

Characterisation of an oocyte specific knockout model of Cdh1

Suzanne My-Trinh Tran

PhD,

BSc (Hons) Chemistry,

BSc (Hons) Human Physiology

A thesis submitted in fulfilment
of the requirements for the degree of
Master of Philosophy

April 2012

School of Biomedical Sciences and Pharmacy

University of Newcastle

Callaghan NSW 2308

Australia

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library**, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

****Unless an Embargo has been approved for a determined period.**

Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, or carried out in other institutions. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

Acknowledgements

I would like to acknowledge the following people involved in this research:

- My supervisor, Prof Keith Jones.
- Dr Phoebe Jennings for performing the majority of the kinetochore counts for not only me, but also other members of our research group.
- Dr Janet Holt for her part in the Western blots of cyclin B1 and securin and the primers for chromosomes 15-19.
- Prof Eileen McLaughlin for the ZP3-Cre male mice and the Cre primers.
- Dr Sergio Moreno (Instituto de Biología Molecular y Celular del Cáncer, CSIC / Salamanca University, Campus Miguel de Unamuno, 37007 Salamanca, Spain) for the supplying the initial six male and six female mice used to set up the colony of loxFZR mice.

I would also like to thank The University of Newcastle, Australia for UNIPRS and UNRSC scholarships and the faculty for funding this project.

I would like to extend my thanks to the University of Newcastle's Security for escorting me home safely on countless late nights from the lab, and other staff and students of the university who have been kind to me throughout my time in Australia.

Last but not least, I would like to convey my gratitude for the love and support from my family and friends, both old and new.

Contents

<i>TITLE PAGE</i>	<i>i</i>
<i>STATEMENT OF ORIGINALITY</i>	<i>ii</i>
<i>STATEMENT OF COLLABORATION</i>	<i>iii</i>
<i>ACKNOWLEDGEMENTS</i>	<i>iv</i>
<i>CONTENTS</i>	<i>v</i>
<i>LIST OF FIGURES</i>	<i>xii</i>
<i>LIST OF TABLES</i>	<i>xiv</i>
<i>ABBREVIATIONS</i>	<i>xv</i>
<i>ABSTRACT</i>	<i>xvii</i>

CHAPTER 1 Introduction

1.1 Aims	1
1.2 The cell cycle – A highly co-ordinated and controlled sequence of events	3
1.2.1 Entry into the Mitotic Phase of the Cell Cycle is based on the activity of cyclin B-CDK1	3
1.2.2 The ubiquitin ligases SCF and APC/C ensure unidirectional progression through mitosis	5
1.2.3 Targets of APC/CCdh1	8
1.2.4 The temporal activities of activators of APC/C in Mitotic Cell cycle.....	8
1.2.5 The Early Mitotic Inhibitor 1 (Emi1) acts as a pseudo substrate, inhibiting the activities of APC/CCdc20 and APC/CCdh1 in the Mitotic Cell cycle	10

1.2.6	The Activities of APC/C are inhibited by the Spindle Assembly Checkpoint until all kinetochores are biorientated	11
1.3	The Cell Biology of Oocytes	12
1.3.1	Overview of Meiosis	12
1.3.2	Oocyte is arrested at GV stage inside the ovary	14
1.3.3	Follicular and oocyte development in-vivo in mouse ovary	16
1.3.4	Maintenance of GV arrest –requires high levels of cAMP and the temporal inhibition of CDK1	18
1.3.5	Maintenance of GV arrest is assisted by different sub-localization of CDK1 regulators (Wee1B, Myt1 and Cdc25B)	18
1.3.6	Maintenance of GV arrest requires regulation of cyclin B1, by continual degradation and GV exclusion	20
1.3.7	Maintenance of GV arrest requires the activity of APC/CCdh1 to keep levels of cyclin B1 low	21
1.3.8	Maintenance of prophase I arrest and meiotic maturation in porcine oocyte are similar to mouse	23
1.4	Meiotic Regulation of APC/CCdh1	24
1.4.1	Regulation of APC/CCdh1 during prophase I arrest by Emi1 or CDC14B can modulate the timing of GVB	24
1.4.2	Regulation of APC/CCdh1 during prophase I arrest by competitive inhibition of cyclin B1 by securin can modulate the timing of GVB....	25
1.4.3	APC/CCdh1 gets help from one of the SAC components, BubR1	26
1.4.4	SAC in female meiosis I	27
1.4.5	APC/C ^{Cdh1} prevents aneuploidy by targeting Cdc20 in prometaphase	29
1.5	Studies on Cdh1 knockout mice	30
1.5.1	Cdh1 degradation of cyclin B1 in the placenta prevents embryonic lethality	30
1.5.2	APC/C Cdh1 is essential for genomic stability	32

1.5.3	Generation of oocyte-specific knockouts of Cdh1 using Cre/lox P technology	33
1.6	Aneuploidy in eggs	36
1.6.1	Introduction	36
1.6.2	Mechanism of aneuploidy in MII eggs	37
1.6.3	Karyotyping studies of human oocytes	38
1.6.4	Techniques use for the aneuploidy assessment of human oocytes ...	41
1.6.5	Analysis of aneuploidy via kinetochore counts	43
1.7	Perspectives	44

CHAPTER 2 Materials and Methods

2.1	Animal Ethics	46
2.2	Animals and Husbandry	46
2.3	Mice Strains	46
2.3.1	B6CBF1 hybrids	47
2.3.2	C57Bl/6 strain	47
2.3.3	Transgenic strains	48
2.3.4	LoxFZR mice	48
2.3.5	93knwZp males	50
2.3.6	The experiment cohort is generated in the FZRZP3 colony	50
2.3.7	Transference of loxFZR mice onto a pure Black 6 background.....	52
2.3.8	Generation of the experimental cohort on a pure Black 6 background and establishment of the FZRZP3B6 colony	55
2.4	Genotyping of the Experiment Cohort	55
2.4.1	Genomic DNA extraction	55
2.4.2	Genotyping of FZRZP3 progeny	57
2.5	Preparation of Media	59
2.5.1	Source of ultra-pure water	60

2.5.2	Preparation of M2 media from stocks	60
2.5.3	Preparation of M2 media from constituents.....	61
2.5.4	Preparation of Minimum Essential Medium	64
2.5.5	Preparation of media containing hyaluronidase	65
2.5.6	Preparation of media containing milrinone	65
2.6	Ovarian Priming and Superovulation	66
2.6.1	Preparation of PBS	66
2.6.2	Preparation of Pregnant Mare Serum Gonadotrophin	66
2.6.3	Human Chorionic Gonadotrophin	67
2.6.4	Hormonal priming of ovaries	67
2.6.5	Superovulation of female mice	67
2.7	Collection of oocytes and <i>in-vitro</i> maturation	68
2.7.1	Collection and denudation of CEOs	68
2.7.2	Collection of <i>in-vivo</i> matured eggs	68
2.7.3	In-vitro maturation (IVM)	69
2.8	Buffers and solutions for preparation of the egg for analysis	70
2.8.1	Polyvinylpyrrolidone	70
2.8.2	PHEM Buffer	70
2.8.3	Preparation of 4% paraformaldehyde in PHEM buffer.....	71
2.8.4	Preparation of fixing and permeabilisation solution	71
2.8.5	Preparation of Antibody Buffer	72
2.8.6	Preparation of blocking solution	72
2.8.7	Preparation of SDS protein sample buffer for Western blotting	72
2.9	Treatment of GV oocytes collected from mice.....	73
2.9.1	Protocol for the Western Blot of GV oocytes	73
2.10	Post-IVM treatment of oocytes	77
2.10.1	Monastrol treatment of MII eggs	77
2.10.2	Fixation and permeabilisation of eggs for immuno-staining	77
2.10.3	Immunocytochemical Labelling of kinetochores	78
2.10.4	Aneuploidy analysis via Kinetochore counts	79

2.10.5 Chromosome spread of MII eggs	79
2.11 Preparation of Ovaries for histology	80
2.12 One-pot PCR protocol for primers against chromosomes 15-19	81
2.13 Statistical Analysis	83

CHAPTER 3 Studies on Cdh1 using a ZP3-Cre driven oocyte specific knockout model

3.1 Introduction	84
3.2 Results	85
3.2.1 Establishment of oocyte-specific Cdh1 knockout mice	85
3.2.2 Confirmation of Cdh1 loss in oocytes of Δ/Δ mice	90
3.2.3 Ovaries of Δ/Δ mice appeared normal and healthy	92
3.2.4 Fewer CEOs were collected from Δ/Δ ovaries	95
3.2.5 The number of secondary and antral follicles in Δ/Δ ovaries assessed by counting on ovarian sections.....	98
3.2.6 Enhanced GVB following loss of Cdh1	99
3.2.7 Increased susceptibility to meiotic resumption following loss of Cdh1.....	104
3.2.8 Cdh1 Δ/Δ oocytes have five fold higher cyclin B levels than controls.....	108
3.2.9 Cdh1 Δ/Δ and control oocytes have similar levels of expression for securin and Cdc25B	108
3.3 Discussion	112
3.3.1 Generation of an oocyte specific knockout model of Cdh1	112
3.3.2 Increased precocious in-vivo meiotic resumption following loss of Cdh1	114
3.3.3 Maintenance of an intact cumulus partially rescues precocious in-vitro meiotic resumption following loss of Cdh1	116

3.3.4	Cyclin B increased 5 fold in Cdh1 Δ/Δ oocytes.....	117
3.3.5	Lower cyclin B1 levels in Cdh1 deficient MEFs.....	118
3.3.6	Cdh1 in neurones is associated with memory and learning.....	118
3.3.7	Cdc25B levels are unaffected by Cdh1 ablation in oocytes	119
3.3.8	Securin levels are unaffected by Cdh1 ablation in oocytes	120
3.3.9	Further work – Analysis of levels of other substrates of APC ^{Cdh1} in Δ/Δ oocytes	121

CHAPTER 4 Loss of Cdh1 and delayed IVM lead to increased aneuploidy

4.1	Introduction	123
4.2	Results	123
4.2.1	Towards establishment of a protocol for aneuploidy analysis.....	124
4.2.2	Towards a ‘one pot’ PCR using primers against certain genes in Chromosome 15-19	125
4.2.3	gDNA content in oocyte is 105 fold less than in liver template	127
4.2.4	Adoption of known protocols for chromosome counting	129
4.2.5	Δ/Δ eggs matured in-vivo are protected from chromosomal anomalies	132
4.2.6	An intact cumulus afforded little benefit for Δ/Δ eggs but reduced the incidence of aneuploidy in fl/fl eggs under dIVM24 conditions	137
4.2.7	Numerical kinetochore anomalies were predominantly even.....	138
4.3	Discussion	138
4.3.1	Towards establishment of a protocol for aneuploidy analysis.....	138
4.3.2	Assessment of aneuploidy analysis methods used.....	141
4.3.3	Advantages of a PCR based protocol for aneuploidy analysis.....	141

4.3.4 Detrimental in-vitro conditions (dIVM24) were responsible for high
aneuploidy in denuded oocytes rather than loss of Cdh1142

4.3.5 An intact cumulus has little benefit following loss of Cdh1 in in-vitro
conditions143

4.3.6 Mechanism of aneuploidy is predominantly by non-disjunction.....144

4.3.7 Comparison of aneuploidy results with studies on human eggs.....146

4.4 Summary and Conclusion147

List of Figures

CHAPTER 1

Figure 1.1	The mitotic cell cycle.....	4
Figure 1.2	Ubiquitin-Proteasome Pathway.....	6
Figure 1.3	Overview of meiosis I.....	13
Figure 1.4	Stages of follicular development.....	15
Figure 1.5	Regulation of MPF (CDK1-cyclin B1).....	19
Figure 1.6	Mechanism of Cdh1 knockout using Cre/loxP technology.....	34
Figure 1.7	Reductional division of homologous chromosomes and abnormal segregation.....	40

CHAPTER 2

Figure 2.1	Identification of the transgenic mouse by ear marks.....	49
Figure 2.2	Generation of the experiment cohort.....	51
Figure 2.3	Alternative Breeding strategy yielded Δ/Δ mice and fl/fl at a faster rate.....	53
Figure 2.4	Percentage Black 6 inheritance for LoxFZR mice backcrossed with pure C57Bl/6.....	54

CHAPTER 3

Figure 3.1	An example of a PCR gel showing genotypes of experimental mice...91
Figure 3.2	Western blot for Cdh1 in fl/fl, Δ /wt and Δ/Δ oocytes.....93
Figure 3.3	Average ovary weights of unprimed control and Δ/Δ mice.....94
Figure 3.4	Ovarian sections from fl/fl and Δ/Δ mice.....96
Figure 3.5	Box and Whisker plot of number of CEOs collected from primed control, Δ /wt and Δ/Δ mice.....97
Figure 3.6	Counts of secondary, antral and atretic follicles in control and Δ/Δ ovaries.....100

Figure 3.7	Percentage of oocytes within CEOs from control, Δ /wt and Δ / Δ mice that have undergone GVB.....	101
Figure 3.8	Percentage of abnormal oocytes in ovaries of control and Δ / Δ mice	103
Figure 3.9	Percentage GVB after 12 h incubation in MEM with arresting agent, milrinone (1 μ M or 10 μ M).....	105
Figure 3.10	Time of GVB for control and Δ / Δ oocytes when released from 10 μ M milrinone arrest.....	107
Figure 3.11	Percentage GVB in CEOs and denuded oocytes (DO) after 12 h incubation in milrinone containing media.....	109
Figure 3.12	Levels of cyclin B1 following loss of Cdh1 in oocytes.....	110
Figure 3.13	Securin levels do not change following loss of Cdh1 in oocytes.....	111
Figure 3.14	Levels of Cdc25B following loss of Cdh1 in oocytes.....	113
CHAPTER 4		
Figure 4.1	Agarose gel displaying discrete PCR products of different sizes for each of the five chromosomes 15-19.....	126
Figure 4.2	Optimisation gel displaying discrete PCR products of different sizes for each of the five chromosomes 15-19 in the same lane.....	128
Figure 4.3	Gel of PCR product from 10 fold dilutions of liver gDNA sample against mGAPDH primers.....	130
Figure 4.4	Two examples of Chromosome spreads.....	131
Figure 4.5	Technique used to count kinetochores.....	133
Figure 4.6	Schematic representation of <i>in-vivo</i> ; <i>in-vitro</i> ; 12 h and 24 h delayed <i>in-vitro</i> maturation (dIVM12 and dIVM24) conditions.....	135
Figure 4.7	Increased percentage aneuploidy following Cdh1 loss.....	136
Figure 4.8	Breakdown of aneuploidy results based on even and odd kinetochore numbers.....	139

List of Tables

Table 1.1	<i>APC/C^{Cdh1} substrates in mitosis.....</i>	<i>9</i>
Table 2.1	<i>Primers used to genotype the experiment cohort.....</i>	<i>58</i>
Table 2.2	<i>Composition of M2 Stocks.....</i>	<i>62</i>
Table 2.3	<i>Composition of M2 Media.....</i>	<i>63</i>
Table 2.4	<i>Antibodies used in the immunodetection step of Western Blot.....</i>	<i>75</i>
Table 2.5	<i>DNA sequence of primers against Chromosomes 15 to 19.....</i>	<i>82</i>
Table 3.1	<i>The frequency of fl/fl, fl/wt, Δ/wt and Δ/Δ genotypes in the first 180 female pups.....</i>	<i>87</i>
Table 3.2	<i>The number of female pups generated with fl/fl and Δ/Δ genotypes from a total of 470 female mice.....</i>	<i>89</i>

Abbreviations

Approx.	approximately
BSA	bovine serum albumin
cAMP	cyclic adenosine 3'-5 monophosphate
CCD	charge coupled device
CEO	cumulus enclosed complex
Cntl	control
dbcAMP	dibutyl cAMP
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Disodium Salt Dihydrate or Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-Amino ethyl ether)-N,N, N',N'-tetra acetic acid
EtBr	Ethidium bromide
F	female
FCS	foetal calf serum
fl	floxed
FSH	follicle stimulating hormone
GV	germinal vesicle
GVB	germinal vesicle breakdown
H&E	Hematoxylin & Eosin
h	hour
hCG	human chorionic gonadotrophin
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
Het	heterozygote
IVM	<i>in vitro</i> maturation
KO	knockout
LH	luteinizing hormone
MEM	minimal essential medium

mg	milligrams
MgCl	magnesium chloride
MI	meiosis I
MII	meiosis II
Mil	milrinone
Milli-Q	Distilled water filtered through 0.22 um Millipak®Express Millipore
Min	minutes
PBE	polar body extrusion
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PFA	paraformaldehyde
Pgcs	primordial germ cells
PIPES	piperazine-N,N'-bis(ethanesulfonic acid)
PKA	protein kinase A
PMSG	pregnant mare's serum gonadotrophin
PVP	polyvinylpyrrolidone
Rpm	revs per minute
Rt	room temperature
SDS	sodium dodecyl sulfate
sec	seconds
TAE	Tris-Acetate-EDTA
µm	micrometer
µM	micromolar
V	volts
Wt	wildtype
Zona	zona pellucida

Abstract

Cdh1, a co-activator of the Anaphase Promoting Complex (APC) has recently been shown to be important in germinal vesicle stage arrest and in the prevention of aneuploidy during the first meiotic division of mouse oocytes. However, this was through antisense knockdown approaches done *in-vitro*. Therefore, here I generated an oocyte-specific knockout of Cdh1 (Δ/Δ) to explore this further. In this way, Cdh1 protein was specifically deleted only in germinal vesicle (GV) stage oocytes from growing follicles. Fewer cumulus enclosed oocytes were observed from Δ/Δ mice, of which significant numbers had undergone GVB. Furthermore, significantly more meiotically advanced, fragmented or degenerate oocytes were observed in knockouts. Denuded Δ/Δ GV oocytes also displayed a propensity for spontaneous GV breakdown (GVB) which could be partially rescued by maintaining an intact cumulus mass. Δ/Δ oocytes also underwent accelerated GVB on release from arresting media. Western Blots revealed a 5 fold increase in cyclin B1 levels following loss of Cdh1, whereas other substrates of APC^{Cdh1}, securin and Cdc25B, remained unchanged.

In-vivo and *in-vitro* matured metaphase II (MII) oocytes were analysed for aneuploidy rates. *In-vivo* matured knockout oocytes had higher, but not statistically significant, rates of aneuploidy than controls. Denuded oocytes that underwent IVM also had a

higher incidence of aneuploidy in knockouts and in this group this was highly significant. In summary, data from the *in-vivo* knockout model supports those of the *in-vitro* antisense approach and provide further evidence for the role of Cdh1 in both GV arrest and the prevention of aneuploidy, at least in the situation where oocytes are cultured *in-vitro*.

