digestion: lanes 3, 5, 7.

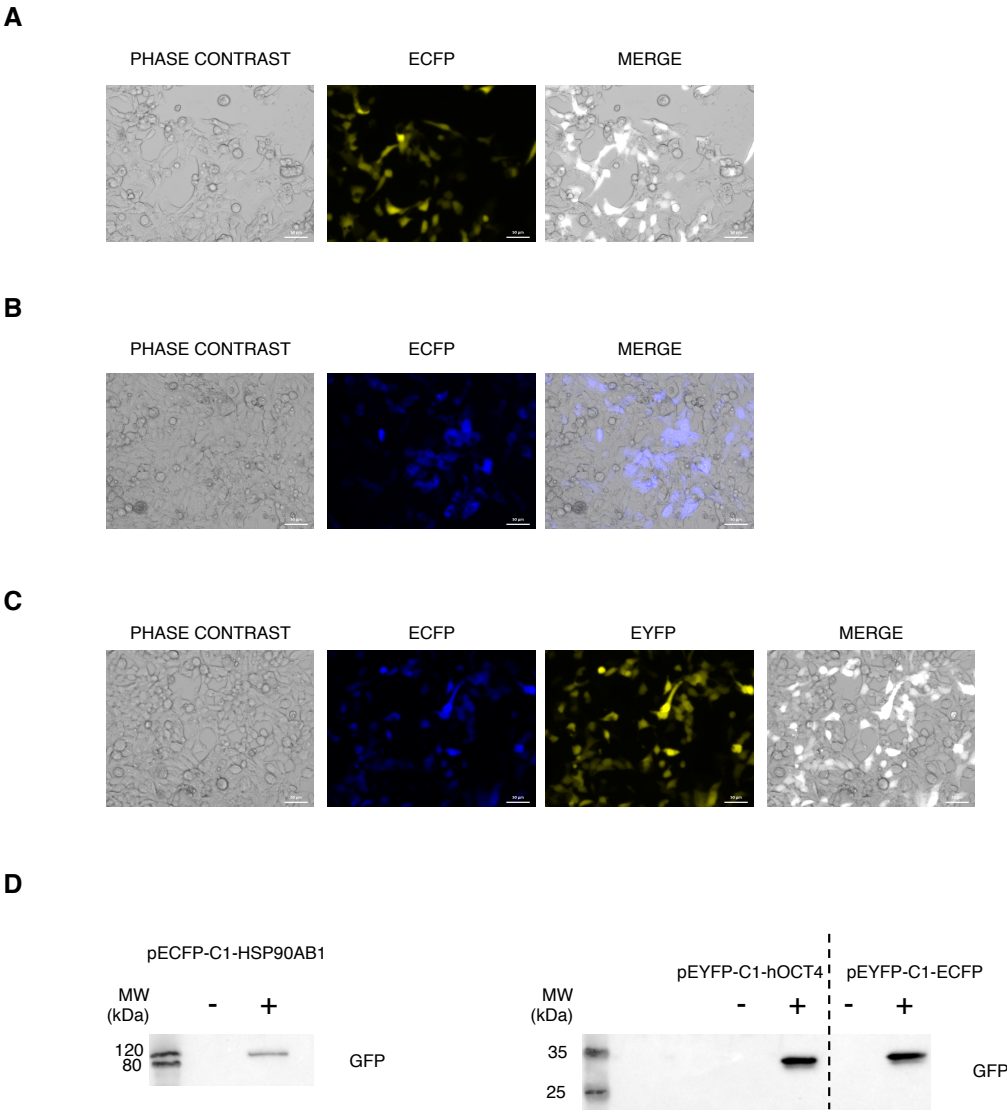


Figure A.4 | Verification of generated fluorescent fusion construct vectors pEYFP-C1-hOCT4, pECFP-C1-HSP90AB1, and pEYFP-C1-ECFP. FRET constructs pEYFP-C1-hOCT4 (A), pECFP-C1-HSP90AB1 (B) and FRET positive control pEYFP-C1-ECFP (C) were analyzed using fluorescence microscopy (A, B and C respectively) and Western blot (D). HEK293FT cells were transfected with pECFP-C1-HSP90AB1 (A), pEYFP-C1-hOCT4 (B) and FRET positive control pEYFP-C1-ECFP (C) using X-tremeGENE (Roche) for 48 hours before fluorescence visualization using a Zeiss AxioVert.A1 FL-LED fluorescence microscope (20x objective magnification). Scale bars: 100 μ m. (D) After fluorescence microscopy analyses cells were harvested for Western blot analyses probing for GFP.