Commonwealth of Australia
Copyright Regulations 1969

Warning

This material has been reproduced and communicated to you by or on behalf of The University of Adelaide pursuant to Part VB of the Copyright Act 1968 (the Act).

The material in this communication may be subject to copyright under the Act. Any further reproduction or communication of this material by you may be the subject of copyright protection under the Act.

Do not remove this notice.

External Copyright permission (if applicable)
The Role of Cyclin Dependent Kinase 2 (Cdk2) in the Proliferation and Differentiation of Pluripotent Embryonic Stem Cells

A thesis submitted to the University of Adelaide, Australia
for the degree of Doctor of Philosophy

Elaine Stead B. Sc. (Hons)

Department of Molecular Biosciences (Biochemistry)
University of Adelaide, Australia
Adelaide, South Australia

August 2002
Summary

A detailed study of cell cycle regulation during early embryogenesis to date has been limited to primitive developmental systems such as Xenopus and Zebrafish. However, there has been little analysis of early embryonic cells of more complex developmental systems such as mammals which undergo unique spatial organization during embryogenesis. While there has been preliminary analysis of the cell cycle regulation of mouse oocytes and 2 and 4 cell stage embryos, little is known about the mechanisms that regulate proliferation and the cell cycle after this time. Of particular interest, is the period just prior to implantation until gastrulation. Within this period, the rapidly proliferating pluripotent cells of the embryo undergo significant proliferative changes associated with cellular differentiation.

The aim of this thesis was to characterise the regulators mechanisms which underlie the rapid proliferation rate of pre-gastrulation pluripotent cell populations. However, due to size, relative inaccessibility and the technical limitations involved in performing this analysis in the embryo, this analysis was performed using ES and EPL cells, which are in vitro representations of pre and post-implantation pluripotent cell populations in the embryo. It was determined that both ES and EPL cells have a rapid proliferation rate, characterised by a truncated G1 phase. Underpinning the rapid proliferation rate is constitutive and elevated levels of cyclin dependent kinase 2 (Cdk2) activity throughout the cell cycle.

Additionally, this thesis aimed to address how cell cycle regulation is altered, both superficially and at a molecular level during the pluripotent embryonic stem cell differentiation. Three embryoid body systems that have been characterised in vitro, ES
embryoid bodies, EPL embryoid bodies and neur ectoderm embryoid bodies, were exploited to investigate changes in the expression or activity of cell cycle regulators and to address the function of these factors as developmental regulators. In each in vitro differentiation system, the cell cycle was remodelled to resemble differentiated cell types where the majority of cells are present in G1 phase. Additionally, levels of Cdk2 activity were significantly reduced during ES cells differentiation in each embryoid body system. ES and EPL cells acquire cell cycle regulation of Cdk2 activity shortly after the withdrawal of LIF, but this does not impact on the absolute levels of Cdk activity or the proportion of time spent in G1. Suppression of Cdk2 activity using chemical inhibitor caused a distinct delay in ES cell differentiation indicating that absolute levels of Cdk2 activity, at least in part, regulate the rate of ES cell differentiation. However, the precise mechanism that underlies this is unknown. It is hoped that this analysis will provide a sound basis for the investigation of the mechanisms which govern cell cycle control and differentiation of pluripotent embryonic stem cells, with respect to the pre-gastrulating mouse embryo, and the differentiation of pluripotent stem cells for therapeutic use.
CHAPTER 1 ........................................................................................................... 5

1.1 THE MAMMALIAN CELL CYCLE ..................................................................... 5

1.2 CYCLIN-DEPENDENT KINASE ACTIVITY CO-ORDINATES THE ORDER OF THE CELL CYCLE ........................................................................................................ 6

1.3 GENERAL REGULATION OF CYCLIN-DEPENDENT KINASE ACTIVITY .......... 6

1.3.1 CDK activation by cyclin binding .................................................................. 7

1.3.2 CDK activation by phosphorylation .............................................................. 8

1.3.3 CDK inhibition by phosphorylation ............................................................. 9

1.3.4 Cyclin dependent kinase inhibitors ............................................................ 10

1.4 REGULATION OF SPECIFIC CDK ACTIVITY IN PROLIFERATING CELLS .................................................................................................................. 12

1.4.1 Regulation of Cdk4/6 .................................................................................. 12

1.4.2 Regulation of Cdk2 .................................................................................... 13

1.4.3 Regulation of Cdk1 .................................................................................... 17

1.5 REGULATION OF THE G1/S PHASE TRANSITION THROUGH THE G1 RESTRICTION POINT (R-POINT) .................................................................. 19

1.5.1 Mitogenic control of G1 progression .......................................................... 19

1.5.2 Regulation of G1/S phase transition .......................................................... 21

1.5.2.1 E2F transcriptional repression of E2F ................................................... 21

1.5.2.2 Transcriptional Repression of E2F ......................................................... 25

1.5.3 Activation of the G1 Checkpoint ................................................................ 25

1.6 THE G1 CHECKPOINT AND CELLULAR DIFFERENTIATION .................... 26

1.6.1 A role for CDK1 during cellular differentiation ......................................... 27

1.6.2 G1 Cyclin-Cdk complexes during cellular differentiation ......................... 27

1.6.2.1 Cyclin D-Cdk4 complexes .................................................................... 27

1.6.2.2 Cyclin E-Cdk2 complexes .................................................................... 29

1.7 THE CELL CYCLE AND EARLY MOUSE EMBRYOGENESIS ...................... 31

1.7.1 Early mouse embryogenesis ....................................................................... 31

1.7.1.1 Pre-implantation development .............................................................. 31

1.7.1.2 Blastocyst formation ............................................................................ 31

1.7.1.3 Post-implantation development ........................................................... 32

1.7.1.4 Gastrulation .......................................................................................... 34

1.7.2 Early cell cycle length during early mouse embryogenesis ...................... 35

1.7.3 Cell cycle regulation during early embryogenesis ...................................... 37

1.7.4 Checkpoint control in early embryogenesis ............................................. 39

1.8 IN VITRO DIFFERENTIATION SYSTEMS FOR THE STUDY OF EMBRYOGENESIS ....................................................................................................... 41

1.8.1 Embryonic Stem (ES) cells ......................................................................... 41

1.8.2 ES embryoid bodies (EBs) ......................................................................... 41

1.8.3 Early Primitive Ectoderm Like (EPL) cells ............................................. 42

1.8.4 EPL embryoid bodies (EPLB) ................................................................. 43

1.8.5 Differentiation of ES cells into neuroectoderm ....................................... 45

1.9 AIM .................................................................................................................. 45

CHAPTER 2 ........................................................................................................... 47

2.1 ABBREVIATIONS .......................................................................................... 47

2.2 TISSUE CULTURE ......................................................................................... 51

2.2.1 Materials .................................................................................................. 51
2.2.2 Tissue Culture plastic ware ................................................................. 51
2.2.3 Buffers .................................................................................................. 51
2.2.4 Solutions .............................................................................................. 52
2.2.5 Cell Culture medium ........................................................................... 52
2.2.6 Cell Lines ............................................................................................. 53
2.2.7 Miscellaneous ....................................................................................... 54
2.3 TISSUE CULTURE METHODS .................................................................. 55
  2.3.1 Gelatinised Tissue Culture plates ..................................................... 55
  2.3.2 Determination of cell number ............................................................ 55
  2.3.3 Thawing stored cell lines ..................................................................... 55
  2.3.3.1 ES cells .......................................................................................... 55
  2.3.3.2 Additional cells lines ....................................................................... 56
  2.3.4 Maintenance of ES and EPII cell lines ............................................. 56
  2.3.5 Maintenance of additional cell lines ................................................. 57
  2.3.6 Preparation of MedII conditioned medium ..................................... 57
  2.3.7 Preparation of Embryoid Bodies (EB's) ............................................. 58
  2.3.7.1 ES Embryoid Bodies ..................................................................... 58
  2.3.7.2 EPII Embryoid Bodies .................................................................. 58
  2.3.7.3 Neurectoderm Embryoid Bodies .................................................. 58
  2.3.8 Harvesting Cells and Embryoid Bodies ......................................... 59
  2.3.9 Cell Synchronisation ........................................................................ 59
    2.3.9.1 Static Cell Synchronisation ........................................................ 59
    2.3.9.2 G1/S Phase Synchronisation/Release of ES Cells .................... 59
    2.3.9.3. Mitotic synchronisation and release of embryoid bodies ........ 60
2.4 MOLECULAR BIOLOGY .......................................................................... 61
  2.4.1 Radiochemicals .................................................................................. 61
  2.4.2 Chemicals ......................................................................................... 61
  2.4.3 Enzymes .......................................................................................... 63
  2.4.4 Buffers .............................................................................................. 63
  2.4.5 Solutions .......................................................................................... 65
  2.4.6 Molecular Biology Kits ..................................................................... 65
  2.4.7 DNA Molecular weight markers ...................................................... 66
  2.4.8 Protein Molecular Weight Markers ............................................... 66
  2.4.9 cDNA Fragments Used For Probe Synthesis .................................. 66
  2.4.10 Primary Antibodies ....................................................................... 67
  2.4.11 Secondary Antibodies ................................................................... 68
  2.4.12 Oligonucleotides .......................................................................... 68
  2.4.13 Miscellaneous ................................................................................ 68
2.5 MOLECULAR METHODS ........................................................................ 69
  2.5.1 Preparation of Whole Cell Extracts ............................................... 69
  2.6 PROTEIN DETECTION METHODS ..................................................... 69
    2.6.1 SDS PAGE Analysis .................................................................... 69
    2.6.2 Western Blotting ....................................................................... 70
2.7 PROTEIN CONCENTRATION DETERMINATION (BRADFORD ASSAY) ... 70
2.8 KINASE ACTIVITY ASSAY .................................................................. 72
  2.8.1 Preparation of Histone H1 ............................................................... 72
  2.8.2 Preparation of Protein A Sepharose .............................................. 72
  2.8.3 Immunoprecipitation of Active Kinase ....................................... 72
  2.8.4 Histone H1 Phosphorylation Assay ............................................. 73
2.9 NORTHERN ANALYSIS ........................................................................................................... 73
2.9.1 RNA Extraction .................................................................................................................. 73
2.9.2 Northern Transfer .............................................................................................................. 74
2.9.3 Radioactively Labelling DNA Probe .................................................................................. 75
2.9.4 Hybridisation and washing ............................................................................................... 75
2.10 GEL SHIFT ANALYSIS ........................................................................................................ 76
2.10.1 Construction of the E2F template .................................................................................... 76
2.10.2 Radioactively labelling and purifying E2F probe ............................................................. 76
2.10.3 E2F binding reaction ....................................................................................................... 77
2.11 FLOW CYTOMETRIC ANALYSIS ....................................................................................... 78
2.11.1 Adherent Cells .................................................................................................................. 78
2.11.2 Aggregated Cells .............................................................................................................. 78
2.12 BRD4 PROLIFERATION ASSAY ......................................................................................... 79

CHAPTER 2 .................................................................................................................................... 80
3.1 INTRODUCTION ..................................................................................................................... 80
3.2 ANALYSIS OF THE CELL CYCLE STRUCTURE OF PLURIPOTENT EMBRYONIC STEM
CELLS ........................................................................................................................................... 81
3.3 CHARACTERIZATION OF CDK ACTIVITY IN PLURIPOTENT CELLS .................................. 83
3.3.1 Analysis of Cdk activity in stably arrested cells ................................................................. 83
3.3.2 Analysis of Cdk regulation in a synchronous ES cell cycle .............................................. 85
3.3.3 CDK2 ACTIVITY DRIVES THE RAPID PROLIFERATION OF PLURIPOTENT ES CELLS... 87
3.3.4 SUMMARY ....................................................................................................................... 90
3.4 MOLECULAR AND BIOCHEMICAL ANALYSIS OF E2F REGULATED TRANSCRIPTION
IN ES CELLS .................................................................................................................................. 91
3.4.1 Transcriptional analysis of E2F target genes in ES cells .................................................. 91
3.4.2 Biochemical analysis of ternary E2F complexes in ES cells ........................................... 94
3.5 SUMMARY ............................................................................................................................ 97

CHAPTER 4 ................................................................................................................................... 100
4.2 CELL CYCLE REGULATION DURING ES EMBRYOID BODY (ESEB)
DIFFERENTIATION ..................................................................................................................... 101
4.2.1 Differentiation of ESEB's is associated with a lengthened G1 phase ................................ 101
4.2.2 Cdk activity is down regulated with pluripotency during ESEB differentiation ............... 102
4.2.3 Down regulation of Cdk activity and cell cycle remodeling are functions of ES cell differentiation ......................................................................................................................... 104
4.3 CELL CYCLE REGULATION DURING EPL EMBRYOID BODY (EPELB) DIFFERENTIATION ................................................................................................................... 105
4.3.1 The cell cycle is remodelled during EPELB differentiation ............................................ 106
4.3.2 Cdk activity is down regulated with pluripotency during EPELB differentiation .......... 107
4.3.3 Cell cycle remodelling and down regulation of Cdk activity are functions of
EPL cell differentiation ............................................................................................................... 109
4.4 CELL CYCLE REGULATION DURING DIFFERENTIATION OF ES CELLS INTO NEUROTODERM ................................................................................................................... 110
4.4.1 Remodelling of the cell cycle is delayed as ES cells differentiate into neuroectoderm ... 111
4.4.2 Cdk activity is down regulated independently of pluripotent status during neuroectoderm formation ......................................................... 112
4.4.3 Summary .................................................................................. 114

CHAPTER 5 .................................................................................. 118
5.1 INTRODUCTION ......................................................................... 118
5.2 THE ROLE OF ELEVATED Cdk2 LEVELS DURING ES AND EPL CELL
DIFFERENTIATION ....................................................................... 119
5.2.1 ES cells differentiated as embryoid bodies ................................. 119
5.2.2 EPL cells differentiated as embryoid bodies .............................. 121
5.3 THE ACQUISITION OF CELL CYCLE REGULATED Cdk2 ACTIVITY DURING ES CELL
DIFFERENTIATION ...................................................................... 123
5.3.1 Cdk2 activity becomes cell cycle regulated during EPL/E2 differentiation
................................................................................................. 126
5.3.2 Cell cycle regulation of Cdk activity is not a consequence of culturing ES
cells as embryoid bodies .................................................................. 128
5.4 SUMMARY ................................................................................ 130

CHAPTER 6 ................................................................................ 134
6.1 INTRODUCTION ......................................................................... 134
6.2 CONSERVED FEATURES CELL CYCLE CONTROL IN PLURIPOTENT CELLS .... 134
6.3 AN UNUSUAL MODE OF Cdk ACTIVITY IN PLURIPOTENT CELLS .......... 135
6.4 CAN Cdk ACTIVITIES ACCOUNT FOR SHORT GAP PHASES IN PLURIPOTENT CELLS? ............................................................. 136
6.5 ARE CELL DIVISION RATES AND CELL CYCLE STRUCTURE SEPARABLE FEATURES OF
PLURIPOTENCY? .......................................................................... 139
6.6 Cdk REGULATION AND ITS IMPACT ON E2F TARGET GENES .............. 138
6.7 CONSERVED ASPECTS OF Cdk REGULATION DURING PLURIPOTENT CELL
DIFFERENTIATION ..................................................................... 139
6.8 CELL CYCLE REGULATION OF Cdk ACTIVITIES AND CELL CYCLE REMODELLING
ARE SEPARABLE FEATURES OF PLURIPOTENT CELL DIFFERENTIATION .... 141
6.9 TEMPORAL ACTIVATION OF PLURIPOTENT CELL DIFFERENTIATION IS RATE LIMITED BY Cdk2 LEVELS .................. 143

APPENDIX

CHAPTER 7 ................................................................................ 146
THESIS AMMENDMENTS .............................................................. 176