Perry, Rebecca Jane (2010) *An investigation of the effect of oestrogen on longitudinal growth.* MD thesis.

http://theses.gla.ac.uk/1881/

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

An Investigation of the Effect of Oestrogen on Longitudinal Growth

Rebecca Jane Perry

MB ChB, MRCPCH

Thesis submitted for the degree of Doctor of Medicine

The University of Glasgow Faculty of Medicine

August 2009

Research conducted in the

Section of Child Health

Division of Developmental Medicine

© Rebecca J. Perry 2009

Dedication

This thesis is dedicated to my husband, Keith and my son, Rory. I would like to thank Keith for his sound advice and unwavering support during the whole MD process, and not least for keeping Rory entertained and out of mischief.

Abstract

In children, maintenance of longitudinal growth is a complex process regulated by numerous hormonal and genetic factors, environment and nutrition. Among these, sex steroids are of vital importance and the most obvious demonstration of their effect on growth is the initiation, maintenance and decline of the pubertal growth spurt. In both sexes it is now accepted that oestrogen is the critical hormone in controlling the acceleration of growth during puberty and fusion of the growth plate. Although abnormalities of growth during puberty are common, the exact mechanisms that control the beginning and cessation of pubertal growth at the growth plate are poorly understood. The aim of the first part of my thesis was to characterise the effects of oestradiol at the level of the growth plate chondrocyte. The ATDC5 cell line, a murine chondrocyte model which progresses through the maturational stages of chondrogenesis and differentiation was initially used to investigate the effects of oestradiol *in vitro*. Thereafter, more physiological models of murine growth, primary chondrocytes and organ cultures, were used in an attempt to gain a better understanding of oestradiol action at the growth plate. In the absence of readily available physiological models of human growth, the effects of oestradiol on the human C28/I2 chondrocyte cell line were studied. The classical oestrogen receptors, ERα and ERβ, were shown to be expressed in both murine and human chondrocyte cell lines. Oestradiol and related chemicals, which alter the function of the oestrogen receptors (ER), were exploited to tease out the different functions of each ER in the growth plate. In the absence of foetal bovine serum, oestradiol had no effect on proliferation, differentiation or apoptosis of chondrocyte cells in monolayer culture or on the growth of the foetal metatarsal culture system. In addition,

oestradiol did not convey a protective effect on chondrocytes exposed to the pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in monolayer culture. However, endogenous oestrogen appears to play an important role in maintaining chondrocyte proliferation in monolayer culture and mineralisation in metatarsal culture as reflected by the inhibitory effects of Faslodex, the non-specific ER antagonist, on chondrocytes and metatarsals in culture. In the presence of methyl-piperidino-pyrazole (MPP), a selective ERα antagonist, and raloxifene, a selective oestrogen receptor modulator with higher ERβ binding affinity, a reduction in chondrocyte proliferation and increase in apoptosis was observed in murine and human chondrocytes. Similarly, a marked reduction in linear growth occurred when foetal murine metatarsals were exposed to MPP and raloxifene in combination. A less marked reduction in growth was observed in MPP-treated metatarsals. These findings suggest that the oestrogen receptors may have opposing actions in the growth plate with ERβ acting like a brake on chondrocyte growth and ERα promoting growth. ERβ may regulate cell proliferation through control of cell cycle modulators affecting G_1/S phase transition as MPP and raloxifene in combination reduced cyclin E and p53 levels on Western blot analysis.

The aim of the second part of my thesis was to investigate the effect of oral oestrogen on linear growth in girls with primary ovarian insufficiency (POI). A retrospective review of girls with POI treated at a tertiary endocrinology clinic over an 11 year period was performed. As expected the majority of girls with POI had Turner syndrome (TS; 83.7%). Non-TS associated POI was rare and the leading cause was iatrogenic secondary to the effects of total body irradiation for bone

marrow transplantation (12.8%). A significant proportion of these girls developed POI after full pubertal development so few cases were available to investigate the effect of oestrogen on growth. The oral oestrogen regime followed in individual patients with TS was highly variable so it was not possible to assess the effects of dose on height velocity (HV). The second clinical study examined the effect of oestrogen on growth in TS girls who received a standardised course of oral ethinylestradiol (EE2) for pubertal induction and a standard dose of growth hormone (10 mg/m^2/week). These girls participated in a prospective randomised double-blind placebo-controlled multi-centre study of growth promoting treatment in TS. The girls were initially randomised to oxandrolone or placebo at 9 years of age and further randomised to oral EE2 at 12 or 14 years of age. Oxandrolone and delaying pubertal induction to 14 years of age both improved final height but there was a negative interaction suggesting that the interventions are antagonistic. Low dose EE2 (2 μg/day) maintained HV for 1 year when it was introduced at 12 years of age. Thereafter, the natural deceleration in TS girls was augmented with subsequent increases in EE2. In contrast, the same low dose EE2 commenced at 14 years of age led to a reduction in HV which increased further with subsequent EE2 dose increases. Therefore TS girls who received oral EE2 to induce pubertal development did not show growth acceleration. In contrast, girls who underwent spontaneous puberty did exhibit a growth spurt. These differences observed in pubertal growth may be attributable to a less severe skeletal dysplasia in girls with preserved ovarian function but this is less likely because the degree of short stature and dysmorphology scores at enrolment between TS girls with spontaneous puberty and POI were similar. Therefore spontaneous puberty may be more growth-enhancing during

puberty than artificial induction with oral EE2 but final height obtained is unchanged.

The laboratory effects of oestradiol found in this thesis suggest that ERα may stimulate or maintain growth, and ERβ may inhibit growth. The obvious question is how these observations might be involved in the complex relationship between puberty, oestrogen and height velocity in humans. As affinity studies show that the half maximal effective concentration (EC_{50}) of ERα is achieved at slightly lower concentrations of oestradiol than ERβ it is conceivable that the ERα effect could predominate at lower systemic oestradiol concentrations and that ERβ could become more important at higher concentrations for example in later puberty. Alternatively, it is possible that the expression of ERα reduces or ERβ increases in the growth plate after reaching peak height velocity.

Declaration

I declare that the work contained within this thesis is my own original work. This work has not been previously submitted for a higher degree. All sources of information have been acknowledged.

Part I – Oestrogen action at the level of the growth plate

The laboratory based studies were performed in laboratory 513 (Bone Biology Group) in the Roslin Institute. I was the principal investigator and responsible for study design, experimental analysis, data collection and statistical analysis. I received regular input from Colin Farquharson, on a weekly basis, with regards to study design and future experiment planning and on a monthly basis from Faisal Ahmed in the form of project meetings. Elaine Seawright, laboratory technician/manager demonstrated several of the techniques used and maintained the standard operating procedures folder. Colin Farquharson demonstrated dissection of metatarsals from Swiss mice embryos for metatarsal organ culture.

Part II – Effect of pubertal induction with oestrogen on growth in girls with primary ovarian insufficiency

I was the principal investigator in the first clinical study (chapter 7) which was a retrospective review. I was responsible for the study design along with Malcolm Donaldson (supervisor of clinical/second part of thesis) and also case note review, data collection/analysis and statistical analysis.

I was a collaborator in the second clinical study entitled the "United Kingdom Turner Study" which is a randomised, double-blind placebo-controlled study of growth promoting treatment in Turner syndrome organised by the British Society for Paediatric Endocrinology and Diabetes. I was responsible for analysing the data with regards to the effect of oestrogen on height velocity, pubertal progression and bone maturation. This was not part of the original study protocol and ethical permission was therefore obtained from Multi-Centre Research Ethics Committee to analyse the data (see Appendix 1). I performed the statistical analysis based on advice obtained from David Young (Statistician, Research & Development department, Royal Hospital for Sick Children, Glasgow). The main objectives of the study were to examine the influence on final height of a) oxandrolone versus placebo from 9 years of age and b) delaying pubertal induction with oral ethinylestradiol from 12 to 14 years. The statistical analysis of the principal study outcomes (including assessment for interaction between the two treatments) was performed by Professor Tim Cole, Centre of Paediatric Epidemiology and Biostatistics, University College London Institute of Child Health, London.

Rebecca Jane Perry

Acknowledgements

I would like to express my gratitude for the support and advice that I received from the following people, without which the work and results of this study would not have been possible: Colin Farquharson (Principal Investigator, Bone Biology Group) and Faisal Ahmed (Honorary Senior Lecturer) my supervisors for the laboratory research performed at the Roslin Institute (University of Edinburgh); Malcolm Donaldson (Senior Lecturer) my supervisor for the clinical research performed in the Section of Child Health. Professor Laurence Weaver (Samson Gemmell Professor of Child Health) my postgraduate advisor and Professor Mike Connor (Head of Division) my principal supervisor.

I would like to thank all the staff at the Roslin Institute for making me feel very welcome. In particular, I would like to pay tribute to Elaine Seawright (Technician/ Laboratory Manager, Bone Biology Group) who taught me several techniques, the Staff of the Small Animal Unit for their animal husbandry who made many of my experiments possible and Tenovus Scotland for their generous financial support.

In Yorkhill, I would like to thank Emma Jane Gault (Turner Research Assistant), Wendy Paterson (Auxologist/Database Manager), Catherine Milmore (Dr Donaldson's secretary) and David Young (Statistician, Research & Development department) for their invaluable assistance with the clinical studies.

Refereed Publications

Perry RJ, Farquharson C, Ahmed SF. The role of sex steroids in controlling pubertal growth. Clinical Endocrinology 2008; 68:4-15.

Published Meeting Abstracts

Perry RJ, Farquharson C, Ahmed SF. Oestrogen action at the level of the growth plate. Hormone Research 2006; 65 (supplement 4): 27.

Perry RJ, Gault EJ, Donaldson MDC. The impact of oestrogen and oxandrolone on pubertal progression and height velocity in Turner syndrome. Hormone Research 2009; 72 (supplement 3): 464-5.

Unpublished Meeting Abstracts

Perry RJ, Farquharson C, Ahmed SF. The effect of oestrogen on the growth plate. European Society for Paediatric Endocrinology Growth Plate Working Group, ESPE Annual Scientific Meeting, Rotterdam July 2006.

Awards

Small Research Grant from Tenovus Scotland £8000 to cover laboratory consumables

European Society for Paediatric Endocrinology 45th Annual Scientific Meeting – Rotterdam, June 2006

Travel grant to attend meeting and present "Oestrogen action at the level of the growth plate"

Royal Hospital for Sick Children Research Prize Day – Glasgow, November 2006

Second prize for presentation entitled "Oestrogen action at the growth plate"

List of abbreviations

AF-1	Ligand-independent activation function-1
AF-2	Ligand-dependent activation function-2
AIF	Apoptosis inducing factor
AIRE	Autoimmune regulator
ALL	Acute lymphoblastic leukaemia
ALP	Alkaline phosphatase
ALS	Acid labile substance
ALSKO	Acid labile substance knockout mouse
AML	Acute myeloid leukaemia
ANDRKO	Androgen receptor knockout mouse
ANOVA	Analysis of variance
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
AR	Androgen receptor
ArKO	Aromatase knock out mouse
BA	Bone age
BERKO	Oestrogen receptor-β knockout mouse
17βHSD	17β-hydroxysteroid dehydrogenase
BMI	Body mass index
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
BSPED	British Society for Paediatric Endocrinology and Diabetes

CAIS	Complete androgen insensitivity syndrome
cAMP	cyclic Adenosine monophosphate
CDGA	Constitutional delay of growth and adolescence
cDNA	Complementary deoxyribonucleic acid
CoReg	Coregulator
CPP	Central precocious puberty
CTS	Charcoal treated serum
CYP17A1	17-α hydroxylase/17,20-lyase enzyme
CYP19A1	Cytochrome P450 family 19 subfamily A polypeptide 1
DBD	DNA binding domain
DERKO	Oestrogen receptor-α/-β knockout mouse
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMEM: F12	Dulbecco’s Modified Eagle Medium with Ham’s F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DXA	Dual energy X-ray absorptiometry
E2	17β-oestradiol
EBP	Oestrogen binding proteins
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EE2	Ethinylestradiol

ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Oestrogen receptor
ERα	Oestrogen receptor-α
ERβ	Oestrogen receptor-β
ERE	Oestrogen response element
ERK	Extra-cellular signal related kinases
ERKO	Oestrogen receptor-α knockout mouse
hERKO	Human oestrogen receptor knockout
FBS	Foetal bovine serum
FCR	Fundal-cervical ratio
FH	Final height
FOXL2	Forkhead transcription factor
FSH	Follicle-stimulating hormone
FSHR	FSH receptor
GALT	Galactose-1-phosphate uridylyltransferase
GD	Gonadal dysgenesis
GH	Growth hormone
GHBP	Growth hormone binding protein
GHRH	Growth hormone releasing hormone
GIPP	Gonadotrophin-independent precocious puberty
GLM	General linear model
GNAS1	Guanine nucleotide binding protein, alpha stimulating activity polypeptide
GnRH	Gonadotrophin releasing hormone

GPR30	G Protein-coupled Receptor 30
hCG	Human chorionic gonadotrophin
HCL	Hydrochloric acid
HPG	Hypothalamo-pituitary-gonadal
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
HV	Height velocity
IGF	Insulin-like growth factor
IL-1β	Interleukin-1β
IPT	Isolated premature thelarche
JAK	Janus kinase
kDa	Kilodalton (s)
LBD	Ligand binding domain
LH	Luteinising hormone
LHCGR	LH/choriogonadotrophin receptor
LHRH	Luteinising hormone releasing hormone
LID	Liver-specific IGF-1 gene-deletion mouse
MAPK	Mitogen-activated protein kinase
MPP	Methyl-piperidino-pyrazole
MREC	Multi-centre research ethics committee
mRNA	Messenger RNA
NO	Nitric oxide
OVX	Ovariectomised
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PHV	Peak height velocity
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
POI	Primary ovarian insufficiency
pNPP	p-Nitrophenyl phosphate
RHSC	Royal Hospital for Sick Children
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription
^{35}S	Radioactive sulfate-35
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SERM	Selective oestrogen receptor modulator
SHBG	Sex hormone binding globulin
SHOX	Short stature homeobox
SOCS	Suppressor of cytokine signalling
SOX-9	SRY-related High Motility Group-box gene 9
SP	Spontaneous puberty
SPSS	Statistical package for the social sciences
SRY	Sex-determining region Y

STAR	Steroidogenic acute regulatory protein
STAT	Signal transducers and activators of transcription
TBI	Total body irradiation
TBST	Tris-buffered saline with 0.1% Tween-20
TNFα	Tumour necrosis factor-α
TS	Turner syndrome
TSH	Thyroid stimulating hormone
WT	Wild type

Chapter 1: Introduction and literature review — Page

Preface 2

1.1 Growth in puberty 2

1.1.1 Height velocity and pubertal development 2

1.1.2 Oestrogen and the pubertal growth spurt 3

1.1.3 Growth plate fusion 4

1.2 Conditions affecting pubertal growth 5

1.2.1 Early pubertal development 5

1.2.1.1 Central precocious puberty 6

1.2.1.2 Gonadotrophin-independent precocious puberty 7

1.2.2 Delayed pubertal development 8

1.2.2.1 Primary delay of growth and puberty 9

1.2.2.2 Secondary delay of growth and puberty 9

1.2.2.3 Hypogonadotrophic hypogonadism 10

1.2.2.4 Hypergonadotrophic hypogonadism 10

1.2.2.5 Turner syndrome 11

1.2.2.6 Hypogonadism with other endocrinopathy 12

1.3 Oestrogen family 13

1.4 Oestrogen receptor structure and function 15

1.5 Systemic effects of sex steroids on GH-IGF-1 axis 18

1.5.1 GH-IGF-1 axis during puberty 18

1.5.2 Effect of androgens 19

1.5.3 Effect of oestrogens 20

1.6 Local effects of sex steroids on the growth plate 21

1.6.1 Effect of growth hormone 21

1.6.2 Effect of androgens 22

1.6.3 Effect of oestrogens 23

1.7 Lessons learned from oestrogen receptor knockouts 25

1.8 Aims and Strategy 28

1.8.1 Oestrogen action at the level of the growth plate 28

1.8.2 Effect of oral oestrogen on longitudinal growth in girls 29

Part I – Oestrogen action at the level of the growth plate

Chapter 2: Effect of oestradiol on murine ATDC5 chondrocyte cell line

2.1 Introduction **31**
2.2 Hypothesis **35**
2.3 Aims **35**
2.4 Materials and Methods **36**
2.4.1 Materials **36**
2.4.2 Cell culture **36**
2.4.2.1 Preparation of cell culture reagents **36**
2.4.2.2 Maintenance and differentiation of ATDC5 cells **36**
2.4.2.3 Freezing and thawing cells **37**
2.4.3 RNA methods **38**
2.4.3.1 Isolation of total RNA from cells **38**
2.4.3.2 Reverse transcription **38**
2.4.3.3 Polymerase chain reaction **39**
2.4.4 Protein concentration determination **40**
2.4.5 Cell proliferation and differentiation assays **40**
2.4.5.1 [^{3}H]Thymidine incorporation assay **40**
2.4.5.2 Alcian Blue staining of cell monolayer **41**
2.4.5.3 Alkaline phosphatase assay **41**
2.4.6 Apoptosis assay **42**
2.4.7 Statistical analysis **42**
2.5 Results **43**
2.5.1 Expression of classical oestrogen receptors in ATDC5 cells **43**
2.5.2 Effect of E2 on cell proliferation during chondrogenesis and terminal differentiation **43**

2.5.3 Effect of E2 on ALP activity and proteoglycan production **45**
2.5.4 Effect of co-incubating E2 and pro-inflammatory cytokines on chondrocyte proliferation and apoptosis **46**
2.6 Discussion **48**
2.7 Conclusions **54**

Chapter 3: Effect of oestradiol on human C28/I2 chondrocyte cell line

3.1 Introduction **57**
3.2 Hypothesis **57**
3.3 Aims **58**
3.4 Materials and Methods **58**
3.4.1 Materials **58**
3.4.2 Methods **58**
3.4.2.1 C28/I2 chondrocyte cell culture **58**
3.4.2.2 Chondrocyte proliferation assay **59**
3.4.2.3 Apoptosis assay **59**
3.4.2.3 Semi-quantitative RT-PCR **60**
3.4.3 Statistical analysis **60**
3.5 Results **61**
3.5.1 Gene expression **61**
3.5.2 Effect of oestradiol **61**
3.5.3 Effect of oestradiol-related chemicals **63**
3.6 Discussion **67**
3.7 Conclusions **68**

Chapter 4: Effect of oestradiol on physiological models of murine growth

4.1 Introduction **71**
4.2 Hypothesis **72**
4.3 Aims **72**

4.4 Materials and Methods **73**
4.4.1 Foetal murine metatarsal organ culture **73**
4.4.2 Morphometric analysis of metatarsals **73**
4.4.3 Sex determination of mouse embryos **74**
4.4.4 Isolation of primary murine chondrocytes **76**
4.4.5 Primary murine chondrocyte cell culture **76**
4.4.6 Chondrocyte proliferation assay **77**
4.4.7 Statistical analysis **77**
4.5 Results **77**
4.5.1 Longitudinal growth of foetal metatarsal culture system **77**
4.5.2 Mineralising zone growth of foetal metatarsal culture system **81**
4.5.3 Primary murine chondrocytes **84**
4.6 Discussion **86**
4.7 Conclusions **90**

Chapter 5: Investigation of growth inhibitory effect of Raloxifene and MPP

5.1 Introduction **93**
5.2 Hypothesis **94**
5.3 Aims **95**
5.4 Materials and Methods **95**
5.4.1 ATDC5 cell culture **95**
5.4.2 Western blotting analysis **95**
5.5 Results **97**
5.6 Discussion **98**
5.7 Conclusions **99**

Chapter 6: Effect of oestradiol on mineralisation

6.1 Introduction **102**
6.2 Aims **102**
6.3 Materials and Methods **103**

6.3.1 Foetal metatarsal organ culture **103**
6.3.2 Non-radioactive calcium phosphate deposition assay **103**
6.3.3 ALP activity in ATDC5 chondrocytes **103**
6.3.4 Statistical analysis **104**
6.4 Results **105**
6.4.1 Calcium content of metatarsals treated with Faslodex or E2 **105**
6.4.2 ALP activity in ATDC5 chondrocytes treated with Faslodex or Raloxifene **106**
6.5 Discussion **106**
6.6 Conclusions **109**

Part II – Effect of pubertal induction with oestrogen on growth in girls with primary ovarian insufficiency

Chapter 7: Effect of oral oestrogen on longitudinal growth in girls with primary ovarian insufficiency

7.1 Introduction **111**
7.2 Aims **115**
7.3 Subjects and Methods **115**
7.3.1 Subjects **115**
7.3.2 Methods **116**
7.3.3 Ethical approval **117**
7.3.4 Statistical analysis **117**
7.4 Results **118**
7.4.1 POI associated with Turner syndrome **119**
7.4.2 Non-Turner syndrome associated POI **122**
7.5 Discussion **124**
7.6 Conclusions **129**

Chapter 8: Effect of oral Ethinylestradiol on longitudinal growth in girls with Turner syndrome

8.1 Introduction **131**
8.2 Hypothesis **133**
8.3 Aims **133**
8.4 Subjects and Methods **133**
8.4.1 Subjects **134**
8.4.2 Study design **135**
8.4.3 Statistical analysis **136**
8.5 Results **137**
8.5.1 Effect of Oxandrolone on final height **139**
8.5.2 Effect of age of Ethinylestradiol initiation on final height **139**
8.5.3 Effect of Oxandrolone and Age of Ethinylestradiol initiation on FH **141**
8.5.4 Effect of GH duration prior to Ethinylestradiol on final height **144**
8.5.5 Effect of Ethinylestradiol on pubertal progression **146**
8.5.6 Effect of Ethinylestradiol on height velocity **147**
8.5.7 Effect of Ethinylestradiol on bone maturation **155**
8.6 Discussion **157**
8.7 Conclusions **161**

Chapter 9: General discussion and future work

9.1 General discussion **164**
9.2 Future work **171**

Reference List **174**
Appendix 1: Ethics Letter **200**
Appendix 2: Smoothed reference centile curves for uterine length **201**
Appendix 3: Smoothed reference centile curves for fundal cervical ratio **202**

List of Figures **Page**

Chapter 1

1.1 Principal pathways in steroid biosynthesis **14**

1.2 Overall distribution of human ERα and ERβ in different tissues **15**

1.3 The structural organisation of nuclear receptors **16**

1.4 Comparison of the primary structures of ERα and ERβ **17**

1.5 Models of oestrogen action **24**

Chapter 2

2.1 ER-α and ER-β expression in ATDC5 cells **43**

2.2 Effect of E2 on ATDC5 proliferation **44**

2.3 Effect of E2 on ATDC5 differentiation **46**

2.4 Effect of co-incubating E2 with Il-1β or TNFα on ATDC5 proliferation **47**

2.5 Effect of co-incubating E2 with Il-1β or TNFα on ATDC5 apoptosis **47**

Chapter 3

3.1 ER-α and ER-β expression in C28/I2 cells **61**

3.2 Effect of E2 on C28/I2 proliferation in the presence of FBS **62**

3.3 Effect of E2 on C28/I2 proliferation in serum-free medium **63**

3.4 Effect of E2 alone and in combination with Tamoxifen, Raloxifene or Faslodex on C28/I2 proliferation **64**

3.5 Effect of E2, Raloxifene or MPP alone and in combination on C28/I2 proliferation **64**

3.6 Effect of E2, Faslodex, Raloxifene or Raloxifene & MPP on apoptosis of C28/I2 cells **66**

Chapter 4

4.1 Foetal murine metatarsal culture model **74**

4.2 SRY gene expression to determine sex of mice embryos **75**

4.3 Effect of E2 or Faslodex on longitudinal growth of murine metatarsals **78**

4.4 Effect of Raloxifene on longitudinal growth of murine metatarsals **79**

Chapter 4 continued

4.5 Effect of Raloxifene, MPP or Raloxifene & MPP on longitudinal growth of murine metatarsals **80**

4.6 Effect of Raloxifene, MPP or Raloxifene & MPP on longitudinal growth of male or female murine metatarsals **80**

4.7 Effect of E2, Faslodex or Raloxifene on mineralising zone growth of metatarsals **82**

4.8 Effect of MPP, Raloxifene or Raloxifene & MPP on mineralising zone growth of metatarsals **83**

4.9 Primary murine chondrocytes after Alcian Blue staining **84**

4.10 Effect of E2 on primary murine chondrocyte proliferation **85**

4.11 Effect of Faslodex or Raloxifene & MPP on primary murine chondrocyte proliferation **85**

Chapter 5

5.1 Western Blot: effect of Raloxifene, MPP or Raloxifene & MPP on cell cycle proteins **97**

5.2 Control of the G_1/S cell cycle checkpoint **100**

Chapter 6

6.1 Effect of E2 or Faslodex on calcium concentration of metatarsals **105**

6.2 Effect of Faslodex or Raloxifene on ALP activity in ATDC5 cells **106**

Chapter 7

7.1 Aetiology of POI in patients who attended RHSC from 1989 to 2000 **118**

7.2 Latest bone age performed in TS girls who required pubertal induction with corresponding daily oral EE2 dose **121**

7.3 Initial diagnosis of patients with POI associated with late effects of cancer-related treatment including TBI **122**

Chapter 8

8.1	Flow diagram of progress through UK Turner Study	**138**
8.2	Height SD score for chronological age at enrolment and final height according to EE2 randomisation	**140**
8.3	Height SD score for chronological age at enrolment and final height according to Oxandrolone and EE2 randomisation	**141**
8.4	Difference in height SD score from enrolment to final height according to Oxandrolone randomisation	**143**
8.5	Delta height SD score in girls treated with EE2 according to Oxandrolone, E2 randomisation and GH duration before EE2	**145**
8.6	Height velocity of girls treated with EE2 at 12 years	**147**
8.7	Height velocity of girls treated with EE2 at 12 years according to Oxandrolone randomisation and/or GH duration before EE2	**148**
8.8	Height velocity of girls treated with EE2 at 14 years	**149**
8.9	Height velocity of girls treated with EE2 at 14 years according to Oxandrolone randomisation and/or GH duration before EE2	**150**
8.10	Height velocity according to age of pubertal induction	**152**
8.11	Height velocity according to EE2 dose	**152**
8.12	Height velocity of girls with spontaneous puberty	**153**
8.13	Height velocity of girls with spontaneous puberty compared to girls who required artificial induction with oral EE2	**153**
8.14	Height gain during puberty of girls with SP compared to girls who required artificial induction with oral EE2 at 12 or 14 years	**154**
8.15	Bone maturation of girls who started EE2 at 12 or 14 years from 11-17 years of age	**155**
8.16	Bone maturation of girls who started EE2 at 12 or 14 years from 12-15 years of age	**156**
8.17	Bone maturation of girls who started EE2 at 12 or 14 years from 12-15 years of age according to Oxandrolone randomisation	**156**

List of Tables **Page**

1.1	Characteristics of female murine knockout models	**26**
1.2	Characteristics of male murine knockout models	**27**
2.1	Effects of E2 on chondrocytes *in vitro*: summary of published data	**33**
2.2	Primer sequences and product sizes for murine ERα and ERβ genes	**39**
3.1	Primer sequences and product sizes for human ERα and ERβ genes	**60**
4.1	Primer sequences and product sizes for murine SRY gene	**75**
5.1	Primary antibodies used for western blotting analysis	**96**
5.2	Secondary antibodies used for western blotting analysis	**96**
7.1	Causes of ovarian follicular depletion	**113**
7.2	Causes of ovarian follicular dysfunction	**114**
7.3	Characteristics of all girls with TS who attended RHSC 1989-2000	**119**
7.4	Characteristics of TS girls who attended RHSC 1989-2000 who have reached final height	**120**
7.5	Oral ethinylestradiol regimes employed to induced puberty in girls with POI associated with TS	**120**
7.6	Uterine development of girls with TS before and after puberty	**121**
7.7	Characteristics of girls with POI associated with TBI and BMT treated with oral EE2	**123**
7.8	Oral ethinylestradiol regimes used in girls with POI associated with TBI and BMT	**123**
8.1	Dysmorphology scoring system	**135**
8.2	Participant characteristics according to Oxandrolone randomisation	**139**
8.3	Participant characteristics according to EE2 randomisation	**140**
8.4	Participant characteristics according to Oxandrolone and EE2 randomisation	**142**
8.5	Participant characteristics according to EE2 randomisation and GH duration before EE2	**145**
8.6	Pubertal progression with age at each Tanner breast stage achieved	**146**

Chapter 1

Introduction and literature review

Chapter Contents

Preface
1.1 Growth in puberty
1.1.1 Height velocity and pubertal development
1.1.2 Oestrogen and the pubertal growth spurt
1.1.3 Growth plate fusion
1.2 Conditions affecting pubertal growth
1.2.1 Early pubertal development
1.2.1.1 Central precocious puberty
1.2.1.2 Gonadotrophin-independent precocious puberty
1.2.2 Delayed pubertal development
1.2.2.1 Primary delay of growth and puberty
1.2.2.2 Secondary delay of growth and puberty
1.2.2.3 Hypogonadotrophic hypogonadism
1.2.2.4 Hypergonadotrophic hypogonadism
1.2.2.5 Turner syndrome
1.2.2.6 Hypogonadism with other endocrinopathy
1.3 Oestrogen family
1.4 Oestrogen receptor structure and function
1.5 Systemic effects of sex steroids on GH-IGF-1 axis
1.5.1 GH-IGF-1 axis during puberty
1.5.2 Effect of androgens
1.5.3 Effect of oestrogens
1.6 Local effects of sex steroids on the growth plate
1.6.1 Effect of growth hormone
1.6.2 Effect of androgens
1.6.3 Effect of oestrogens
1.7 Lessons learned from oestrogen receptor knockouts
1.8 Aims and Strategy
1.8.1 Oestrogen action at the level of the growth plate
1.8.2 Effect of oral oestrogen on longitudinal growth in girls

Preface

In children, maintenance of longitudinal growth is a complex process regulated by several hormonal and genetic factors, environment and nutrition. Among these, sex steroids are of crucial importance and the most obvious demonstration of their effect on human growth is the initiation, maintenance and decline of the pubertal growth spurt. This accelerated period of growth accounts for about 20% of final adult height. Although abnormalities of growth during puberty are very common, the underlying mechanisms that govern the beginning and cessation of pubertal growth at the level of the growth plate are poorly understood. The effect of sex steroids on growth can either be studied at the systemic level or at the local level of the epiphyseal growth plate. In both sexes it is now accepted that oestrogen is the critical hormone in controlling the acceleration of growth during puberty and fusion of the growth plate.

1.1 Growth in puberty

1.1.1 Height velocity and pubertal development

There are at least three distinct phases of post-natal growth. The infantile phase is the first phase with rapid height velocity (HV) during foetal life followed by rapid deceleration up to 3 years of age. The childhood phase follows which has a slowly decelerating velocity until puberty. The pubertal phase shows acceleration in HV and reaches a peak on average 22 months after initiation. HV then rapidly decreases and ceases.[1] This pubertal growth comprises 15-20% of final height (FH) and precedes fusion of the growth plates. Both the amplitude of the pubertal spurt and peak HV vary negatively with the age of onset of puberty.[1-3] Concordance is shown between the pubertal growth spurt and clinical pubertal development.[1;4;5] The tempo of

pubertal growth shows sexual dimorphism. In girls, growth acceleration is usually seen early with peak HV occurring at Tanner breast stage B2 in 40%, B3 in 30%, B4 in 20% and B1 in 10%.[1] In contrast, boys show an acceleration of growth usually during the second year of pubertal development with peak HV occurring at Tanner genital stage G3 in 60%, stage 4 G4 in 28%, stage 2 G2 in 8% and stage 5 G5 in 4%.[1] This dimorphic pattern may be related to the delay in boys reaching the level of oestrogen required from the aromatisation of testosterone. Aromatisation is mediated by aromatase (an enzyme of the cytochrome P450 group) whose function is to mediate the conversion of androgens to oestrogens.

1.1.2 Oestrogen and the pubertal growth spurt

The pubertal growth spurt is primarily due to increased secretion of the sex steroids, oestrogens and androgens. It has been widely accepted that oestrogen mediates pubertal bone growth in females, however it has only been recently established that oestrogen, and not testosterone, mediates the same function for males. The growth patterns observed in certain rare syndromes suggest that, in humans, oestrogen is responsible for the initiation of the pubertal growth spurt and closure of the growth plate in both sexes. Inactivating mutations in either the oestrogen receptor-α (ERα) gene or the aromatase gene in males have resulted in a lack of a pubertal growth spurt, absent epiphyseal closure with resultant tall stature (taller than predicted). The individual with a defective ERα is unique.[6] He presented with tall stature, normal male sexual maturation, osteoporosis and open epiphyses. Treatment with oestrogen did not produce epiphyseal fusion nor improve his bone mineral density. A similar phenotype was described in a 24-year old man with congenital aromatase deficiency

who had tall stature, a bone age (BA) of 14 years and osteoporosis despite high testosterone levels.[7] Treatment with conjugated oestrogens for 6 months led to fusion of the growth plates and increased bone mineral density.[8] Aromatase deficiency has now been described in others and they all show persistent linear growth in adulthood with open epiphyseal growth plates.[9;10] Although data are limited, individuals with aromatase deficiency seem to have an absent growth spurt and fusion of the growth plates is reported after treatment with exogenous oestrogen but not with testosterone.[9] 46,XY girls with complete androgen insensitivity syndrome (CAIS; gonads *in situ*) do have a pubertal growth spurt, which tends to occur at a similar age and of similar amplitude to unaffected girls, thus, demonstrating that oestrogen is able to increase HV in the absence of androgen action.[11]

Pubertal growth is influenced by the amount of body fat. In a longitudinal study of normal children an additional body mass index (BMI) point (+1 kg) was reported to decrease the growth spurt by a mean of 0.5 cm and 0.9 cm, in girls and boys, respectively. However, FH was not affected due to the growth acceleration observed in childhood.[12]

1.1.3 Growth plate fusion

It has been widely accepted that longitudinal growth ceases in late adolescence as a result of epiphyseal fusion, an abrupt event in which growth plate cartilage is replaced by bone tissue. However, careful observation suggests that growth ceases before fusion occurs[13;14] which suggests that fusion is the result of growth

cessation.[13;15] The deceleration in longitudinal growth was previously attributed to a systemic mechanism. However, recent evidence indicates that it is caused by a local mechanism within the growth plate. In growth plate transplantation studies, the growth rate of the transplanted growth plate depended on the age of the donor animal, not that of the recipient.[16] This intrinsic mechanism has been referred to as "growth plate senescence".[17] The senescent decline in growth rate is mainly due to a decrease in the rate of chondrocyte proliferation in the growth plate.[18] Young rabbits treated with systemic dexamethasone for a 5-week period showed a reduction in longitudinal bone growth.[19] After recovery, their growth plates showed a delay in senescent decline in the growth rate, chondrocyte proliferation rate, growth plate height, proliferating zone and hypertrophic zone height.[19] This programmed senescence appears to be a function of proliferation. Oestrogen is reported to accelerate the normal process of growth plate senescence, leading to earlier exhaustion of the growth plate, and as a consequence earlier fusion.[13] Although, this hypothesis was not supported by a recent study of castrated male rabbits[20] in which oestrogen was shown to decrease proliferation in the resting zone chondrocytes. This latter study examined younger, male rabbits which were treated for a shorter period of time (two weeks compared to eight weeks) and the plasma oestrogen levels achieved were much lower.

1.2 Conditions affecting pubertal growth

1.2.1 Early pubertal development

Early pubertal development is traditionally defined by the onset of puberty before the age of 8 years in girls or 9 years in boys. The occurrence of menarche before the age

of 10 years also indicates sexual precocity. In the USA these criteria have been reconsidered due to a community-based study of 17,000 girls who showed a trend towards earlier breast development.[21] However, age at menarche is unaltered, suggesting that the tempo of puberty in the early developers may be slower. It has been proposed that the age of 7 years for white girls and 6.5 years for the African American population should be used as a cut-off for defining early puberty.[22] In boys, the cut-off is unchanged. In Europe, the original criteria are still valid as there is no evidence of a recent reduction in age at onset of puberty in either gender.[23] Precocious puberty can be classified as central precocious puberty (CPP) or gonadotrophin-independent precocious puberty (GIPP). CPP involves the premature activation of the hypothalamic-pituitary-gonadal (HPG) axis. In GIPP the presence of sex steroids is independent of pituitary gonadotrophin release.

1.2.1.1 Central precocious puberty

CPP has an incidence of 1 in 5-10 000 children with a female to male ratio of greater than 20:1.[24] The spectrum of idiopathic CPP contains transient, alternating, slowly progressive and rapidly progressive forms.[25] Ninety-five percent of girls with CPP have idiopathic CPP whereas over 50% of boys have an identifiable aetiology.[26] Acceleration of growth is almost invariable in CPP but these children will also show premature ossification and fusion of the growth cartilage and early cessation of growth. Girls who present before 6 years are reported to lose 12-15 centimetres in FH whereas those who present after 6 years lose 7-10 centimetres.[27] The risk of short stature is higher in children with associated growth hormone (GH) insufficiency or limited potential to grow due to cranial or craniospinal irradiation.

Early puberty may result in a greater sitting height to leg length ratio at FH as an indicator of the premature bone maturation.

1.2.1.2 Gonadotrophin-independent precocious puberty

GIPP is characterised by pubertal sex steroid levels with pre-pubertal or suppressed gonadotrophins. Excess secretion of the hormone leads to hypertrophy of the target tissue as well as acceleration of growth and bone maturation. The source of hormone production can be gonadal, adrenal (tumour or congenital adrenal hyperplasia), ectopic (gonadotrophin- or human chorionic gonadotrophin-producing tumours) or exogenous. Additionally the McCune-Albright syndrome produces discordant sexual development and is characterised by irregular pigmented café-au-lait patches and polyostotic fibrous dysplasia. Pubertal signs are usually discordant with early bleeding in girls and no evidence of gonadotrophin cyclicity. In this condition, growth acceleration may also be due to hyperthyroidism or GH excess. It occurs due to mosaicism for a sporadic post-zygotic activating mutation in the *GNAS1* (guanine nucleotide binding protein alpha stimulating activity polypeptide) which prevents down regulation of cyclic adenosine monophosphate (cAMP) signalling in endocrine tissues leading to overactivity.[28] In males, testoxicosis or familial male precocious puberty can lead to pubertal changes and growth acceleration. The testes are often small for the degree of virilisation.[29] In all of these conditions the excess sex steroid production leads to advanced epiphyseal maturation which can predict the timing of the onset of central puberty as there is remarkable synchrony between the onset of central puberty and skeletal maturation across the various disorders of puberty.[30] Central puberty may occur due to exposure of the hypothalamus to high levels of sex

steroids; this phenomenon is known as “priming”.

Although puberty tends to be delayed in hypothyroid children, severe longstanding hypothyroidism may cause GIPP probably mediated by thyroid stimulating hormone (TSH) stimulation of the follicle-stimulating hormone (FSH) receptor.[31] Characteristically there is testicular enlargement without significant virilisation in boys and breast development, uterine bleeding and multicystic ovaries in girls. Thyroxine treatment can lead to resumption of the normal consonance of puberty and catch-up growth.[32] However, FH may be reduced attributable to a rapid advance in epiphyseal maturation.[33]

1.2.2 Delayed pubertal development

Delayed puberty is the failure to develop secondary sexual characteristics by a certain age usually set as two standard deviations from the mean. In girls, pubertal delay is usually defined as a lack of breast development by 13 years, lack of pubic hair by 14 years, lack of menarche by 16 years or greater than 5 years between thelarche and menarche. In boys, puberty is considered delayed if testicular enlargement does not occur by 14 years, lack of pubic hair by 15 years, or more than 5 years are required to complete genital enlargement. Children with delayed puberty continue to grow at a pre-pubertal rate. Natural slowing of linear growth, just before onset of puberty may be exaggerated, emphasising the difference in size from peers who are accelerating in growth.

1.2.2.1 Primary delay of growth and puberty

This condition, often referred to as constitutional delay in growth and adolescence (CDGA), typically affects boys. The clinical features include relative short stature for chronological age, delayed puberty and delayed bone maturation in otherwise healthy adolescents. Because the timing of the onset of puberty, pubertal growth spurt, and epiphyseal fusion are determined by a child's skeletal age (biological age) children with CDGA are often referred to as "late developers". A relatively short upper body segment is common at presentation and persists at attainment of FH.[34] Although FH is predicted to be normal based on BA estimation, adolescents with CDGA are usually shorter than their mid-parental height.[34-37] There is often a family history of delayed puberty[38] or a personal history of atopy.[39] The decrease in FH may be explained by the short stature at onset of puberty, shorter duration between onset of puberty and pubertal growth spurt and compromised peak HV.[40] A short course of testosterone enanthate or low doses of oxandrolone can accelerate the pubertal growth spurt without altering FH.[41;42] However, FH in boys with CDGA may be improved by treatment with aromatase inhibitors by delaying bone age progression.[43]

1.2.2.2 Secondary delay of growth and puberty

A delay in puberty can be seen in virtually any chronic disease of childhood.[44] Several factors influence the degree to which growth and pubertal development are affected. These include age, duration of illness and its severity, nutritional status and medications. This form of delayed puberty is often mistaken for CDGA but may be more profound and protracted depending on the underlying condition.

1.2.2.3 Hypogonadotrophic hypogonadism

Hypogonadism can be classified according to the serum gonadotrophin levels; high levels indicate primary gonadal failure and low levels indicate disorders at the hypothalamo-pituitary level. In hypogonadotrophic hypogonadism, such as Kallman's syndrome, puberty may be completely absent. The pubertal component of the infancy-childhood-puberty model will be lacking so childhood growth will continue at its slowly declining rate. In untreated individuals growth will continue until the third decade and FH may actually be taller than average due to this persistence of the childhood component of growth. However, this results in abnormal body proportions with a relatively longer lower body segment. Short stature may be seen initially relative to chronological age but a normal FH is achieved after sex steroid replacement.[45]

1.2.2.4 Hypergonadotrophic hypogonadism

This condition results from a primary defect of the gonads which renders them unresponsive to gonadotrophins. A similar growth pattern to those affected by hypogonadotrophic hypogonadism is observed. However, the commonest cause of primary hypogonadism in boys is Klinefelter syndrome (47,XXY) which is associated with tall stature. Often there is a normal onset of puberty but pubertal arrest can occur at any stage. If spontaneous puberty does occur then the magnitude and timing of pubertal growth is reported to be normal, with a mean FH of 186 centimetres. The tall stature may be attributable to the extra sex chromosome. Although testes are of normal size and consistency at birth they fail to grow normally during puberty and seldom exceed 4 millilitres. Testicular involution then occurs and

androgen deficiency occurs. Plasma testosterone levels are significantly lower by 16 years of age.[46] In boys with prenatal testicular atrophy for example due to "the vanishing testes syndrome" the childhood component of growth is reported to be normal. Whereas, in girls the commonest cause of primary hypogonadism is ovarian dysgenesis in association with Turner syndrome.

1.2.2.5 Turner syndrome

Turner syndrome (TS) is a congenital disorder caused by the loss of all, or a critical part, of one X chromosome and is associated with a combination of characteristic features of which the most common are short stature and ovarian dysgenesis. It is estimated to affect around 3% of all female foetuses[47] but the majority of foetuses with TS are spontaneously miscarried and only 1% survive to term.[48] The number of females born with TS is estimated to range from 25 to 210 per 100 000 live births.[49] Individuals with TS commonly present with short stature or delayed puberty. Other organs and systems may be affected resulting in congenital malformations and characteristic physical features such as webbed neck, broad chest and cubitus valgus (widened carrying angle). The 45,X karyotype accounts for 50-70% of cases and is associated with the classical, more dysmorphic, phenotype and a higher proportion of foetal deaths than live births indicating that a foetus with 45,X is less likely to survive to term than other TS karyotypes.[50] The remaining cases of TS are associated with a range of karyotypic abnormalities which include abnormalities of the second X chromosome and mosaicism. Mosaicism occurs when some of the cells in the body have a TS chromosome component, while others have a different chromosome component which may be normal (45,X/46,XX) or abnormal

(45,X/47,XXX). As such a small proportion of 45,X foetuses survive to term it is likely that all TS patients are actually mosaics and may only be detected if multiple tissues are analysed using specialised staining techniques.[51] Short stature is the most consistent feature of TS. The cause of this remains unknown but is thought to be due to a primary bone defect. Some of the height deficit is accounted for by haplo-insufficiency of the short stature homeobox (SHOX) gene. Although there is no evidence of classical GH deficiency[52;53] it has been suggested that partial GH and/or insulin-like growth factor (IGF) insensitivity at the level of the growth plate may play a role.[52-54] Ovarian insufficiency is common and typically affects around 95% of females with TS.[55] This usually occurs during the first few months or years of life. However, spontaneous puberty can occur in girls with TS and in a recent Italian study the incidence was 16% which is higher than previously thought.[56] It was more frequently associated with mosaicism than with the 45,X karyotype. However in those with spontaneous puberty, premature ovarian failure frequently ensues and the occurrence of natural pregnancy is low (less than 5%).[47;56] Girls with TS usually demonstrate mild growth retardation *in utero* followed by slow growth during infancy with a delayed onset of the childhood component of growth and then slow growth during childhood and adolescence with absence of the pubertal growth spurt resulting in reduced FH.[47;53;57] The average height of untreated women with TS is 20 centimetres lower than the mean for normal women.[58]

1.2.2.6 Hypogonadism with other endocrinopathy

Delayed puberty is often seen in patients with GH deficiency or a GH receptor defect. A common feature to both these conditions is low or lack of IGF-1 function.

This highlights the possible role of IGF-1 in the regulation of puberty.[59;60] In Type 1 Diabetes Mellitus pubertal delay has also been described. However, this was not confirmed in more recent studies where FH was unaffected. Nonetheless, the pubertal growth spurt in Type 1 Diabetes Mellitus may be attenuated.[61]

Primary hypothyroidism can also be associated with hypogonadotrophic hypogonadism which is reversible with thyroxine treatment.[62] Although children with primary hypothyroidism may continue to grow for a longer period after therapy is initiated, FH can still be restricted.[63]

Cushing's disease is associated with a delayed onset or mid-pubertal arrest of puberty which is reversible after removal of the source.[64;65] GH deficiency is common following treatment of childhood onset Cushing's disease and may persist for many years.[66] Early investigation for diagnosis and treatment of GH deficiency is thus advocated.[67] FH within target range can be achieved but excess adiposity remains a potential long-term complication.[67]

1.3 Oestrogen family

Oestrogens are a family of hormones produced mainly in the ovary. There are three main types: oestradiol, oestriol and oestrone. Oestradiol is the principle hormone active in pre-menopausal women and is produced by conversion of androgenic steroids by 17β-hydroxysteroid dehydrogenase (17βHSD) and aromatase (Figure 1.1). In contrast, oestriol is only found in low levels apart from during pregnancy as it is produced by the placenta. The foetal adrenal cortex produces

dehydroepiandrosterone sulphate (DHEAS) which is then converted into oestriol. Levels of oestriol are used for screening for congenital abnormalities and forms part of the triple/quadruple test (low levels suggest an abnormality). The third hormone oestrone, is a weak oestrogen, but the only one present in any quantity in post-menopausal women. Oestrone is produced by conversion of androstenedione by aromatase. Its derivative, oestrone sulphate, is long-lived and serves as a reservoir for oestrone and can be converted into the more active oestradiol by 17β-HSD.

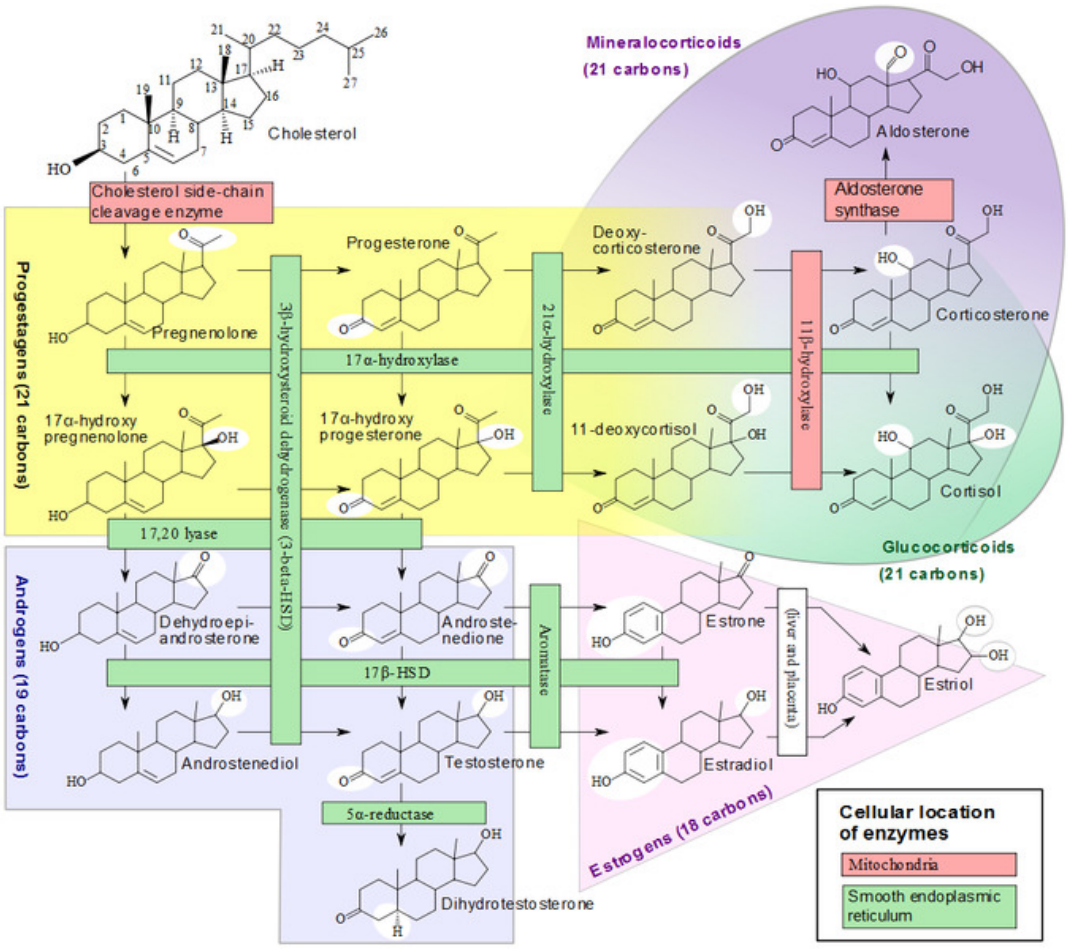


Figure 1.1 – Principal pathways in steroid biosynthesis
(http://en.wikipedia.org/wiki/File:Steroidogenesis.svg)

1.4 Oestrogen receptor structure and function

The execution of the biological effects of oestrogen is mediated by two isoforms of the nuclear ER, namely, ERα and ERβ. They are encoded by separate genes; ERβ is localised on human chromosome 14 whereas ERα is found on chromosome 6. The receptors are widely distributed throughout the body and have distinct tissue expression patterns (Figure 1.2). The ERs belong to the steroid/thyroid hormone superfamily of nuclear receptors, of which members share a common structural architecture. They are composed of 3 independent but interacting functional domains: the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (LBD) (Figure 1.3).

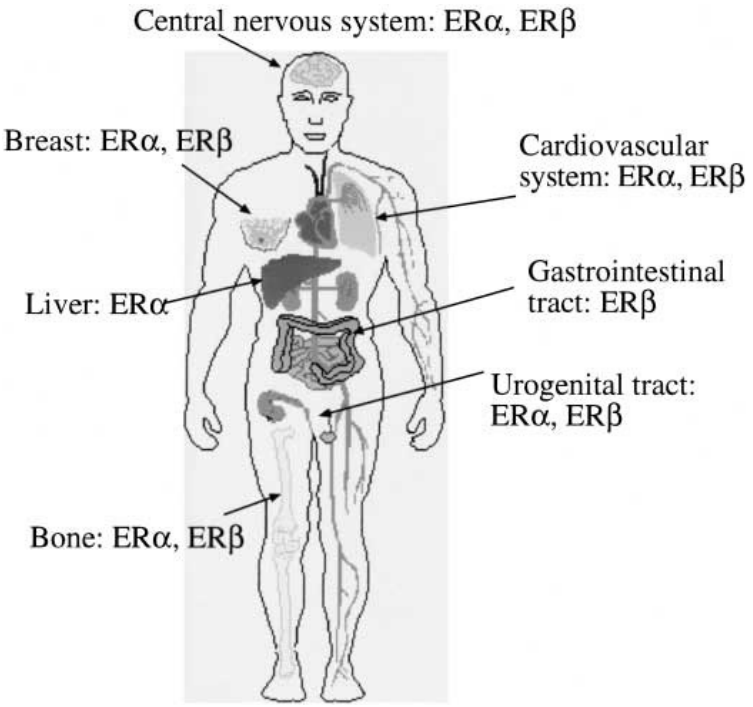


Figure 1.2 – Overall distribution of human ERα and ERβ in different tissues
From Gustafsson JA, 1999, Journal of Endocrinology, 163, 379-383
© Society for Endocrinology 1999. Reproduced by permission.[68]

The A/B domain encodes a ligand-independent activation function-1(AF-1) which is responsible for activation of transcription and co-factor binding. ERα is very active in stimulation of reporter-gene expression from a variety of oestrogen response element (ERE)-reporter constructs in different cell lines[69] but ERβ has negligible activity of the AF1 domain under the same conditions. The C/D domain is the DNA binding domain (DBD) and contains a two zinc finger structure which plays an important role in receptor dimerisation and in binding of receptors to specific DNA sequences. These domains are highly conserved in ERα and ERβ showing 97% homology (Figure 1.4). The D/F domain contains the ligand binding pocket as well as the ligand-dependent activation function-2 (AF-2) domain that directly contacts coactivator peptides. Upon ligand binding the ER dimerises and migrates to the nucleus where it interacts with specialised sequences of DNA in the promoter region of certain genes, with subsequent activation or inhibition of transcription.[70] The genomic sequences capable of binding the ER-ligand complex include the ERE as well as more recently described alternative sequences.[71;72] Oestrogens produce agonist effects through these interactions to varying degrees in oestrogen responsive tissues.

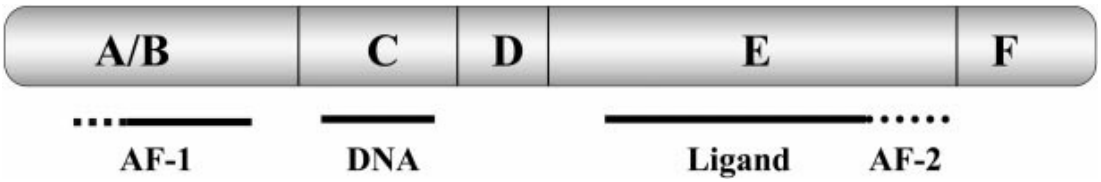


Figure 1.3 – The structural organisation of nuclear receptors
From Nilsson *et al*, 2001 used with permission.[73]

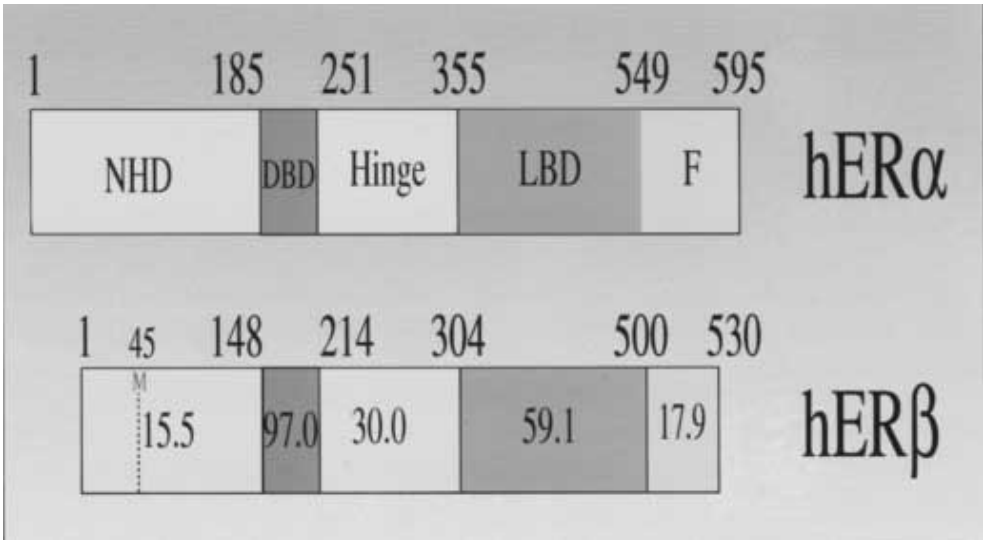


Figure 1.4 – Comparison of the primary structures of ERα and ERβ The figures above the receptor representations indicate the number of amino acids, with number 1 being the most N-terminal. The numbers within the ERβ receptor represent the degree of homology (%) between respective domains in the two receptors. From Gustafsson JA, 1999, Journal of Endocrinology, 163, 379-383 © Society for Endocrinology 1999. Reproduced by permission.[68]

A variety of non-steroidal ligands also bind the ER, but, these compounds produce distinct ER:ligand complex conformations[74] and are known as selective oestrogen receptor modulators (SERMs). They interact with the ER to produce tissue specific oestrogen agonist or antagonist responses. Tamoxifen is an ER agonist in bone and uterus but an antagonist in the breast and has been a safe and effective adjuvant endocrine therapy for breast cancer for almost 20 years. Raloxifene has oestrogen-like agonist effects in bone and cholesterol metabolism in ovariectomised (OVX) rats[75] and postmenopausal women.[76] However, raloxifene fails to produce oestrogen agonist effects on the uterus of OVX rats[75] or postmenopausal women[76] allowing raloxifene to completely antagonise oestrogen effects in the uterus. This lack of significant oestrogen agonist activity in the uterus distinguishes raloxifene

from other SERMs which are partial agonists in the uterus**.** The relative binding affinity of oestrogen to ERα is about 10 times higher than that of raloxifene in the ER-positive MCF-7 breast cancer cell line.[77] Tamoxifen binds equally to ERα and ERβ[78], while raloxifene binds with about 2-fold greater affinity to ERβ than ERα.[79] Therefore differences in binding do not account for the difference in the activity of tamoxifen and raloxifene with ERs. Structural studies have revealed that tamoxifen and raloxifene produce different conformations of ERα with raloxifene causing a greater increase in the mobility of Helix 12 of the LBD.[80] The resulting 3-dimensional structure determines which coactivators and/or corepressors are recruited to the promoter. Thus, the relative level of corepressors and coactivators in a particular tissue is a critical determinant of SERMs agonist/antagonist activity. The ERα/ERβ ratio varies between tissues and which isoforms predominates also affects SERM activity. Thus the physiological response to a particular SERM results from a combination of factors including its chemical structure and the cellular and promoter context in which the SERM acts.

1.5 Systemic effects of sex steroids on GH-IGF-1 axis

1.5.1 GH-IGF-1 axis during puberty

The striking increase in HV during puberty is under complex endocrine control. GH increases growth at puberty through the stimulation of IGF-1 production. During puberty the pulsatile secretion of GH increases (1.5- to 3- fold) along with a greater than 3-fold increase in serum IGF-1 levels. Peak IGF-1 levels occur at 14.5 years in girls and one year later in boys.[81] The rise in mean 24-hour GH levels results from an increase in the maximal GH secretory rate and also in the mass of GH per

secretory burst.[82;83] The increase in GH secretion during puberty shows a sexually dimorphic pattern that parallels the change in HV. In girls, an increase in circulating GH is seen relatively early in puberty at Tanner breast stage 2 with peak levels coinciding with Tanner breast stages 3-4. In boys, this increase in GH is seen later with the peak occurring at Tanner genital stage 4.[84] After secondary sexual development is complete, GH and IGF-1 levels fall to pre-pubertal levels in both sexes. The secretion of GH is mediated by two hypothalamic hormones: growth hormone releasing hormone (GHRH) and somatostatin. GHRH has a stimulatory effect whereas somatostatin has an inhibitory effect. These hypothalamic influences are tightly regulated by an integrated system of neural, metabolic and hormonal factors.[85] In animal studies, performed principally in rodents, sex steroids influence GH synthesis and secretion with effects on both the hypothalamus and anterior pituitary. In the neonatal period, sex steroids influence the number of GHRH neurons which will be present in the adult hypothalamus and also their response to post-pubertal steroids.[86] Post-pubertally, androgens modify hypothalamic somatostatin synthesis whereas oestrogens modify GHRH synthesis. In addition, both neonatal and post-pubertal steroids influence the secretory pulsatility of anterior pituitary hormone release by altering hypothalamic synaptic organisation.[86] At the level of the pituitary, sex steroids modify the response of somatotrophs to somatostatin.[86]

1.5.2 Effect of androgens

Dihydrotestosterone (DHT) and oxandrolone increase HV in boys with delayed puberty without any alteration of serum GH/IGF-1.[87-90] In contrast, testosterone, typically, increases HV in association with an increase in GH/IGF-1.[90;91] This

ability of testosterone to stimulate GH secretion is principally due to its conversion to oestrogen by aromatisation. This is supported by a study in pubertal boys who showed a reduction in GH and IGF-1 when treated with tamoxifen.[92] The non-aromatisable androgens (DHT and oxandrolone) increase HV independent of GH/IGF-1 suggesting that "pure androgens" may stimulate growth through other mechanisms, possibly via a direct action on the androgen receptor within the growth plate cartilage.[93]

1.5.3 Effect of oestrogens

The stimulation of growth by oestrogen is largely dependent on pituitary GH and is mediated via the oestrogen receptors, ERα and ERβ, which are expressed in the anterior pituitary as well as in the hypothalamus. GH and oestrogen levels show positive correlations in pre-pubertal girls and boys.[94;95] Endogenous oestrogen in peri-pubertal children increases GH sensitivity.[96] Priming with oestrogen for GH-stimulation testing has been shown to augment GH release in normal adolescents.[97] Furthermore, GH secretion is reduced when oestrogen signalling is blocked.[92;98] This tight relationship between oestrogen and GH status is further demonstrated by the strong correlation between oestrogen and GH concentrations throughout normal female puberty.[99] GH levels are higher in women compared to men.[100;101] As boys and girls with IGF-1 deficiency (Laron syndrome) do not have a discernible pubertal growth spurt[102] a major part of the stimulation of growth by oestrogen is through the GH/IGF-1 axis. The effect of exogenous oestrogen depends on its form. If oestrogen treatment is administered orally (for hormone replacement therapy), large, supra-physiological doses are required as oestrogen is actively metabolised by the hepatic

cytochrome system. This supra-physiological concentration in the portal circulation perturbs many aspects of hepatic function. The liver is the major site of GH-regulated metabolism and the main source of IGF-1.[103] Oral oestrogen administration leads to increased circulating GH levels and a reduction in IGF-1 production. The high oestrogen concentration in the portal circulation impairs hepatic IGF-1 production and increases the concentrations of GH binding protein which binds to GH and thereby blunts its action. There is also stimulation of the synthesis of angiotensinogen, clotting factors, lipoproteins and the binding proteins for several steroid hormones. These negative metabolic sequelae are not seen with transdermal administration.[104]

1.6 Local effects of sex steroids on the growth plate

1.6.1 Effect of growth hormone

In addition to its systemic effects GH also has a direct action at the level of the growth plate. GH enhances the recruitment of resting zone chondrocytes and local IGF-1 production in the growth plate.[105-108] The relative contribution of systemic versus local IGF-1 on longitudinal growth remains unclear. However, they both appear to have an impact on longitudinal growth (in mice at least). The liver-specific IGF-1 gene-deletion mouse model (LID) has normal growth but circulating IGF-1 levels are only reduced by 75%. When the LID mice were crossed with the acid labile substance (ALS) gene-deleted mice (ALSKO) the LID/ALSKO mice had a further reduction in circulating IGF-1 levels, to 85-90% of normal levels, and showed early postnatal growth retardation.[109] But tissue IGF-1 may still play a role since the total IGF-1 knockout mice show more marked growth retardation.

However, basal IGF-1 production by growth plate chondrocytes is reported to be minimal.[110;111]

Sex steroids are likely to have a direct action on chondrocytes since the androgen receptor (AR) and both ERα and ERβ, have been demonstrated in growth plate tissue at the mRNA and protein level in several species, including rat, rabbit and human.[93]

1.6.2 Effect of androgens

The AR has been demonstrated in all layers of the human growth plate at different ages with no significant gender variation.[112-115] Several studies support a direct stimulatory effect of androgens on growth plate cartilage. *In vitro* studies have shown that DHT regulates proliferation and differentiation of cultured human epiphyseal chondrocytes, probably by promoting local IGF-1 synthesis and increasing IGF-1 receptor expression.[116] The sex-specific response of rat costochondral growth zone chondrocytes to testosterone requires its further metabolism to DHT and this DHT effect in the male growth plate is maturation-state dependent.[117] Similarly *in vivo,* a non-specific ER blocker (Faslodex) did not prevent the advancement of bone maturation in mice treated with testosterone.[118] Also, direct injections of testosterone into the rat tibial epiphyseal growth plate increased growth plate width without alteration of IGF-1 production.[119] Furthermore, DHT stimulates longitudinal bone growth in OVX rats[120] and testosterone increases growth plate width in castrated, hypophysectomised male rats.[121]

1.6.3 Effect of oestrogens

Classical ERs have been demonstrated in all maturational zones of the human growth plate during development and puberty. However, there is conflicting evidence from *in vitro* studies on the effect of oestrogen on chondrocyte proliferation and differentiation.[122-129] Some of the discrepancies may be explained by the demonstrated ability of chondrocytes to synthesise oestrogen themselves.[129-131] Activation of the ERs by locally produced oestrogen could minimise or eliminate the effect of exogenous oestrogen. To complicate matters further, oestrogen signalling can also occur via non-genomic pathways (Figure 1.5). In the genomic or classical pathway, oestrogen or SERMs bind to the ER by binding to ERE regulatory sequences in target genes and recruiting coregulatory proteins such as coactivators as described earlier (section 1.4). Non-genomic or rapid effects of oestrogen may also occur through the ER located in or adjacent to the plasma membrane which may necessitate the presence of adaptor proteins which target the ER to the membrane. Activation of the membrane ER leads to a rapid change in cellular signalling molecules and stimulation of kinase activity, which may in sequence affect transcription. Finally, other non-ER membrane-associated oestrogen-binding proteins (EBPs) may also generate an intracellular response. The new oestrogen receptor GPR30 (G Protein-coupled Receptor 30) is expressed in the human growth plate and down-regulated during pubertal progression.[132] This new G-protein coupled receptor may explain some of the previous “non-genomic” actions of oestrogen and is also involved in regulating calcium levels.[133] Furthermore, some of the rapid responses to oestrogen, including activation of protein kinase C (PKC), are limited to cells from female animals.[126;134;135] *In vivo*, oestrogen inhibits chondrocyte cell

division in the proliferating zone of the rat growth plate.[136-138] The age-related decrease in size of the hypertrophic chondrocytes[139;140] is enhanced by oestrogen.[138] In rats, the withdrawal of oestrogen by OVX stimulates longitudinal bone growth.[141] This increase in bone length is associated with increased chondrocyte proliferation[131], growth plate width[131] and IGF-1 production.[120;131] Similarly in humans, tall girls treated with high dose oestrogen display a rapid reduction in HV but have only a modest decrease in serum IGF-1 suggesting a direct, non-GH-dependent, effect of oestrogen.[142] Moreover, children with precocious puberty and GH deficiency can have a pubertal growth spurt.[143]

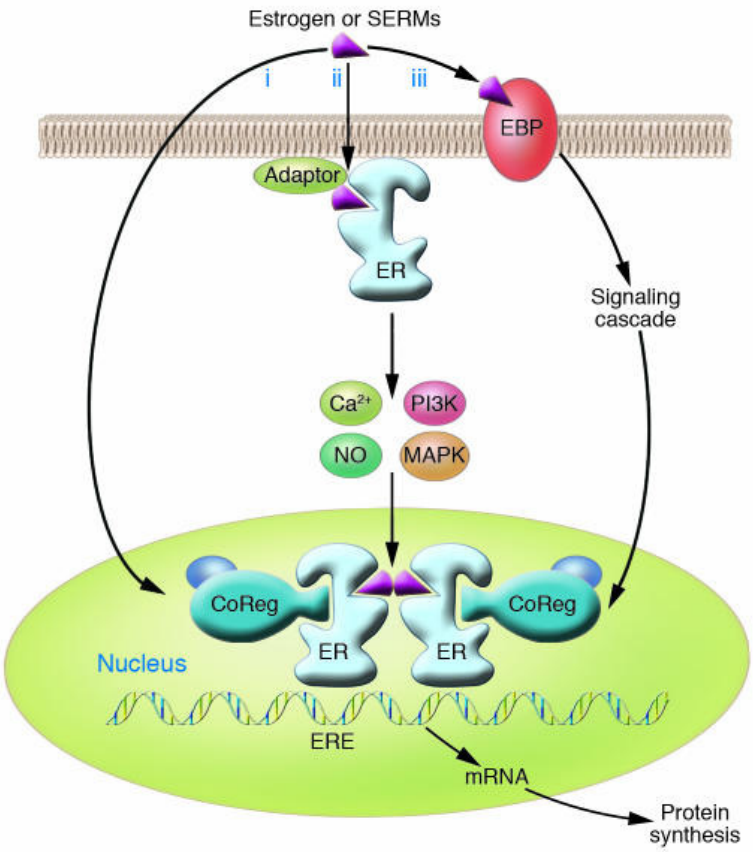


Figure 1.5 – Models of oestrogen action From Deroo *et al*, 2006.[144] Key: Genomic pathway, i; Non-genomic pathway via ii) membrane-associated ER or iii) non-ER membrane-associated oestrogen-binding proteins (EBPs); PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; NO, nitric oxide, CoReg, coregulator; ERE, oestrogen response element.

1.7 Lessons learned from ER knockouts in mouse and man

Meta-analysis of longitudinal bone length in these knockouts has shown that the role played by the ERs in association with bone length may depend on sex and age (Tables 1.1 and 1.2).[145-158] ERβ has an inhibitory effect on longitudinal bone growth only in adult female mice.[146;147;151] It has been hypothesized that this inhibitory action is only seen in the presence of elevated serum oestrogen. This is supported by the finding that adult female ERβ knockout (BERKO) mice have the highest serum oestrogen levels[152] during which period the inhibitory effect of ERβ is exclusively seen. Furthermore, female ERα knockout (ERKO) mice have shorter bones where ERβ is present compared to female ERα/ERβ knockout (DERKO) where both ERs are absent.[146;147] Both of these strains have markedly elevated serum oestrogen levels due to an inactive feedback loop.[146]

Knocking out ERβ does not affect femur length in male mice of any age.[151;152;156] Finally, in the presence of high levels of oestrogen, ERβ induces fusion of the growth plate in older female mice.[147;148] Knocking out ERα does not have an effect on the growth of the younger animal but may inhibit growth in the older mouse.[145-150;156;157] There is no evidence of sexual dimorphic effects. The patient lacking ERα (hERKO) carries a mutation at residue 157 in the protein, which would allow the short, 46-kDa spliced form of ERα to be synthesized.[159] This is one possible explanation for discordance in phenotype between mice and man.

Table 1.1 – Characteristics of female murine knockout models

Knock-out Model	Sex steroid levels	Serum IGF-1	Body weight	Axial growth (crown-rump length)	GP fusion at 18-mo	Appendicular growth (femur length)			Ref.
						A (30-60d)	B (70-240d)	C (>/=1y)	
ERKO*	-	↓ 23%	↑	↔	-	↔	↓		(145)
	E2 ↑ 550% T ↑	↓	↑	↔	-	↔	↓ ERKO < DERKO < WT < BERKO		(146)
	-	-	-	↔	Fused (F & Tib)		↓ ERKO < DERKO < WT < BERKO	↓ ERKO < DERKO < WT = BERKO	(147)
					Fused (Tib)			↓	(148)
ERKO‡	E2 ↑ 10x T ↑	-	↔	-	-		↔		(149)
							↔		(150)
BERKO*	-	-	↑	-	-	↔	↑	↔	(151;152)
	E2 ↔ T ↔	↑	↔	↑ 140d (3%)	-	↑ 60d	↑		(146)
	-	-		↑ 4-mo ↔ 18-mo	Unfused		↑	↔	(147)
BERKO‡	E2 ↔ T ↔	-	↔	-	-		↔		(149)
DERKO*	E2 ↑ 251% T ↑	↔	↑	↔	-	↔ 60d	↓		(146)
	-	-	-	↑ 9-mo, 18-mo	Unfused		↓	↓	(147)
DERKO‡	E2 ↔ T ↔	-	↔	-	-		↔		(149)
ArKO	T ↑ 10x	-	-	-	-	-	-	-	(153)
	T ↑	-	-	-	-		↔		(154)
	-	↑	↑	-	-	↓	↔		(155)

ERKO, ERα knockout; BERKO, ERβ knockout; DERKO, ERα & ERβ knockout; ArKO, Aromatase knockout; E2, oestradiol; T, testosterone; GP, growth plate; F, femoral; Tib, tibial; WT, wild type. *Mouse strain generated by Dr Oliver Smithies (ERKO have remaining ERα activity [AF-2] whereas BERKO have no ERβ activity); ‡Mouse strain generated by Dr Pierre Chambon (ERKO have no ERα activity whereas BERKO have remaining ERβ activity). d, days; mo, months; y, years; %, percentage change.

Table 1.2 – Characteristics of male murine knockout models

Knock-out Model	Sex steroid levels	Serum IGF-1	Body weight	Axial growth (crown-rump length)	Appendicular growth (femur length)			Ref.
					A (30-60d)	B (70-240d)	C (>/=1y)	
ERKO*	-	↓	↓	↓	↔	↓ ERKO = DERKO < WT = BERKO		(156)
	T ↑	-	↔	-		↓		(157)
							↓	(148)
ERKO‡	T ↑4x E2 ↔	-	↔	-		↔		(149)
	-	-	-	-		↓		(150)
BERKO*	-	-	↔	-	↔	↔	↔	(151;152)
	-	↔	↔	↔	↔	↔		(156)
BERKO‡	E2 ↔ T ↔	-	↔	-		↔		(149)
DERKO*	-	↓	↓	↓	↓ ERKO = DERKO < WT = BERKO	↓ ERKO = DERKO < WT = BERKO		(156)
DERKO‡	T ↑4x E2 ↔	-	-	-		↓ DERKO < WT = BERKO = ERKO		(149)
ArKO	T ↑	-	-	-	-			(153)
	T ↔	-	-	-		↓		(154)
	-	↓	↓	-	↔	↓		(155)
ANDRKO	T ↓	-	-	-		↑		(158)
	-	-	-	-		↑		(148)

ERKO, ERα knockout; BERKO, ERβ knockout; DERKO, ERα & ERβ knockout; ArKO, Aromatase knockout; ANDRKO, AR knockout; E2; oestradiol; T, testosterone; WT, wild type. *Mouse strain generated by Dr Oliver Smithies (ERKO have remaining ERα activity [AF-2] whereas BERKO have no ERβ activity); ‡Mouse strain generated by Dr Pierre Chambon (ERKO have no ERα activity whereas BERKO have remaining ERβ activity). d, days; mo, months; y, years.

1.8 Aims and Strategy

1.8.1 Oestrogen action at the level of the growth plate

The aim of the laboratory component of my thesis is to investigate the effect of oestradiol (E2) at the level of the growth plate chondrocyte. The ATDC5 chondrocyte cell line, an *in vitro* model of chondrocyte proliferation, will be studied for the first part of the project. This murine model is characterised by chondrocyte that progresses through the maturational stages of chondrogenesis and differentiation. Thereafter, the more physiological models of murine growth; primary chondrocytes and organ cultures, will be utilised in an attempt to gain a better understanding of oestradiol action at the level of the growth plate. Finally, in the absence of readily available physiological models of human growth, the effects of E2 on the human C28/I2 cell line will be investigated. E2 and related chemicals, in particular those which alter the function of the classical ERs, will be employed to try to tease out the different functions of each ER in the growth plate. My specific aims are:

a) To confirm the presence of the oestrogen receptors, ERα and ERβ in chondrocytes

b) To characterise the effects of E2 on the murine ATDC5 cell line

c) To characterise the effects of E2 on physiological models of murine growth (primary chondrocytes and foetal metatarsal culture system)

d) To characterise the effects of E2 on the human C28/I2 cell line

1.8.2 Effect of oral oestrogen on longitudinal growth in girls

The aim of the clinical component of my thesis is to investigate the effect of oral oestrogen therapy on linear growth in girls with absent puberty due to primary ovarian insufficiency. A retrospective review of girls with primary ovarian insufficiency treated at a tertiary endocrinology clinic at the Royal Hospital for Sick Children, Glasgow over an eleven year period will be performed. My specific objectives are:

a) To identify the incidence and prevalence of primary ovarian insufficiency within the West of Scotland

b) To investigate the aetiology of primary ovarian insufficiency in a tertiary referral centre

c) To describe oestrogen treatment prescribed and its effect on height velocity, bone maturation and final height

The second clinical study will examine the effect of oestrogen on linear growth in a cohort of girls with TS who received a standardised course of oral ethinylestradiol (EE2) for pubertal induction and a standard dose of GH therapy as part of the UK Turner Study. My specific aims are:

1) To assess the effect of a standardised oral oestrogen regime on HV, bony maturation and FH in girls with TS treated with a standard dose of GH therapy

2) To identify what dose of oestrogen is associated with i) peak height velocity and i) bony fusion

Chapter 2

Effect of oestradiol on murine ATDC5 chondrocyte cell line

Chapter Contents

2.1 Introduction
2.2 Hypothesis
2.3 Aims
2.4 Materials and Methods
2.4.1 Materials
2.4.2 Cell culture
2.4.2.1 Preparation of cell culture reagents
2.4.2.2 Maintenance and differentiation of ATDC5 cells
2.4.2.3 Freezing and thawing cells
2.4.3 RNA methods
2.4.3.1 Isolation of total RNA from cells
2.4.3.2 Reverse transcription
2.4.3.3 Polymerase chain reaction
2.4.4 Protein concentration determination
2.4.5 Cell proliferation and differentiation assays
2.4.5.1 [^{3}H]Thymidine Incorporation assay
2.4.5.2 Alcian Blue staining of cell monolayer
2.4.5.3 Alkaline phosphatase assay
2.4.6 Apoptosis assay
2.4.7 Statistical analysis
2.5 Results
2.5.1 Expression of classical oestrogen receptors in ATDC5 cells
2.5.2 Effect of E2 on cell proliferation during chondrogenesis and terminal differentiation
2.5.3 Effect of E2 on ALP Activity and proteoglycan production
2.5.4 Effect of co-incubating E2 and pro-inflammatory cytokines on chondrocyte proliferation and apoptosis
2.6 Discussion
2.7 Conclusions

2.1 Introduction

A direct action of E2 on chondrocytes is suggested by the finding of both classical ERs in all maturational zones of the human growth plate during development and puberty. However, *in vitro* studies examining the role of E2 on chondrocyte proliferation and differentiation have conflicting results (Table 2.1). To date stimulation of epiphyseal chondrocyte proliferation has been described[123;124] as well as an inhibition[125-127] and more recently no effect.[128;129] E2 has also been reported to stimulate incorporation of radioactive sulfate-35 (^{35}S) into proteoglycans in rabbit and human chondrocytes implying increased chondrocyte differentiation.[160;161]

Our group has previously demonstrated that the pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα), exert diverse inhibitory effects on ATDC5 chondrocyte dynamics and metatarsal growth.[162] Exposure to TNFα and IL-1β led to a reduction in the proliferation of ATDC5 chondrocytes and a decrease in the expression of aggrecan and collagen types II and X. Treatment with TNFα or IL-1β also led to increased apoptosis of ATDC5 cells as determined by caspase-3 activity and was significantly greater in cells treated with TNFα compared to IL-1β.[162]

E2 has been reported to play a protective role against cytokine-induced inflammation in a mouse model of multiple sclerosis (experimental autoimmune encephalomyelitis). Treatment with low-dose E2 has been shown to significantly inhibit the clinical signs and histopathological lesions of experimental autoimmune encephalomyelitis and is being used in clinical trials to treat multiple sclerosis.[163]

This protective effect of E2 is mediated through ERα.[163] A similar effect is seen in human vascular endothelial cells where E2 enhances growth and reduces TNFα induced apoptosis.[164] Similarly, in a mouse model of inherited glaucoma E2 treatment protected retinal ganglion cells through inhibition of ganglion cell apoptosis.[165]

The established murine ATDC5 chondrogenic cell line allows the characterisation and comparison of effects of different E2 concentrations on specific maturational stages of growth plate chondrocyte maturation including cell proliferation and differentiation. It has been shown to undergo the temporal sequence of events that occur during longitudinal bone growth *in vivo* and thereby provides an excellent model to study the molecular mechanisms underlying the regulation of growth plate maturation and endochondral bone formation. [166-168]

Table 2.1 – Effects of E2 on chondrocytes *in vitro*: summary of published data

Chondrocyte					E2		FBS	Effects of E2	Ref.
Cell type	**Source**	**Site**	**Age**	**Sex**	**Dose**	**Days**			
HCS-2/8 cell line	**Human**	Human ChondroSarcoma Humeral bone	Adult 72 y Japanese	Male	10^{-10} to 10^{-6}M 0.1 nM to 1µM	0-12 d	0.2/1 & 5% CTS	**Cell number**: No effect **Cell proliferation**: No effect	(129)
Primary cells	**Rat**	Costochondral cartilage 2 subpopulations (resting zone and growth zone cells)	1-mo	Both	10^{-10} to 10^{-7} M	24 h	1% FBS	Growth zone cells Males: no effect but Females: - **Cell proliferation**: ↓ 10^{-9}-10^{-7} M - **ALP activity**: ↑ 10^{-9}-10^{-8}M# - **Sulfate incorporation**: ↑ 10^{-9}-10^{-8} M#	(169)
Primary cells	**Human**	Femoral chondyles/tibial plateaus **Articular** cartilage	Adults 16 y-39 y	Both 3F, 3M at autopsy	10^{-11} to 10^{-7} M	24 h	1% FBS	Males: no effect but Females: - **Cell proliferation**: ↑ 10^{-9}-10^{-7} M - **Sulfate incorporation:** ↑ 10^{-10}-10^{-7} M - **ALP activity:** ↑ 10^{-10}-10^{-7} M - **PKC activity:** ↑ 10^{-9}-10^{-7} M* *Rapid dose-dep blocked by chelerythrine (PKC inhibitor)	(170)
Primary cells	**Cow**	Growth plate cells isolated from femora, tibiae & humeri – separated 5 subpopulations	Fetal 24-33 wks	Both 4F, 3M + 1?	10^{-13} to 10^{-7} M	7-21 d	0% SF	**Cell proliferation:** No effect **Cell viability:** No effect **Cell differentiation:** Only at 10^{-7} M - ALP activity/matrix calcification: ↑ - Type X Collagen synthesis : ↑	(128)
ATDC5 cell line	**Mouse**	Pre-chondrogenic Teratocarcinoma		Male	10^{-10} to 10^{-6} M	3 d	0% SF	**Cell differentiation:** - ALP activity: No effect - ALP activity with BMP-6: No change (11fold ↑)	(171)
Primary cells	**Human**	Iliac crest "growing cartilage"	Children	NS	10^{-12} to 10^{-6} M	4 d	1% FBS	**Cell proliferation:** - 10^{-12} M: No effect; 10^{-10} M: ↑ - 10^{-8}-10^{-6} M: ↓	(172)
Primary cells	**Rat**	Costochondral cartilage 2 subpopulations (resting zone and growth zone cells)	1-mo	Both	10^{-12} to 10^{-6} M	12/24/ 48h	10% FBS	Growth zone cells Males: no effect but Females: - **Cell number**: ↓ 10^{-9}-10^{-8} M - **Cell proliferation**: ↓ 10^{-9}-10^{-6}M (12-48 h) - **ALP activity**: ↑ 10^{-9}-10^{-7} M biphasic; only occurred in presence of FBS	(126)

Chondrocyte					E2		FBS	Effects of E2	Ref.
Cell type	**Source**	**Site**	**Age**	**Sex**	**Dose**	**Days**			
Primary cells	**Human**	Epiphyseal cartilage chondrocytes isolated from long bones, vertebra or nasal cartilage	Children 4-mo to 7y	Both	10^{-11} to 10^{-8} M	20 h	0% FBS	Only female chondrocytes treated with E2 **[^{35}S] Sulfate incorporation:** - 4-12 months: No effect - 2-7 y: ↑ 10^{-11}-10^{-8} M	(160)
Primary cells	**Rat**	Epiphyseal cartilage	Young rats	Both	$3x10^{-8}$ M	24 h	10% PR	**CK specific activity:** ↑ CK (blocked by Tamoxifen) **Cell proliferation:** ↑ [^{3}H]thymidine	(123)
Primary cells	**Rat**	Epiphyseal cartilage (zones wider in Vitamin D deficiency)	16-18d Vitamin D deficient rats	NS	$3x10^{-9}$-10^{-8} M	24 h	10% PR	**CK specific activity:** ↑ CK (blocked by Tamoxifen) **Cell proliferation:** ↑ [^{3}H]thymidine 40%	(122)
Primary cells	**Rabbit**	Epiphyseal **articular** chondrocytes from long bone cartilage	Postnatal 2-80 d	Both	10^{-12} to 10^{-8} M	20 h	0% FBS	**[^{35}S] Sulfate incorporation:** Highest basal incorporation in foetal cells. After birth ↑ 0-25d then ↓ (similar in male/female cells) **E2 at 10^{-8} M** Female Male - 3-5 d: 26% ↑ 18% ↑ - 5-30 d: No effect* No effect* - 35-62 d: 62% ↑ 20-32% ↑ - 62-75 d: No effect No effect *Corresponded to max basal incorporation at 10-30 d **Dose response curve** Female Male - 10^{-12} M: No effect No effect - 10^{-11}-10^{-9} M: ↑ No effect - 10^{-8} M: ↑ ↑	(161)
Primary cells	**Rabbit**	Epiphyseal **articular** chondrocytes from long bones	Foetal 20 d (Born ~22 d)	Mixture	10^{-8} M	20 h	0% FBS	**[^{35}S] Sulfate incorporation:** Foetal cells (pooled male and female cells): ↑ 79%	(161)

CTS, charcoal treated serum; SF, serum free; FBS, foetal bovine serum; PR, phenol red; BMP-6, bone morphogenic protein-6; PKC, protein kinase C; NS, not specified; h, hours; d, days; wks, weeks; mo, months; y, years; F, female; M, male.

2.2 Hypothesis

It was hypothesised that E2 would have a concentration-dependent effect on chondrocyte proliferation with an increase at low concentrations and a decrease at higher concentrations. Secondly it was hypothesised that E2 would attenuate the effects of cytokines on the ATDC5 chondrocytes and thereby play a protective role against cytokine-induced inflammation.

2.3 Aims

I. Confirm the presence of the classical oestrogen receptors, ERα and ERβ by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in proliferating and terminally differentiating ATDC5 cells

II. Analyse the effect of different E2 concentrations on chondrocyte proliferation in ATDC5 cells during chondrogenesis and terminal differentiation

III. Analyse the effect of E2 on alkaline phosphatase activity and proteoglycan production of ATDC5 cells during terminal differentiation

IV. Compare the effect of co-incubating E2 and cytokines, IL-1β or TNFα, with cytokines alone on chondrocyte proliferation and apoptosis in ATDC5 cells

2.4 Materials and Methods

2.4.1 Materials

All chemicals were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated. PCR oligonucleotide primers were purchased from MWG Biotech (Ebersberg, Germany). Water soluble cyclodextrin-encapsulated 17β-oestradiol (Sigma) was used for all *in vitro* experiments.

2.4.2 Cell culture

2.4.2.1 Preparation of cell culture reagents

Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM: F12), containing 4500g/L glucose and L-glutamine, was purchased from Invitrogen, (Invitrogen, Paisley, UK). The DMEM: F12 was phenol red-free as phenol red has been shown to exhibit oestrogenic activity.[173] All tissue culture reagents were prepared in a sterile category 2 hood. The maintenance medium of DMEM: F12 (Invitrogen) was supplemented with 5% heat-inactivated foetal bovine serum (FBS; Invitrogen), 10µg/ml human transferrin, 3 x 10^{-8} M sodium selenite, sodium pyruvate (1mM; Invitrogen) and gentamicin (50µg/ml; Invitrogen). The differentiation medium consisted of maintenance medium supplemented with insulin (10µg/ml). All media was filter-sterilised through a 0.22µM filter and stored at 4°C.

2.4.2.2 Maintenance and differentiation of ATDC5 cells

The ATDC5 chondrocyte cell line was obtained from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al.*[166] Cells were maintained in 175cm^2 tissue culture flasks (Greiner Bio-One Limited, Stonehouse, Gloucestershire,

UK) at a density of 250,000 cells/flask in maintenance medium. For individual experiments semi-confluent cultures were passaged with trypsin ethylenediaminetetraacetic acid (EDTA) and cultured (day 0) at a density of 6,000 cells/cm^2 in differentiation medium in multi-well plates (Costar, High Wycombe, UK). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air and the medium was changed every second or third day after reaching confluency (day 6).

2.4.2.3 Freezing and thawing cells

To freeze cells a monolayer was stripped with trypsin EDTA and counted using a haemocytometer. The cells were then centrifuged at 2000 revolutions per minute (rpm) for 5 minutes and resuspended in the appropriate volume of cell freezing buffer to give a cell concentration of 2-4 x 10^6 cells per ml. The cells were stored at a temperature of -80°C for 4-7 days within a cryovial (Corning, Surrey, UK) and then moved to -150°C for longer term storage. Frozen cells were thawed at 37°C and added drop wise to 10 ml of culture medium. The cell suspension was then mixed and spun at 2000 rpm for 5 minutes to remove the dimethyl sulfoxide (DMSO) which is a major component of the freezing buffer. The cell pellet was resuspended in culture medium and transferred to a 175cm^2 tissue culture flask (Greiner Bio-One Limited).

2.4.3 RNA Methods

2.4.3.1 Isolation of total RNA from cells

To isolate ribonucleic acid (RNA) from a cell monolayer the cells were scraped directly in Ultraspec™ RNA isolation reagent (Biotecx, Houston, USA; 1 millilitre per 25 cm^2) and transferred to a nuclease-free universal container. The cells were homogenised with an electric homogeniser in five 10-second bursts. Between each burst the container was returned to ice to prevent the build up of heat. The homogenised lysate was then passed through a 2-mililitre syringe and 25-gauge needle repeatedly to create a uniform lysate to which chloroform (200µl per millilitre) was added. After extraction with chloroform, RNA in the aqueous phase was purified with isopropanolol and bound to 50µl RNA Tack resin, before washing with 75% ethanol. The RNA was then eluted from the resin pellet in 100µl nuclease free water to which ribonuclease (RNase) inhibitors (Promega, Southampton, UK) and deoxyribonuclease (DNase) to degrade any contaminating DNA (Ambion, Warrington, UK) were added. For each sample the 260/280 ratio was determined and the samples diluted to a concentration of 50ng/µl and stored at -70°C until required.

2.4.3.2 Reverse transcription

Reverse transcription PCR allows the production of complementary deoxyribonucleic acid (cDNA) from any RNA template. Aliquots of 500ng RNA (or an equivalent volume of water as a control) were reverse transcribed in 20µl reactions with 200ng random hexamers and 200U Superscript II reverse transcriptase using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The following PCR cycle was used: 25°C for 10 minutes, 42°C for 50 minutes and 70°C

for 15 minutes for annealing, elongation and termination respectively. The cDNA was stored at -20°C until required for analysis.

2.4.3.3 Polymerase chain reaction

E2 was added to the cells during both chondrogenesis (days 6-10) and terminal differentiation phases (days 13-17) at a final concentration of 10^{-6} M. Cells were grown in the presence of E2 for 4 days, at which point RNA was extracted, reverse transcribed and analysed for ERα and ERβ gene expression by semi-quantitative RT-PCR.[174-176] PCR was performed in 20µl reactions containing cDNA equivalent to 10ng RNA and 200nM gene-specific primers in 11.1x PCR buffer (Table 2.1).[177] The cycling profile was 1 minute at 92°C (first cycle 2 minutes), 1 minute at 55°C, and 1 minute at 70°C. The number of cycles performed was titrated to ensure that the reactions were in the exponential phase. The reaction products were run on 1.5% agarose gels in the presence of ethidium bromide (250µg/l), and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The protein Classic 18S (Ambion) was used as an internal standard (Table 2.2).

Table 2.2 – Primer sequences and product sizes for murine ERα and ERβ genes analysed by RT-PCR

Gene	Primer sequence Forward primer Reverse primer	Cycles	Product size (base pairs)
Classic 18S	Unknown, purchased commercially from Ambion	20	488
ERα (murine)	5' - GGA GAA TGT TGA AGC ACA AGC - 3' 5' - GGA GCA AAC AGG AGC TTC - 3'	35	433
ERβ (murine)	5' - GGA CAC CTC TCT CCT TTA GC - 3' 5' - CCT TAC ATC CTT CAC AGG ACC - 3'	35	262

2.4.4 Protein concentration determination

The concentration of protein was determined using the BioRad protein assay kit (Bio-Rad Laboraties Ltd) which employs gamma-globulin as a standard and is based on the method described by Bradford.[178] Nine standards of gamma globulin were prepared ranging from 10µg/millilitre to 90µg/millilitre. 160µl of each standard was pipetted in duplicate into a 96 well plate along with a buffer blank. The protein that was to be measured was diluted in the same buffer as gamma-globulin and added to the wells in duplicate. 40µl of dye reagent concentrate was added to each well and mixed. The plate was incubated at room temperature for 5 minutes and the absorbance read at 595 nm by a microplate reader. The absorbencies of the samples were compared to a standard curve generated from the absorbencies from the standards.

2.4.5 Cell proliferation and differentiation assays

2.4.5.1 [^{3}H]Thymidine incorporation assay

E2 was added to cells in 24-well plates at day 7 and day 14 at a final concentration of 10^{-6} M, 10^{-8} M, 10^{-10} M and compared with control cultures which received vehicle (sterile distilled water) only and incubated for a 24 hour period. Murine TNFα (Autogen Bioclear, Calne, Wiltshire, UK) and IL-1β (Autogen Bioclear) were added at a final concentration of 10ng/millilitre to cells cultured in 24-well plates on day 16 in the presence and absence of E2 at a final concentration of 10^{-6} M. Control cells received vehicle only. The cells were then incubated for a 24 hour period. The rate of chondrocyte proliferation was assessed by incubating the cells with 0.2µCi/millilitre [^{3}H]thymidine (37MBq/ml; Amersham Pharmacia Biotech, Little Chalfont,

Buckinghamshire, UK) for the last 2 hours of the incubation period and measuring the amount of radioactivity incorporated into trichloroacetic acid insoluble precipitates.[176]

2.4.5.2 Alcian Blue staining of cell monolayer

Proteoglycan synthesis was assessed by staining with Alcian Blue.[167] The ATDC5 cells were washed twice with phosphate buffered saline (PBS), fixed in 95% methanol for 20 minutes and stained with 1% Alcian Blue 8GX in 0.1M hydrochloric acid (HCl) overnight and rinsed with distilled water. Alcian Blue stained cultures were extracted with 6M Guanidine-HCl for 6 hours at room temperature and the optical density was measured at 630nm using a Jenway 6105 spectrophotometer.[176] Blanks for spectrophotometry used 6M Guanidine-HCl only.

2.4.5.3 Alkaline phosphatase assay

E2 was added to ATDC5 cells at day 13 at a final concentration of 10^{-6} M and the cells were then incubated for 7 days. For analysis of alkaline phosphatase (ALP) activity, cells were rinsed with PBS and lysed with 0.9% NaCl and 0.2% Triton-X 100 and centrifuged at 12000g for 15 minutes at 4°C. The supernatant was assayed for protein content and ALP activity as a measure of cell number and chondrocyte differentiation, respectively. The protein content of the supernatant was measured as described in 2.4.3. Enzyme activity was determined by measuring the cleavage of 10mM p-nitrophenyl phosphate (pNPP) at 410nm. Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein.[176]

2.4.6 Apoptosis assay

Apoptosis was assessed by measuring the cytoplasmic histone-associated DNA fragments (mono- and oligo- nucleosomes) by photometric enzyme-immunoassay (cell death detection ELISA^{PLUS}; Roche Applied Science, Roche Diagnostics, Burgess Hill, UK). IL-1β and TNFα were added to the cells at a final concentration of 10ng/ml in the presence or absence of E2 at a final concentration of 10^{-6} M and compared with control cells. Apoptosis was measured following the manufacturers' protocol in cells cultured in 96-well plates over a 24 hour period starting on day 16.

2.4.7 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA). All data are expressed as the mean ± SEM of at least six replicates within each experiment, and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software package (SPSS Inc., Chicago, USA; version 15). Significance was determined as $P < 0.05$.

2.5 Results

2.5.1 Expression of classical oestrogen receptors in ATDC5 cells

ATDC5 murine chondrocytes expressed ERα and ERβ during both chondrogenesis and terminal differentiation (Figure 2.1). The expression was unaffected by exposure of chondrocytes to E2, at a final concentration of 10^{-6} M for 4 days.

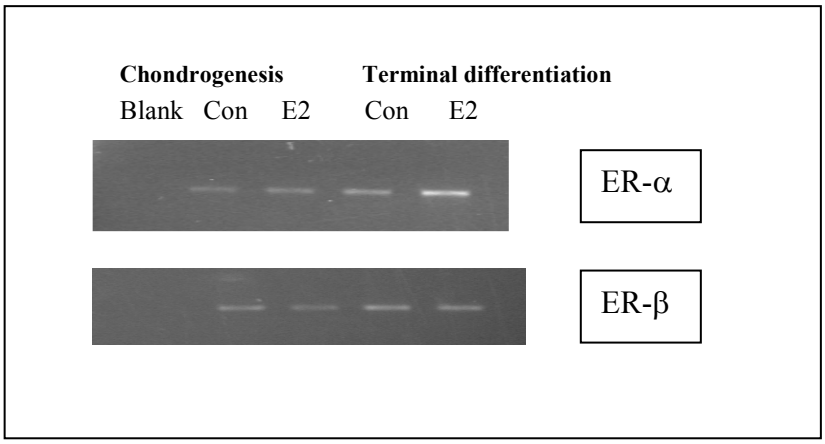
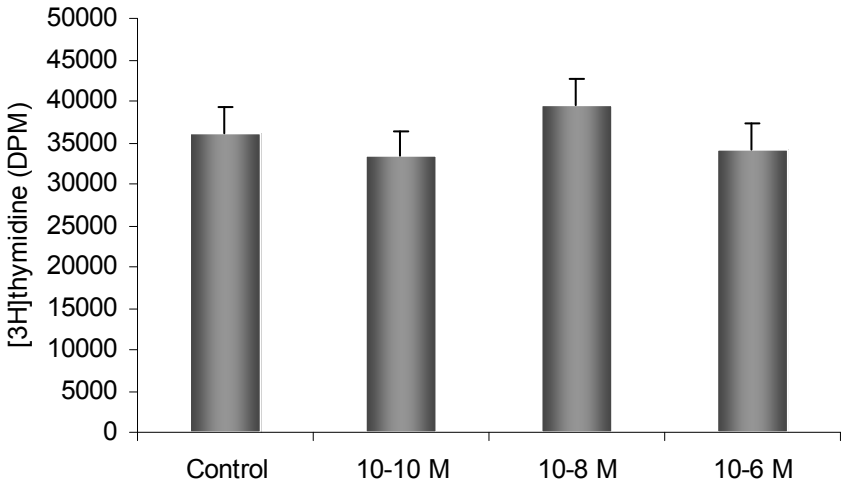


Figure 2.1 – ERα and ERβ expression in ATDC5 cells as detected by semi-quantitative RT-PCR during chondrogenesis and terminal differentiation in cells treated with E2 (10^{-6} M) or in control cells (Con)

2.5.2 Effect of E2 on cell proliferation during chondrogenesis and terminal differentiation

ATDC5 chondrocyte proliferation during chondrogenesis or terminal differentiation was unaffected by exposure to E2 for 24 hours, at a final concentration of 10^{-10} M, 10^{-8} M and 10^{-6} M, as analysed with [^{3}H]thymidine incorporation on day 8 and day 15 (Figure 2.2A, Figure 2.2B respectively). However, as expected there was much lower rates of proliferation in the terminally differentiated cells.

A



B

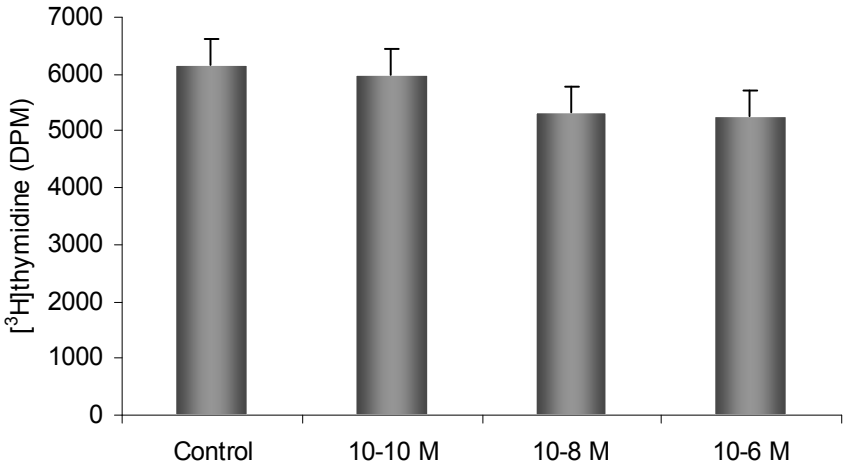


Figure 2.2 – Effect of E2 on ATDC5 proliferation as assessed by [^{3}H]thymidine uptake after 24 hours exposure. (A) ATDC5 proliferation during chondrogenesis. (B) ATDC5 proliferation during terminal differentiation. Data are expressed as means ± SEM (n = 6).

I therefore found that exogenous E2, at physiological and pharmacological concentrations (10^{-10} to 10^{-6} M), had no effect on ATDC5 chondrocyte proliferation. Whilst undertaking this project Chagin *et al* published their findings in the human HCS-2/8 chondrocyte cell line (Table 2.1).[129] The authors reported that exogenous E2 (10^{-10} to 10^{-6} M) had no effect on cell number or thymidine incorporation in HCS-2/8 chondrocytes. However, the chondrocytes themselves were shown to produce E2 which could be of physiological importance. The production of E2 was dependent on the stage of chondrocyte differentiation, being high in proliferative (non-confluent) cells and gradually decreasing in more differentiated and over-confluent cell cultures. Rodd *et al* recently reported a similar lack of response to E2 in primary foetal bovine chondrocytes.[128] Exogenous E2 had no effect on cell number or viability at a range of doses (10^{-13} to 10^{-7} M). However, at a pharmacological concentration (10^{-7} M) E2 induced maturation of prehypertrophic chondrocytes toward the hypertrophic phenotype. This was associated with the appearance of type X collagen and an increase in ALP activity. In addition, Gruber *et al* reported that exogenous E2 (10^{-10} to 10^{-6} M) did not alter ALP activity in ATDC5 cells.[171] Based on these reports, an E2 concentration of 10^{-6} M was employed for all further ATDC5 cell culture experiments.

2.5.3 Effect of E2 on ALP activity and proteoglycan production

In ATDC5 chondrocytes the production of extra-cellular matrix, as reflected by proteoglycan synthesis, was unaltered after exposure to E2 at a final concentration of 10^{-6} M for 7 days (Figure 2.3A). In a similar fashion ALP activity was unaffected by E2 exposure at a final concentration of 10^{-6} M for 7 days (Figure 2.3B).

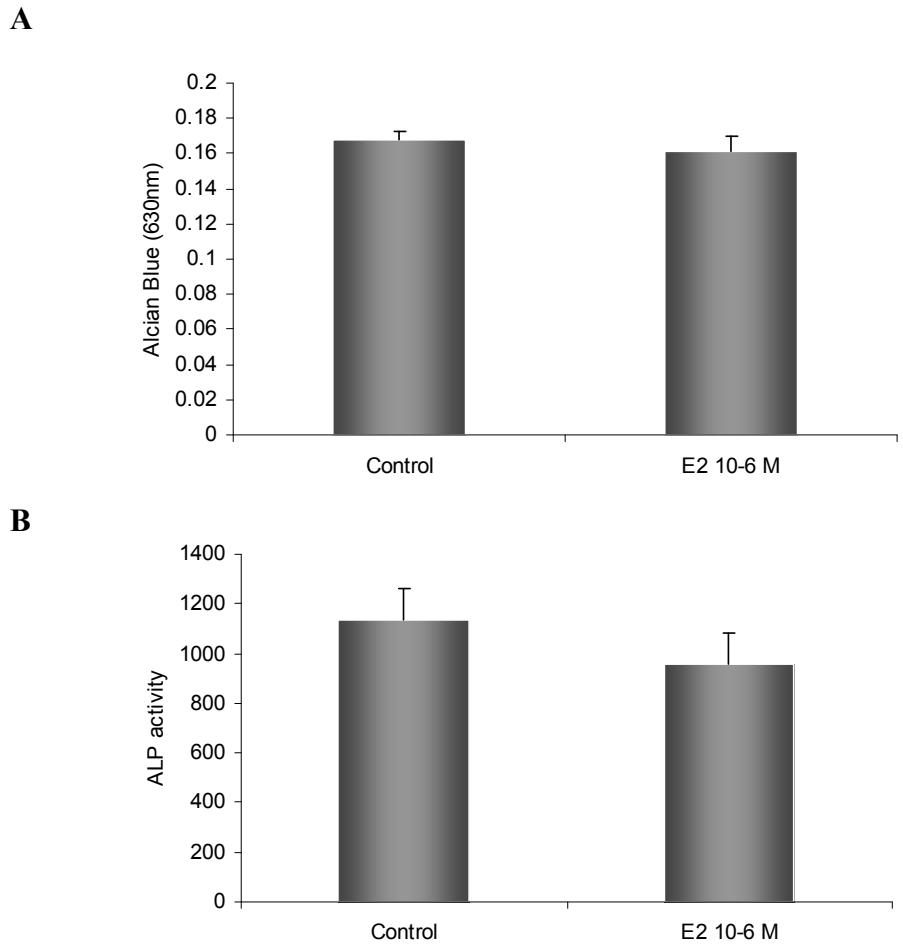


Figure 2.3 – Effect of E2 on ATDC5 differentiation after 7 days exposure at 10^{-6} M A) Proteoglycan production as assessed by Alcian Blue uptake (OD at 630nm) in ATDC5 cells during terminal differentiation. B) ALP activity (nmoles pNPP hydrol/min/mg protein) in ATDC5 cells during terminal differentiation. Data are expressed as means ± SEM (n = 6).

2.5.4 Effect of co-incubating E2 and pro-inflammatory cytokines on chondrocyte proliferation and apoptosis

The cytokines, IL-1β and TNFα (10ng/ml), led to a reduction in proliferation in ATDC5 chondrocytes after 24 hours exposure as analysed with [^{3}H]thymidine incorporation with a reduction in proliferation of 84.5% and 95% respectively (Figure 2.4). The co-incubation of E2 at a final concentration of 10^{-6} M with either of the cytokines (Figure 2.4) did not alter the reduction observed in cell proliferation.

Similarly, both IL-1β and TNFα (10ng/ml) increased apoptosis in the ATDC5 chondrocytes after 24 hours exposure (Figure 2.5). TNFα had a significantly greater effect on apoptosis by 1238% compared with 297%. Exposure of the ATDC5 cells to E2 at a final concentration of 10^{-6} M had no effect on apoptosis either alone or in combination with either of the cytokines (Figure 2.5).

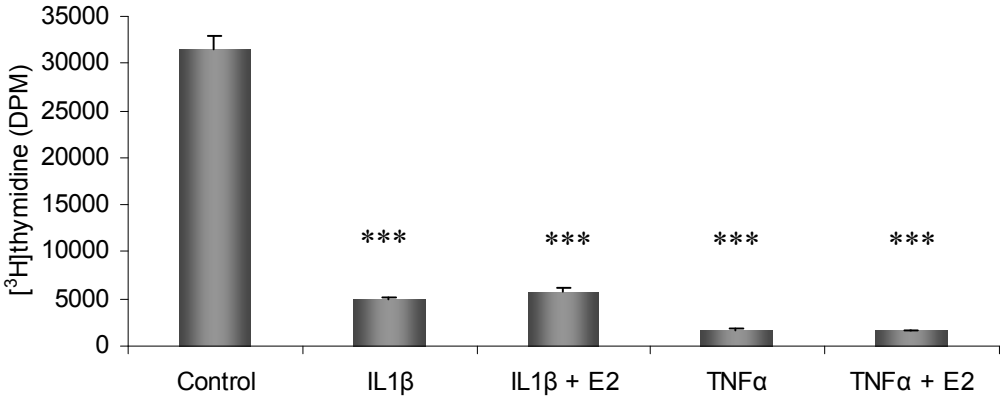


Figure 2.4 – Effect of co-incubating E2 with Il-1β or TNFα on ATDC5 proliferation as assessed by [^{3}H]thymidine uptake after 24 hours exposure. Data are expressed as means ± SEM (n = 6). ***$P < 0.001$ compared with controls.

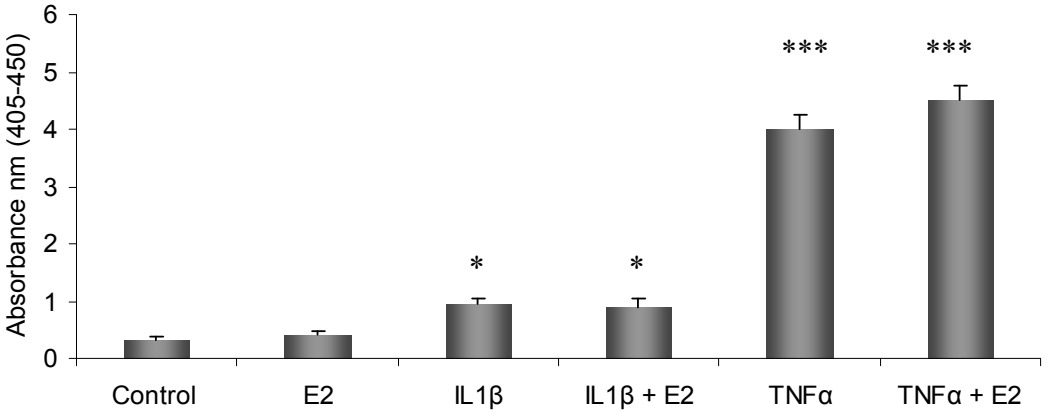


Figure 2.5 – Effect of co-incubating E2 with Il-1β or TNFα on apoptosis in ATDC5 cells as assessed by photometric enzyme-immunoassay (cell death detection ELISA^{PLUS}) after 24 hours exposure. Data are expressed as means ± SEM (n = 6). *$P < 0.05$, ***$P < 0.001$ compared with control cells.

2.6 Discussion

Exogenous E2 had no effect on proliferation, terminal differentiation or apoptosis in the ATDC5 chondrocyte cell line. These findings are in agreement with the recent publications by Rodd *et al* and Chagin *et al.*[128;129] The inconsistent effects of E2 on epiphyseal chondrocytes *in vitro* reported in the literature may be due to differences in the age, gender and species from which the cells have been derived. In addition cell culture conditions play a crucial role as they will influence endogenous E2 production. The presence of FBS in cell media allows E2 to work in concert with other factors or hormones present in the serum. Furthermore, in some of the older studies[122;123] chondrocytes were cultured in the presence of phenol red (a pH indicator) which is now known to have significant oestrogenic activity at the concentrations found in tissue culture media.[179] Recent studies have used serum-free media or media with charcoal treated serum (CTS). Charcoal treatment reduces the concentration of steroid hormones found in the serum. Exogenous E2 has been shown to have no effect on cell number or proliferation in a human chondrocyte cell line [129] and in primary foetal bovine chondrocytes.[128] Likewise no effect was seen on ALP activity in a murine chondrocyte cell line (ATDC5 cell line).[171] In two older studies that also used serum-free media (without phenol red) E2 exposure led to an increase in ^{35}S incorporation in primary human and rabbit chondrocytes which was age-dependent.[160;161] E2 stimulated ^{35}S incorporation in primary human chondrocytes derived from females aged 2-7 years but had no effect on those obtained from girls aged 4-months to 1 year. Male-derived cells were only exposed to DHT.[160] E2 also had an age-dependent stimulatory effect on ^{35}S incorporation in primary rabbit chondrocytes.[161] The maximal response to E2 was observed in foetal

chondrocytes (79%; pooled male and female cells). During the first 5 days of life chondrocytes were moderately responsive with an increase in ^{35}S incorporation which was greater in female derived cells. However, E2 had no effect on cells aged from 5 to 30 days which corresponds to the time period of maximum basal sulfate incorporation in untreated cells. A stimulatory effect of E2 was then observed in cells aged 32-62 days with a greater response in female derived cells. This time period corresponds with the timing of puberty in rabbits.[180] Thereafter cells were unresponsive to E2 (62-75 days). However, in at least one of these studies epiphyseal "articular" chondrocytes were cultured.[161] Articular chondrocytes may respond differently to epiphyseal growth plate chondrocytes and may react more like osteoblasts to E2. In the human osteoblast-like SaOS cell line exposure to E2 led to an increase in proliferation, ALP activity and oestocalcin levels.[181] Fohr *et al* investigated the effect of E2 on four established osteosarcoma cell lines representing different stages of osteogenic maturation and concluded that the response to E2 depends on its osteoblastic commitment rather than its gender.[181]

Whereas, in the presence of FBS, E2 led to a concentration-dependent inhibition of proliferation together with stimulation of differentiation in primary rat chondrocytes (aged 1-month). These responses to E2 were limited to cells from female animals.[126;169] E2 is reported to activate PKC by rapidly increasing intracellular calcium concentration through a capacitative entry mechanism[182] resulting in increased phospholipase C (PLC) activity.[183;184] Further work showed that E2 led to a rapid concentration-dependent activation of mitogen-activated protein kinase (MAPK) dependent on PKC and PLC which was blocked by tamoxifen and did not

require gene transcription or translation. Thus, E2 appears to regulate MAPK through a sex-specific membrane-mediated mechanism that does not involve cytosolic ERs in a traditional sense and extra-cellular signal related kinases 1 and 2 (ERK1/2) and p38 mediate the downstream biological effects of the hormone.[169] In a similar fashion, the response to E2 observed in primary human articular chondrocytes in the presence of FBS was limited to female derived cells.[170] In contrast to epiphyseal chondrocytes a rapid concentration-dependent stimulation of proliferation was observed in articular chondrocytes in response to E2. Chondrocyte differentiation was also promoted with an increase in ALP activity and ^{35}S incorporation. E2 caused a rapid increase in PKC activity in chondrocytes. The E2-dependent stimulation of matrix synthesis is likely to be mediated by PKC as chelerythrine (a general PKC inhibitor) completely blocked its stimulatory effects. Thus, exposure to E2 in the presence of FBS appears to have an inhibitory effect on proliferation in epiphyseal chondrocytes and a stimulatory effect in articular chondrocytes (and osteoblasts). In all, treatment with E2 stimulates chondrocyte differentiation.

To summarise, the ATDC5 cell line may not have responded to E2 as it is male-derived (Professor Hiraki, personal communication) and the previously described rapid responses to E2 in the presence of FBS, including activation of PKC, were limited to cells from female animals[126;134;135] However, this lack of response may be attributable to endogenous E2 production as chondrocytes are able to synthesize E2 which may be physiologically important.[129-131] It is possible that endogenously produced E2 constantly activates ERs and therefore prevents any further effect of exogenous E2.

Exposure of ATDC5 chondrocytes to the pro-inflammatory cytokines, IL-1β and TNFα, led to a comparable reduction in cell proliferation as analysed by thymidine incorporation at a concentration of 10ng/ml (84.5% versus 95% respectively). Similarly, both cytokines increased apoptosis in ATDC5 cells but a significantly greater effect was observed after treatment with TNFα (1238% versus 297%). These inhibitory effects of IL-1β and TNFα on ATDC5 chondrocyte dynamics have been previously documented by our group.[162] In addition, treatment with IL-1β or TNFα (10ng/ml) has previously been shown to decrease proteoglycan synthesis (75% versus 86%) and markedly reduce aggrecan, collagen II and collagen X gene expression.[162] ATDC5 cells treated with IL-1β (10ng/ml) for 2 or 7 days followed by a cytokine-free recovery period of 12 or 7 days respectively showed a reduction of only 12% or 38% in proteoglycan synthesis compared to control cells at the end of the culture period at 14 days.[162] Recovery from TNFα exposure could not be studied in ATDC5 cells because of its toxicity to the cells when added during the chondrogenesis period of maturation.[185] In view of these previous findings the effect of co-incubating E2 with cytokines was investigated in this study during terminal differentiation. The cytokine-induced inhibition of ATDC5 cell proliferation was not affected by co-incubation with E2, at a final concentration of 10^{-6} M. Likewise, the stimulation of apoptosis by the pro-inflammatory cytokines was unaltered by co-incubation with E2. Thus E2 does not appear to have a protective effect on ATDC5 cells against cytokine-induced changes on proliferation or cell survival. This is in contrast to the protective effects of E2 reported in other cell types. In human umbilical vein endothelial cells (HUVEC) exposure to TNFα led to a decrease in DNA synthesis (thymidine uptake) and an increase in apoptosis.[164] This

was associated with an up-regulation in ERK1/2 activity and expression of intercellular and vascular cell adhesion molecules. TNFα effects were significantly attenuated by co-incubation with E2 which included 4 hours of pre-incubation with E2. The inhibitory effect of E2 was blocked by Faslodex suggesting its action was mediated via classical ERs. ERβ is the most likely candidate as HUVEC express ERβ but lack ERα. E2 treatment significantly attenuated ERK1/2 activation but had no affect on other signalling molecules. ERK1/2 are members of the MAPK super family that can mediate cell proliferation and apoptosis. Similarly in bovine carotid artery endothelial cells both E2 and raloxifene analogue (LY117018) prevented caspase-3 dependent apoptosis induced by TNFα through activation of the ERs and ERK1/2 signalling pathway.[186] Cells were pre-incubated with E2 or raloxifene analogue for 30 minutes. E2 treatment is also reported to protect against apoptosis induced by other mechanisms. For example in a cell culture model of Parkinson's disease (PC12 cancer cell line MPP+) E2 exposure suppressed apoptosis and improved cell viability.[187] The beneficial effects of E2 were due to suppression of the pro-apoptotic protein IL-β converting enzyme (also known as caspase 1) and stimulation of the anti-apoptotic protein Bcl-xL. The latter inhibits mitochondrial cytochrome C release into the cytoplasm and subsequent activation of caspase-3. Further, in the murine C8-DIA astrocyte cell line, E2 suppressed hypoxia-induced apoptosis by 50%.[188] The hypoxia-induced apoptosis was associated with an increase in phosphorylated ERK1/2 which was attenuated by pre-incubation with E2. Likewise in primary foetal rat cortical neurones E2 treatment inhibits glutamate-induced apoptosis.[189] Exposure to E2 prevents the release of cytochrome C from mitochrondria into the cytoplasm and subsequent down-regulation of caspase-3. No

pre-incubation with E2 was performed. More recently, a caspase-independent pathway involving calpain (apoptotic protease) and apoptosis inducing factor (AIF) has been shown to mediate some of the apoptotic effects of glutamate in primary rat cortical neurones. This pathway is similarly inhibited by E2. Primary rat cortical neurones express both ERs but ERβ expression is greater than ERα. [190]

In the various cell types studied E2 appears to have an effect on caspase-dependent apoptotic pathways and in particular on the anti-apoptotic protein Bcl-xL and mitochondrial cytochrome C which are both downstream of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway. In addition, effects on the ERK1/2 signalling transduction pathway have been reported. It is possible that E2 is exerting its effect via the suppressor of cytokine signalling (SOCS) family of proteins which are inhibitors of STATs as E2 mediates its inhibition of the GH/JAK/STAT pathway via SOCS-2.[191] In cardiac myocytes SOCS-1 prevented TNF-α induced apoptosis in cardiac myocytes via activation of the ERK1/2 pathway activation.[192] ERK1/2 can mediate both cell proliferation and apoptosis. The Ras–Raf–MEK–ERK signalling cascade controlling cell proliferation has been well studied but the mechanisms involved in ERK1/2-mediated cell death are largely unknown. Activated ERK1/2 is usually translocated to the nucleus but certain proteins are involved in the cytosolic retention of activated ERK1/2.[193] Cytosolic retention of ERK1/2 denies access to the transcription factor substrates that are responsible for the mitogenic response. In addition, cytosolic ERK1/2, besides inhibiting survival and proliferative signals in the nucleus, potentiates the catalytic activity of some proapoptotic proteins in the cytoplasm but further work is required

to further define the function of cytosolic ERK1/2 and its cytosolic substrates that enhance cell death.[193]

Possible explanations for the lack of response we observed when co-incubating oestradiol with cytokines on the ATDC5 cells include differences in cell type with possible differences in cell signalling pathways. In addition, experimental conditions could be important as some but not all of the studies described above included a period of pre-incubation with oestradiol where the cells received oestradiol for a variable period (from thirty minutes to four hours) before the addition of cytokines. This may be relevant as the protective effect of oestradiol may require this period of pre-incubation perhaps to effect a change of cell signalling pathways e.g. up-regulation of certain anti-apoptotic pathways. Lastly, it may be due to the distribution of the ERs within the different cell types and the proportion of ERα compared to ERβ. In chondrocytes both ERs appear to be fairly equally distributed whereas some of the cells described above mainly or only expressed ERβ.

2.7 Conclusions

The murine ATDC5 chondrocyte cell line expresses ERα and ERβ during chondrogenesis and terminal differentiation. Exogenous oestradiol had no effect on proliferation during chondrogenesis or terminal differentiation. The markers of chondrocyte differentiation, ALP activity and Alcian Blue staining, were also unaffected by exposure to oestradiol. Similarly, programmed cell death was unaltered by oestradiol treatment. In addition, oestradiol did not convey a protective

effect on the cytokine-induced inhibition of proliferation and stimulation of apoptosis by TNFα and IL-1β.

Chapter 3

Effect of oestradiol on human C28/I2 chondrocyte cell line

Chapter Contents

3.1 Introduction

3.2 Hypothesis

3.3 Aims

3.4 Materials and Methods

3.4.1 Materials

3.4.2 Methods

3.4.2.1 C28/I2 chondrocyte cell culture

3.4.2.2 Chondrocyte proliferation assay

3.4.2.3 Apoptosis assay

3.4.2.3 Semi-quantitative RT-PCR

3.4.3 Statistical analysis

3.5 Results

3.5.1 Gene expression

3.5.2 Effect of oestradiol

3.5.3 Effect of oestradiol-related chemicals

3.6 Discussion

3.7 Conclusions

3.1 Introduction

The culture of primary chondrocytes of human origin can be difficult as the source of cartilage cannot be controlled leading to variability in chondrocyte preparations. In addition, the large numbers of cells required for individual experiments are not readily available from random operative procedures. Therefore immortalized chondrocytes of human origin have been developed to serve as a reproducible model for studying chondrocyte function. Some of the first human chondrocyte cell lines were established by retroviral-mediated transfection of primary rib chondrocytes with the large T antigen of Simian virus 49.[194] The chondrocytes were derived from costal cartilage from a 15-year old female. The T/C-28a2 cell line was obtained initially, from which the C-28/I2 and T/C-28a4 were derived.[194] These cells retain chondrocytic morphology and maintain continuous proliferation in monolayer culture. Moreover they have been used in numerous studies to investigate chondrocyte-specific response patterns.[194;195] Of these cell lines C28/I2 showed the highest SOX-9 (SRY-related HMG-box gene 9; master gene of chondrocytic cell differentiation) levels and appears to most closely resemble primary chondrocytes with respect to anabolic and catabolic gene expression.[196] However, these cell lines mainly proliferate and show lower expression of genes involved in matrix synthesis and turnover.

3.2 Hypothesis

As E2 had no effect on the male-derived ATDC5 cell line it was hypothesised that the effect of E2 may be limited to female chondrocytes.

3.3 Aims

I. Confirm the presence of the oestrogen receptors, ERα and ERβ, by semi-quantitative RT-PCR in C28/I2 cells

II. Analyse the effect of different E2 concentrations on proliferation in C28/I2 cells

III. Investigate the effect of E2-related chemicals (including specific ER inhibitors) on chondrocyte proliferation and apoptosis

3.4 Materials and methods

3.4.1 Materials

All chemicals were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated. PCR oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). 17β-Oestradiol: cyclodextrin-encapsulated water soluble; tamoxifen: SERM, principally ERα antagonist; raloxifene: SERM, principally ERβ agonist; Methyl-piperidino-pyrazole (MPP) dihydrochloride: selective ERα antagonist.[197] Faslodex: non-specific high affinity ER antagonist (Tocris Bioscience, Bristol, UK).

3.4.2 Methods

3.4.2.1 C28/I2 chondrocyte cell culture

The C28/I2 chondrocyte cell line was kindly supplied by Dr. Goldring (Boston, USA) and maintained as described by Goldring *et al.*[198] Cells were maintained in

T175 tissue-culture flasks (Greiner Bio-Pne GmbH) at a density of 750,000 cells/flask in a maintenance medium of DMEM: F12 without phenol red (Invitrogen) supplemented with 10% FBS (Invitrogen), and gentamicin (50µg/ml Invitrogen). For individual experiments semi-confluent cultures were passaged with trypsin EDTA and cultured (day 0) at a density of 12,500 cells/cm^2 in multi-well plates (Costar) in maintenance medium. Cells were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2 and the medium was changed every second/third day. In selected experiments cells were cultured in serum-free medium.

3.4.2.2 Chondrocyte proliferation assay

E2 and related chemicals were added to the cells at a final concentration of 10^{-6} M in 0.01% DMSO and incubated for a 24 hour period. Control cells received 0.01% DMSO. Chondrocyte proliferation was assessed by incubating the cells with 0.2µCi/ml [^{3}H]thymidine (37MBq/ml; Amersham Pharmacia Biotech) for the last 2 hours of the incubation period and measuring the amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates.[176]

3.4.2.3 Apoptosis assay

Apoptosis was assessed by measuring the cytoplasmic histone-associated DNA fragments (mono- and oligo- nucleosomes) by photometric enzyme-immunoassay (cell death detection ELISA^{PLUS}; Roche Applied Science). E2 and related chemicals were added to the cells at a final concentration of 10^{-6} M in 0.01% DMSO and compared with control cultures which contained 0.01% DMSO only. Apoptosis was

measured following the manufacturers' protocol in cells cultured in 96-well plates over a 24 or 48 hour period starting on day 2.

3.4.2.4 Semi-quantitative RT-PCR

Semi-quantitative PCR was performed as described in chapter 2 (2.4.3) and the sequence of the primer pairs used (human ERα, human ERβ) are shown in Table 3.1.

Table 3.1 - Primer sequences and product sizes for human ERα and ERβ genes analysed by RT-PCR

Gene	Primer sequence Forward primer Reverse primer	Cycles	Product size (base pairs)
Classic 18S	Unknown, purchased commercially from Ambion	20	488
ERα (human)	5'- GAC CGA AGA GGA GGG AGA A - 3' 5' - CCA AGA GCA AGT TAG GAG CAA - 3'	35	460
ERβ (human)	5' - TAG TGG TCC ATC GCC AGT TAT - 3' 5' - GGG AGC CAC ACT TCA CCA T - 3'	35	323

3.4.3 Statistical analysis

Data were analysed by ANOVA. All data are expressed as the mean ± SEM of at least six replicates within each experiment, and statistical analysis was performed using SPSS (version 15). Significance was determined as $P<0.05$.

3.5 Results

3.5.1 Gene expression

The oestrogen receptors, ERα and ERβ, were both expressed in C28/I2 chondrocytes (Figure 3.1).

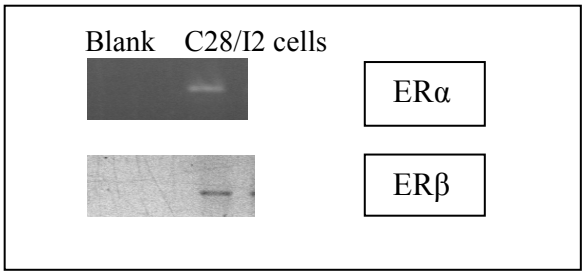


Figure 3.1 – ERα and ERβ expression in C28/I2 cells as detected by semi-quantitative RT-PCR analysis

3.5.2 Effect of oestradiol

Exposure to E2 for 24 hours, in the presence of 10% FBS, led to a significant reduction in cell proliferation as analysed with [^{3}H]thymidine incorporation (Figure 3.2A). Cell proliferation was inhibited in a concentration-dependent manner with a decrease in [^{3}H]thymidine incorporation of 8.4%, 14.7% and 17.2% in C28/I2 cells exposed to E2 at a final concentration of 10^{-10} M, 10^{-8} M and 10^{-6} M respectively ($P<0.05$, $P<0.001$ and $P<0.001$). E2 treatment for 48 hours at a concentration of 10^{-8} M and 10^{-6} M also led to a significant reduction in cell proliferation (32.7% and 28.1% decrease in [^{3}H]thymidine incorporation, $P<0.01$) but had no effect at a final concentration of 10^{-10} M (Figure 3.2B). Whereas, exposure to E2 for 24 hours in serum-free medium had no effect on cell proliferation as analysed with [^{3}H]thymidine incorporation (Figure 3.3). Exposure of C28/I2 cells to E2 for 24 hours in the presence of FBS had no effect on the level of apoptosis as assessed by photometric enzyme-immunoassay (Figure 3.6).

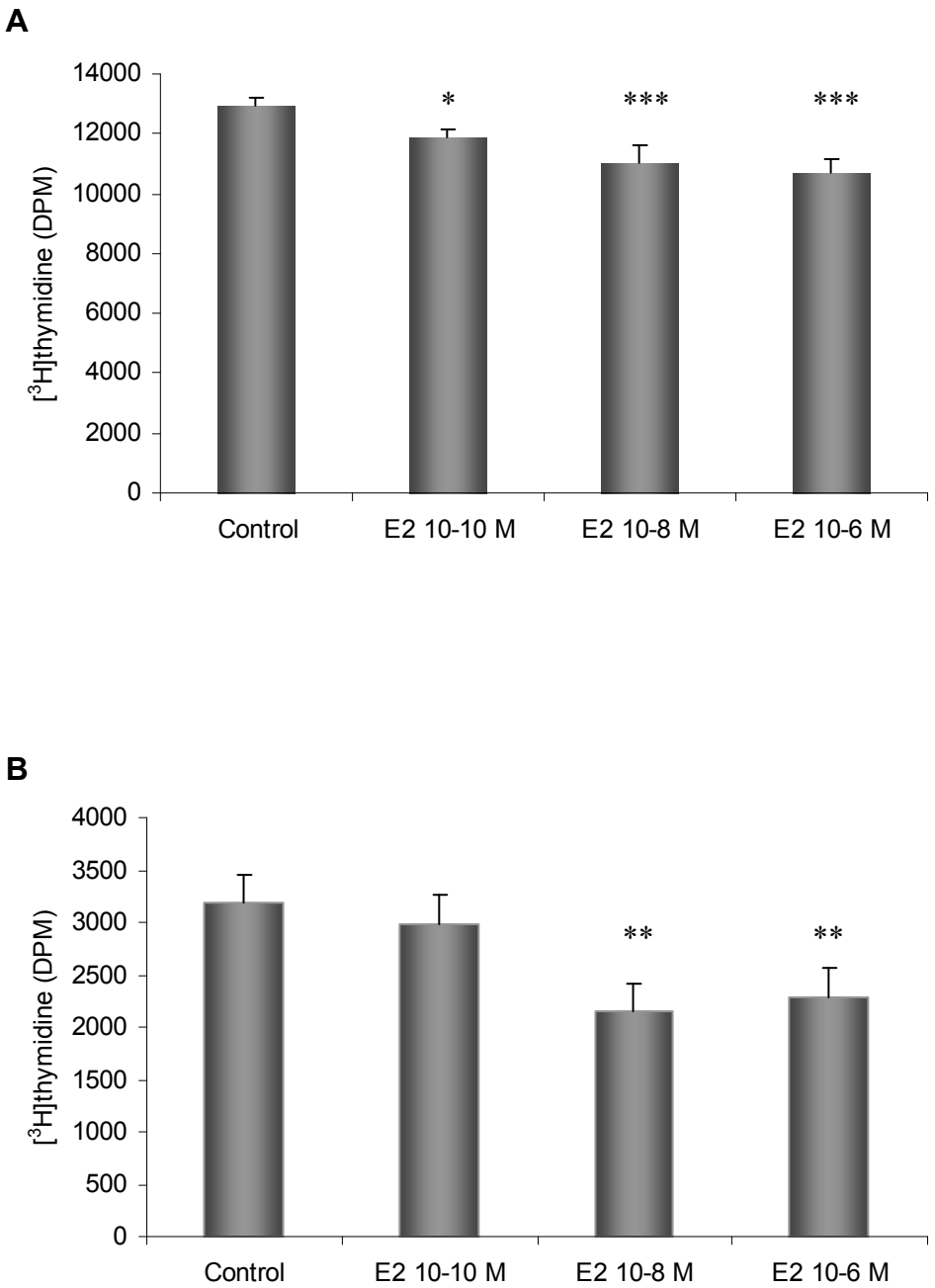


Figure 3.2 – Effect of E2 on C28/I2 proliferation in the presence of FBS as assessed by [^{3}H]thymidine uptake (A) C28/I2 proliferation after 24 hours exposure **P*<0.05, ***P*<0.01, ****P*<0.001 compared with control cells. (B) C28/I2 proliferation after 48 hours exposure **P*<0.05 compared with control cells. All data are expressed as mean ± SEM (n = 6).

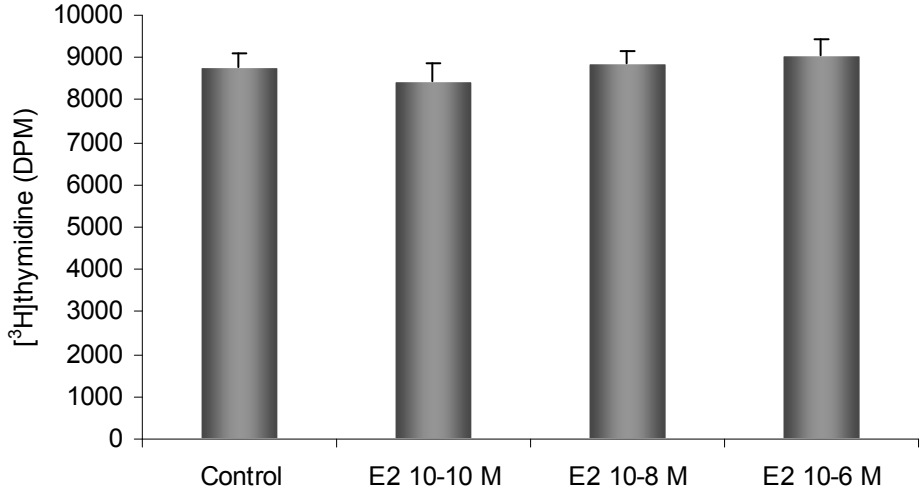


Figure 3.3 – Effect of E2 on C28/I2 proliferation in serum-free medium as assessed by [^{3}H]thymidine uptake after 24 hours. Data are expressed as mean ± SEM (n= 6).

3.5.3 Effect of oestradiol-related chemicals

The E2-induced decrease in proliferation of cells, cultured in medium with 10% FBS, was not observed in the presence of tamoxifen or raloxifene at a final concentration of 10^{-6} M (Figure 3.4). Exposure of Faslodex, the high-affinity non-specific ER antagonist, led to a decrease in cell proliferation when administered either alone (36.6% decrease in thymidine incorporation, $P<0.001$) or in combination with E2 (40.7% decrease, $P<0.001$) (Figure 3.4). The E2-related chemicals, raloxifene and MPP at a final concentration of 10^{-6} M had no effect on cell proliferation when given alone (Figure 3.5). However, exposure to raloxifene and MPP for 24 hours at a final concentration of 10^{-6} M led to a significant decrease in cell proliferation (21.7% decrease in thymidine incorporation, $P<0.001$). This reduction in proliferation was also observed in the presence of E2 at a final concentration of 10^{-6} M (21.7% decrease, $P<0.001$).

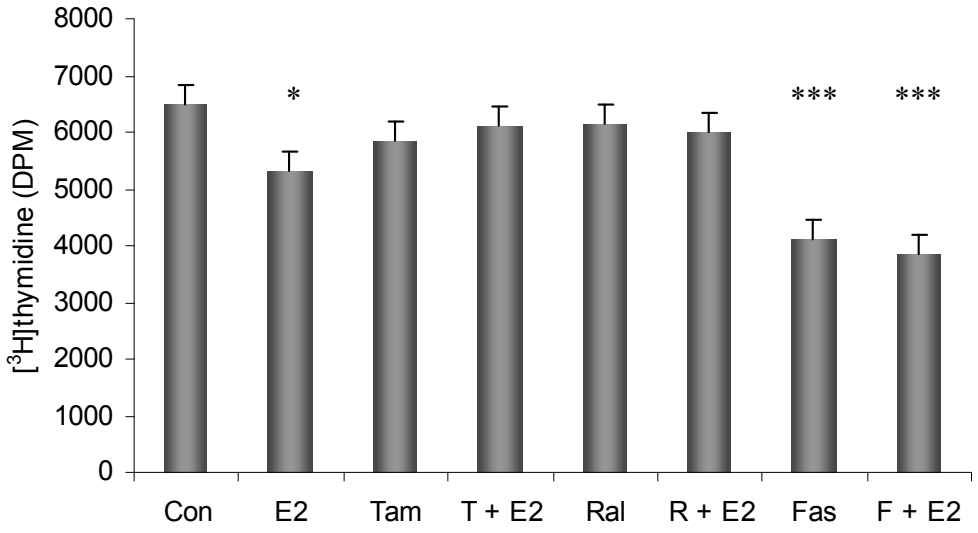


Figure 3.4 – Effect of E2 alone and in combination with Tamoxifen, Raloxifene or Faslodex on C28/I2 proliferation as assessed by [^{3}H]thymidine uptake after 24 hour exposure at 10^{-6} M. Data are expressed as mean ± SEM (n = 6). **P*<0.05, ****P*<0.001 compared with control cells.

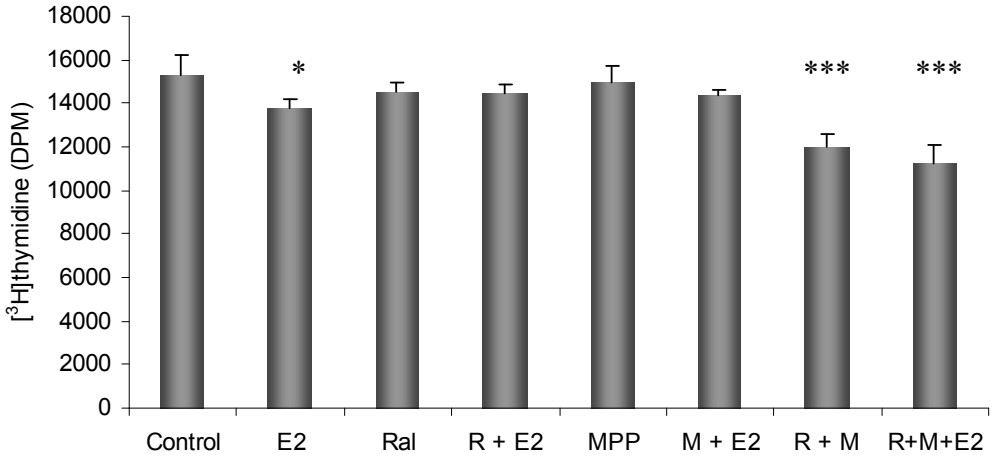
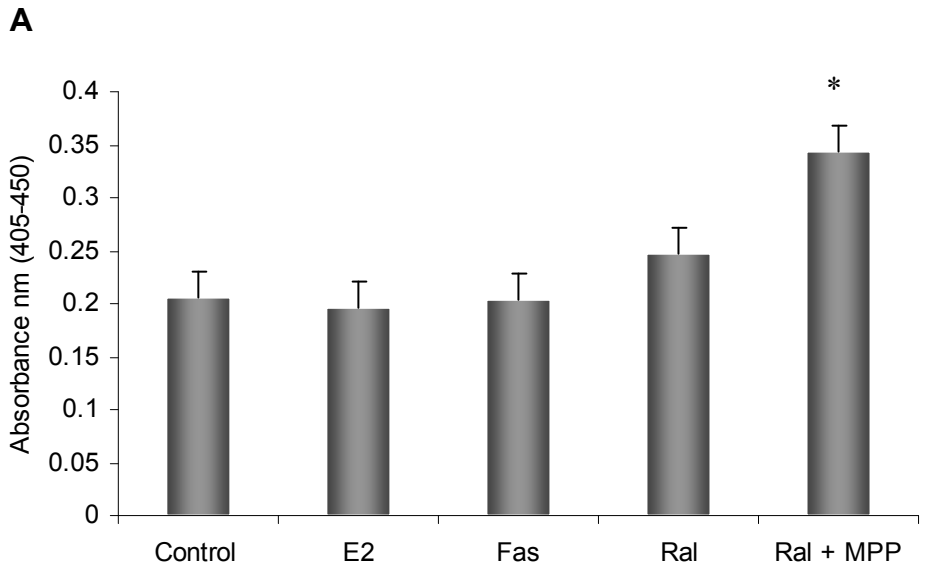


Figure 3.5 – Effect of E2, Raloxifene or MPP alone and in combination on C28/I2 proliferation as assessed by [^{3}H]thymidine uptake after 24 hours exposure. Data are expressed as mean ± SEM (n = 6). **P*<0.05, ****P*<0.001 compared with control cells.

Exposure of C28/I2 chondrocyte cells to E2, raloxifene or Faslodex (at a final concentration of 10^{-6} M) for 24 hours had no effect on the level of apoptosis as assessed by photometric enzyme-immunoassay (Figure 3.6A). However, a significant increase in apoptosis was observed after 24 hour exposure to the combination of raloxifene and MPP at a final concentration of 10^{-6} M (32.5% increase compared to control cells; $P<0.05$). Similarly there was no difference observed in the level of apoptosis after 48 hours of exposure to E2, raloxifene or Faslodex at a final concentration of 10^{-6} M compared to control cells (Figure 3.6B). The combination of raloxifene and MPP at a final concentration led to a significant increase in apoptosis after 48 hours exposure (20% increase compared to control cells; $P<0.05$).



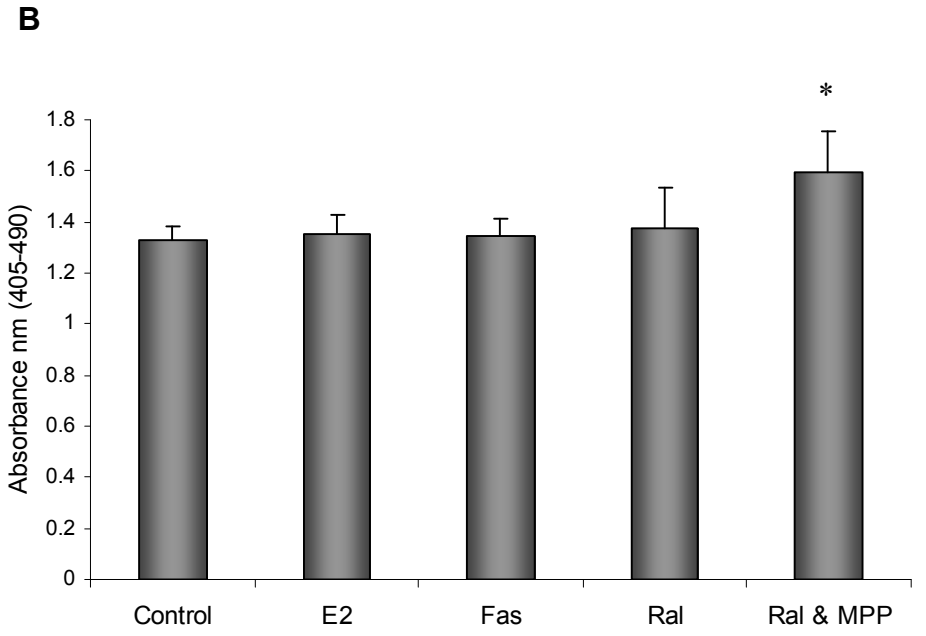


Figure 3.6 – Effect of E2, Faslodex, Raloxifene or Raloxifene & MPP on apoptosis of C28/I2 cells at 10^{-6} M as assessed by photometric enzyme-immunoassay (cell death detection ELISA^{PLUS}). Data are expressed as mean ± SEM (n = 6). A) After 24 hours exposure *P<0.05 compared with control cells. B) After 48 hours exposure *P<0.05 compared with controls.

3.6 Discussion

The reduction in cell proliferation observed after exposure to E2 only occurred in the presence of foetal bovine serum. This suggests that the E2-induced inhibition of proliferation is dependent on a compound found in the serum. However, the response is concentration-dependent which suggests that E2 is having a true effect. This effect was blocked by the addition of tamoxifen, a first generation SERM which functions principally as an ERα antagonist, suggesting that ERα is important in mediating this effect. As tamoxifen blocks the action of PKC it is possible that E2 is exerting its effect via the non-genomic pathway of E2 signalling (PLC/PKC/MAPK/ERK1/2 pathway) in a similar fashion to the sexually dimorphic E2 effects previously reported by McMillan *et al* where a response to E2 was limited to cells from female animals.[169]

Exposure of C28/I2 cells to Faslodex, the high-affinity non-specific ER antagonist, led to a significant reduction in cell proliferation (decrease of 36% compared to controls $P<0.001$). This suggests that endogenous E2 may play an important role in maintaining cell proliferation. However, we found that Faslodex did not alter apoptosis in the human C28/I2 cell line. This is in contrast to the findings of Chagin *et al* where they observed a reduction in proliferation along with an increase in caspase-dependent apoptosis in the human chondrocyte HCS-2/8 cell line after exposure to Faslodex.[129] This effect was concentration-dependant as an increase in apoptosis was only observed after 48 hours when cells were exposed to Faslodex at 10^{-6} M whereas those exposed to a final concentration of 10^{-5} M showed a reduction at 24 hours. Faslodex-induced apoptosis is therefore cell-type dependent. Similar

cell-type dependent apoptotic effects have been described with the glucocorticoid, dexamethasone. Chrysis *et al* reported an increase in apoptosis after exposure of the HCS-2/8 chondrocyte cell line to dexamethasone[199] whereas no effect was observed in the ATDC5 cell line.[185]

Raloxifene, a SERM which functions mainly as an ERβ agonist, had no effect on cell proliferation or apoptosis when used alone. However, when used in combination with a selective ERα antagonist (MPP) a reduction in cell proliferation was observed in association with an increase in apoptosis. As this combination is likely to be exerting its effects via ERβ (as MPP specifically blocks ERα) it suggests that ERβ has an inhibitory effect on cell growth and cell survival and is acting as a "brake". This is supported by studies of other tissue types which also possess classical ERs (ERα, ERβ). In human colon for example, ERβ, the predominant ER subtype expressed, has been observed to be significantly decreased in colonic tumours compared with normal mucosa whereas ERα expression appears to be unaffected.[200;201] Further, ERβ has been shown to be a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components.[202]

3.7 Conclusions

The C28/I2 chondrocyte cell line expresses both classical ERs (ERα and ERβ). Exposure of this human female-derived chondrocyte cell line to E2 led to a concentration-dependent inhibition of proliferation. This effect was indirect as it only occurred in the presence of FBS and did not occur in the male, murine ATDC5 cell line. The effect was blocked by tamoxifen, a PKC inhibitor, suggesting it may be

mediated via the non-genomic pathway of E2 signalling (PLC/PKC/MAPK/ERK1/2 pathway).

Treatment with the non-specific ER antagonist, Faslodex led to a marked reduction in cell proliferation which was not associated with an increase in apoptosis. As Faslodex blocks and downgrades both of the classical ERs it suggests that endogenous E2 plays an important role in maintaining cell proliferation but has little impact on apoptosis in the human C28/I2 cell line.

However, when the function of the ERs were manipulated by selectively blocking ERα with MPP and exposing the cells to raloxifene, an E2-agonist with higher affinity for ERβ, a significant inhibition of cell proliferation and stimulation of apoptosis was observed. This suggests that ERβ may act as a "brake" on the growth of the chondrocyte.

Chapter 4

Effect of oestradiol on physiological models of murine growth

Chapter Contents

4.1 Introduction

4.2 Hypothesis

4.3 Aims

4.4 Materials and Methods

4.4.1 Foetal murine metatarsal organ culture

4.4.2 Morphometric analysis of metatarsals

4.4.3 Sex determination of mouse embryos

4.4.4 Isolation of primary murine chondrocytes

4.4.5 Primary murine chondrocyte cell culture

4.4.5 Chondrocyte proliferation assay

4.4.6 Statistical analysis

4.5 Results

4.5.1 Longitudinal growth of foetal metatarsal culture system

4.5.2 Mineralising zone growth of foetal metatarsal culture system

4.5.3 Primary murine chondrocytes

4.6 Discussion

4.7 Conclusions

4.1 Introduction

The foetal murine metatarsal culture system is an established procedure to directly investigate bone growth and is recognised to provide a more physiological model than the culture of isolated chondrocytes. The growth rate of foetal bones in culture is similar to that found *in vivo* and in contrast to monolayer cell culture the metatarsal culture system maintains cell-cell and cell matrix interactions, and allows the direct assessment of bone growth and histological architecture. Our group has extensive experience of this model, having first published its use for studying childhood growth.[203]

Similarly, the murine primary chondrocyte culture system is an established method of investigating chondrocyte function. In contrast to human primary chondrocyte cells they are easily obtained and retain their differentiated phenotype for several days in culture.[204] These physiological models of murine growth were therefore chosen to further investigate the effect of E2 on chondrocyte function. A limitation of the primary chondrocyte culture system is the large number of cells required for each experiment. For this reason the number of experiments performed with this system was restricted to assessing the effects of E2 and the E2-related chemicals, Faslodex, MPP and raloxifene.

4.2 Hypothesis

The hypothesis of this study was that exogenous E2 would have no effect on linear growth in the metatarsal culture system (as E2 had no effect on chondrocyte cell lines in the absence of FBS) but Faslodex, by blocking endogenous E2 activity, would decrease both primary chondrocyte proliferation and linear growth of metatarsals in culture. Secondly, it was hypothesised that ERβ has an inhibitory effect on chondrocyte proliferation and metatarsal growth. As MPP is a selective antagonist of ERα and raloxifene a SERM that functions mainly as an ERβ agonist it was hypothesised that in combination they would lead to a reduction in primary chondrocyte proliferation and longitudinal growth of metatarsals in culture.

4.3 Aims

I. Analyse the effect of E2 and oestradiol-related chemicals on the longitudinal growth and mineralising zone growth of foetal murine metatarsals maintained in culture

II. Analyse the effect of E2 at varying concentrations on primary murine chondrocyte proliferation

III. Analyse the effect of the oestradiol-related chemicals: Faslodex, MPP & raloxifene on primary murine chondrocyte proliferation

4.4 Materials and methods

The experimental protocol was approved by the Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

4.4.1 Foetal murine metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18-day old embryonic Swiss mice that had been killed by decapitation. Bones were individually cultured at 37°C in a humidified atmosphere of 95% air/5% CO2 in 24-well plates (Costar) for up to 14 days. Each well contained 300 µl of serum-free α-MEM without nucleosides (Invitrogen) supplemented with 0.2% Bovine Serum Albumin Cohn fraction V; 0.1 mmol/l β-glycerophosphate; 0.05 mg/ml L-ascorbic acid phosphate (Wako, Fukuoka, Japan), 0.292 mg/ml L-glutamine (Invitrogen), 0.05 mg/ml gentamicin (Invitrogen) and 1.25 µg/ml fungizone (Invitrogen). E2 (cyclodextrin-encapsulated water soluble) at a final concentration of 10^{-5} M or 10^{-6} M and oestradiol-related chemicals at a final concentration of 10^{-5} M or 10^{-6} M in 0.01% DMSO/0.1% ethanol were added to the bones and the medium was changed every second or third day. The control groups (medium only or 0.01% DMSO/0.1% ethanol) and each experimental group contained 6-9 metatarsals.

4.4.2 Morphometric analysis of metatarsals

Images were taken of the metatarsals every 2 to 4 days of culture using a digital camera attached to a Nikon TE300 microscope. The total length of the bone and the length of the mineralising zone were determined using Image Tool (Image Tool

version 3.00, University of Texas Health Life Science Centre, San Antonio, USA) (Figure 4.1). All results are expressed as a percentage change from harvesting length, which was regarded as baseline to demonstrate the rate of growth over time.

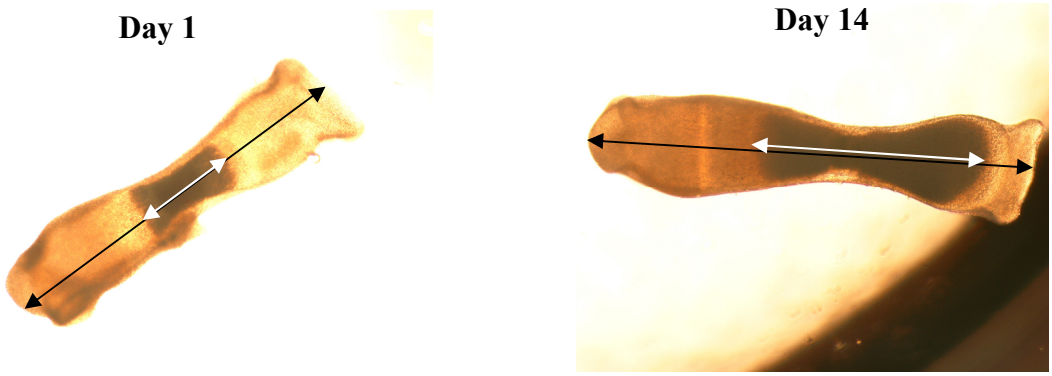


Figure 4.1 - Foetal murine metatarsal culture model. Total length (black arrow) and mineralising zone length (white arrow) are shown in a control metatarsal (A) On the day of harvesting (B) After 14 days of culture.

4.4.3 Sex determination of mouse embryos

In view of the age and size of the mouse embryos it was not possible to determine the sex by direct examination alone. As the sex determining region protein (SRY) gene is encoded by the Y chromosome and is well conserved among species PCR allows the rapid and accurate detection of male DNA. Therefore, DNA was isolated from mouse embryo tail biopsies. Each tail biopsy was placed into a labelled tube containing 1ml of tail digest buffer: 0.3M sodium acetate, 10mM Tris HCl pH 7.9, 1mM EDTA pH 8, 1% SDS and 200µg/ml Proteinase K. The samples were incubated overnight at 37°C and frozen at -20°C for storage until needed. Prior to PCR analysis, samples were centrifuged at 13200 rpm at 4°C for 15 minutes, and then kept on ice. This process was repeated twice, and insured the sodium dodecyl

sulphate (SDS) present in the tail digest buffer was sedimented and did not interfere with the PCR reaction. SRY gene expression was analysed by semi-quantitative RT-PCR as described in chapter 2.0 (2.4.3). The sequences of the primer pairs used are shown in Table 4.1. The efficacy of this system was assessed by analysing the SRY gene expression in DNA from eight mice that had previously undergone tail biopsies for other experiments in whom the sex was known. The results are shown in Figure 4.2.

Table 4.1 - Primer sequences and product size for murine SRY gene

Gene	Primer sequence **Forward primer** **Reverse primer**	Cycles	Product size (base pairs)
Classic 18S	Unknown, purchased commercially from Ambion	20	488
SRY (murine)	5' - TGG GAC TGG TGA CAA TTG TC - 3' 5' - GAG TAC AGG TGT GCA GCT CT - 3'	35	402

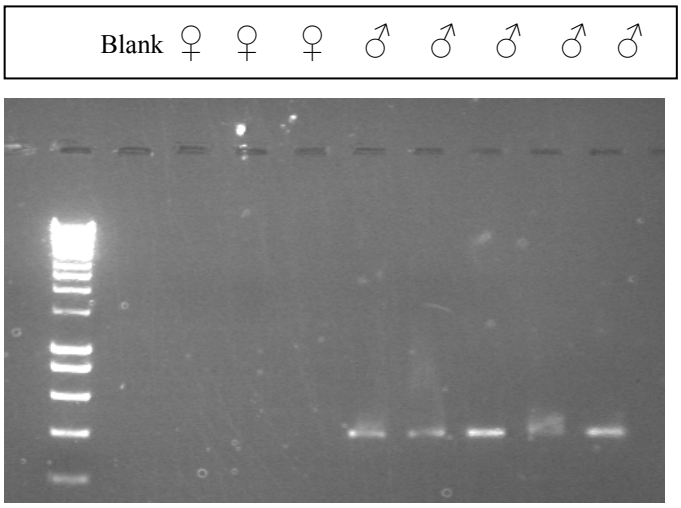


Figure 4.2 – SRY gene expression to determine sex of mice embryos DNA from eight mice in whom the sex was previously known, and depicted above each lane of the PCR gel (♀= female; ♂ = male), was analysed for SRY gene expression by semi-quantitative RT-PCR.

4.4.4 Isolation of primary murine chondrocytes

Primary cultures of chondrocytes from rib growth plates of 3-day old mice were prepared using the culture system developed by Lefebvre et al.[204] The rib cage and sternum were dissected from nine 3-day old mice, rinsed in PBS, and incubated with pronase in PBS (2 mg/ml; DAKO, Glostrup, Denmark) for 30 minutes at 37°C, rinsed again with PBS and then incubated with bacterial collagenase (3mg/ml; collagenase D, Worthington type II) in DMEM at 37°C for about 90 minutes until all soft tissues detached from the cartilages with a few pipettings. Cartilages from male and female mice were pooled and carefully washed several times with PBS and separated from soft tissue residues. They were then digested with collagenase for 3 hours in a petri dish, and undigested bony parts were discarded. Chondrocytes were pelleted by centrifugation and washed twice with DMEM. On average 1.1 x 10^6 chondrocytes were obtained per mouse.

4.4.5 Primary chondrocyte cell culture

Chondrocytes were cultured in 24-well plates in a medium of DMEM: F12 without phenol red (Invitrogen) supplemented with 10% FBS (Invitrogen), gentamicin (50µg/ml Invitrogen) and ascorbic acid (10µg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2.

4.4.6 Chondrocyte proliferation assay

E2 at a final concentration of 10^{-10} M, 10^{-8} M and 10^{-6} M was added to cells cultured in 24-well plates on day 2 and compared with control cultures which contained medium only. Faslodex, raloxifene and MPP were added to the cells at a final concentration of 10^{-6} M in 0.01% DMSO on day 2 and compared with control cultures which contained 0.01% DMSO only. The cells were then incubated for a 24 hour period. The rate of chondrocyte proliferation was assessed by incubating the chondrocytes with 0.2µCi/ml [^{3}H]thymidine (37MBq/ml; Amersham Pharmacia Biotech) for the last 4 hours of the incubation period and measuring the amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates.[176]

4.4.7 Statistical analysis

Data were analysed by ANOVA. All data are expressed as the mean ± SEM of at least six replicates within each experiment, and statistical analysis was performed using SPSS (version 15). Significance was determined as $P<0.05$.

4.5 Results

4.5.1 Longitudinal growth of foetal metatarsal culture system

Foetal murine metatarsals grown in the presence of oestradiol, at a final concentration of 10^{-6} M or 10^{-5} M, paralleled control bone growth (Figure 4.3A, 4.3B). Likewise, foetal murine metatarsals treated with Faslodex, at a final concentration of 10^{-6} M or 10^{-5} M, had unaffected growth compared to controls (Figures 4.3A, B). In a similar fashion, murine metatarsals exposed to raloxifene at a final concentration of 10^{-5} M had unaffected longitudinal growth (Figure 4.4).

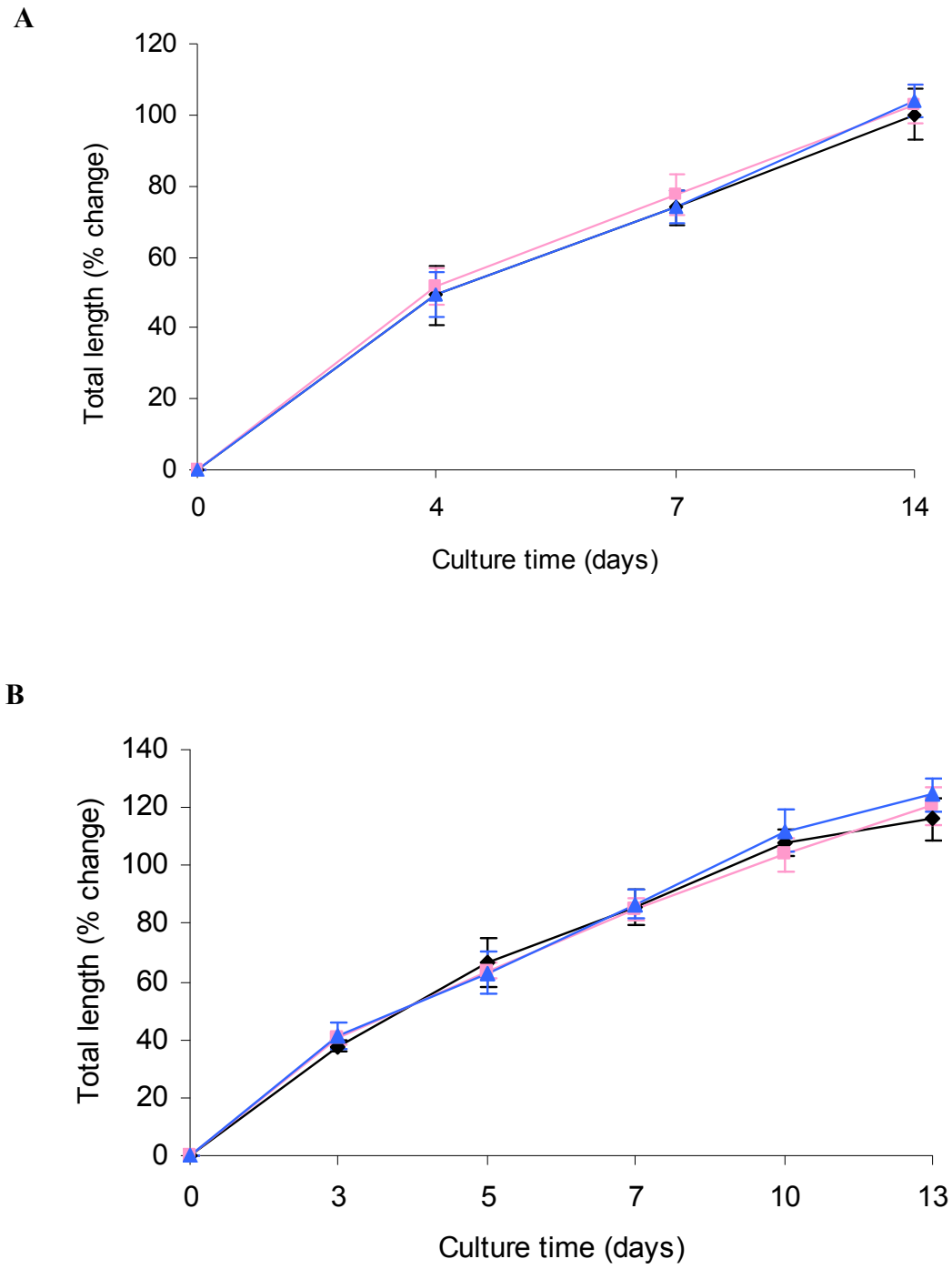


Figure 4.3 – Effect of E2 or Faslodex on longitudinal growth of murine metatarsals isolated from 18-day old foetal Swiss mice (♦= control; ■ = E2; ▲ = Faslodex). A) After 14 days exposure at 10^{-6} M; B) After 13 days exposure at 10^{-5} M. Data are expressed as means ± SEM (n = 6).

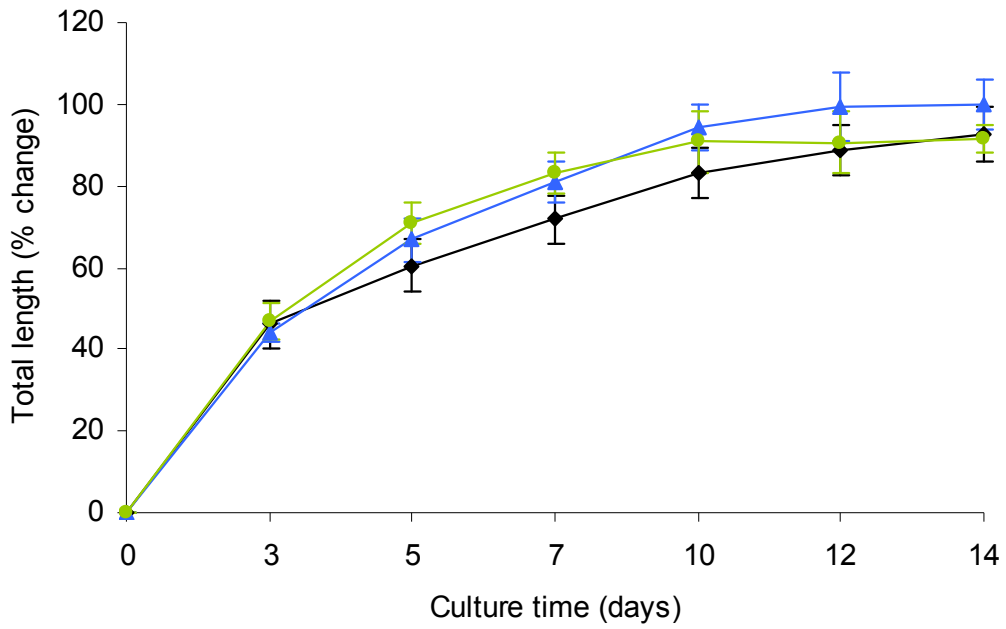


Figure 4.4 – Effect of Raloxifene on longitudinal growth of murine metatarsals isolated from the hind legs of 18-day old foetal Swiss mice and cultured for 14 days at 10^{-5} M (♦= control; ● = Raloxifene; ▲ = Faslodex). Data are expressed as means ± SEM (n = 6).

Whereas, metatarsals treated with the combination of raloxifene and MPP, at a final concentration of 10^{-5} M, were significantly shorter than control bones from day 5 onwards (Figure 4.5). Metatarsals treated with MPP alone, at a final concentration of 10^{-5} M, also showed significant growth retardation (Figure 4.5). The combination of raloxifene and MPP inhibited metatarsal growth to a greater extent than MPP alone with the reduction in total length: 29% vs. 16% on day 3; 55% vs. 25% on day 5; 57% vs. 23% on day 7; 68% vs. 41% on day 10 and 74% vs. 39% on day 14. There was no difference observed in response between metatarsals isolated from female and male embryos (Figure 4.6).

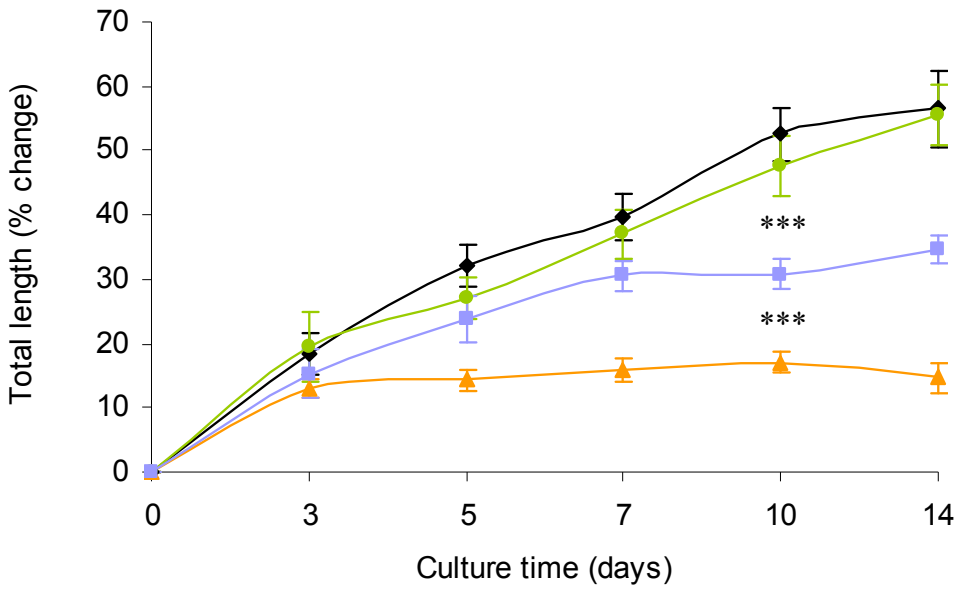


Figure 4.5 – Effect of Raloxifene, MPP or Raloxifene & MPP on longitudinal growth of murine metatarsals cultured over a 14-day period at 10^{-5} M (♦= control; ● = Raloxifene ■ = MPP; ▲ = Raloxifene & MPP. Data expressed as at means ± SEM (n = 6). ***P<0.001 compared with controls.

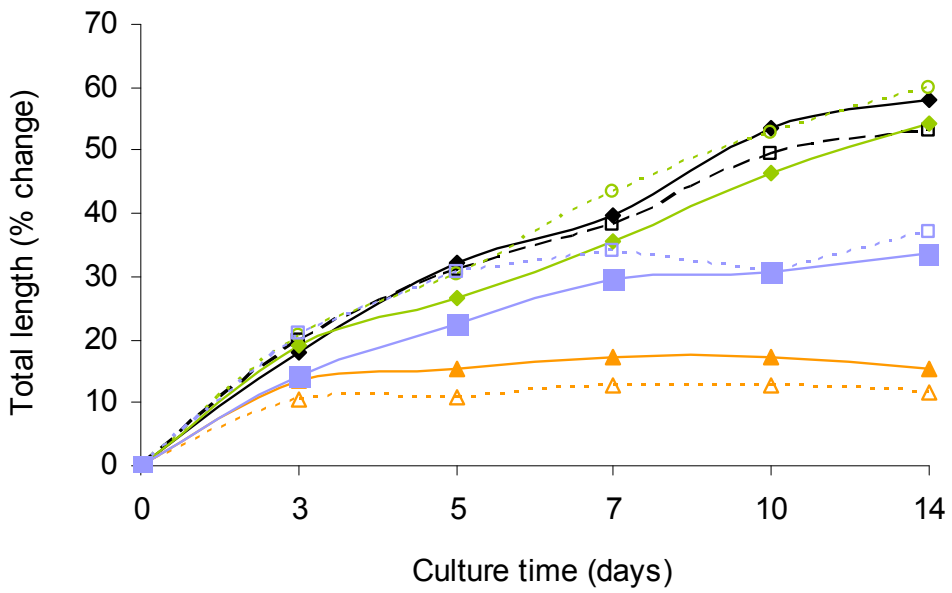


Figure 4.6 – Effect of Raloxifene, MPP or Raloxifene & MPP on longitudinal growth of male or female murine metatarsals (♦= control; ● = Raloxifene ■ = MPP; ▲ = Raloxifene & MPP. Female metatarsals = broken line; male metatarsals = continuous line). Data expressed as means.

4.5.2 Mineralising zone growth of foetal metatarsal culture system

After 14 days of culture E2, at a final concentration of 10^{-6} M and 10^{-5} M, had no effect on the length of the mineralising zone (Figure 4.7A, 4.7B). Whereas, metatarsals treated with Faslodex at a final concentration of 10^{-6} M showed a reduction of the length of the mineralising zone (17.2% reduction vs. control bones, $P<0.05$; Figure 4.7B). A greater reduction of the mineralising zone length was observed in metatarsals exposed to Faslodex at a final concentration of 10^{-5} M (50.9% reduction vs. control bones, $P<0.001$; Figure 4.7B). A reduction of the mineralising zone length was also observed in metatarsals treated with raloxifene and MPP alone at a final concentration of 10^{-5} M (36.1% and 22.2% reduction respectively vs. control bones, $P<0.001$; Figure 4.8A). In addition, a greater reduction was observed when these compounds were used in combination (65.2% reduction vs. control bones, $P<0.001$; Figure 4.8A).

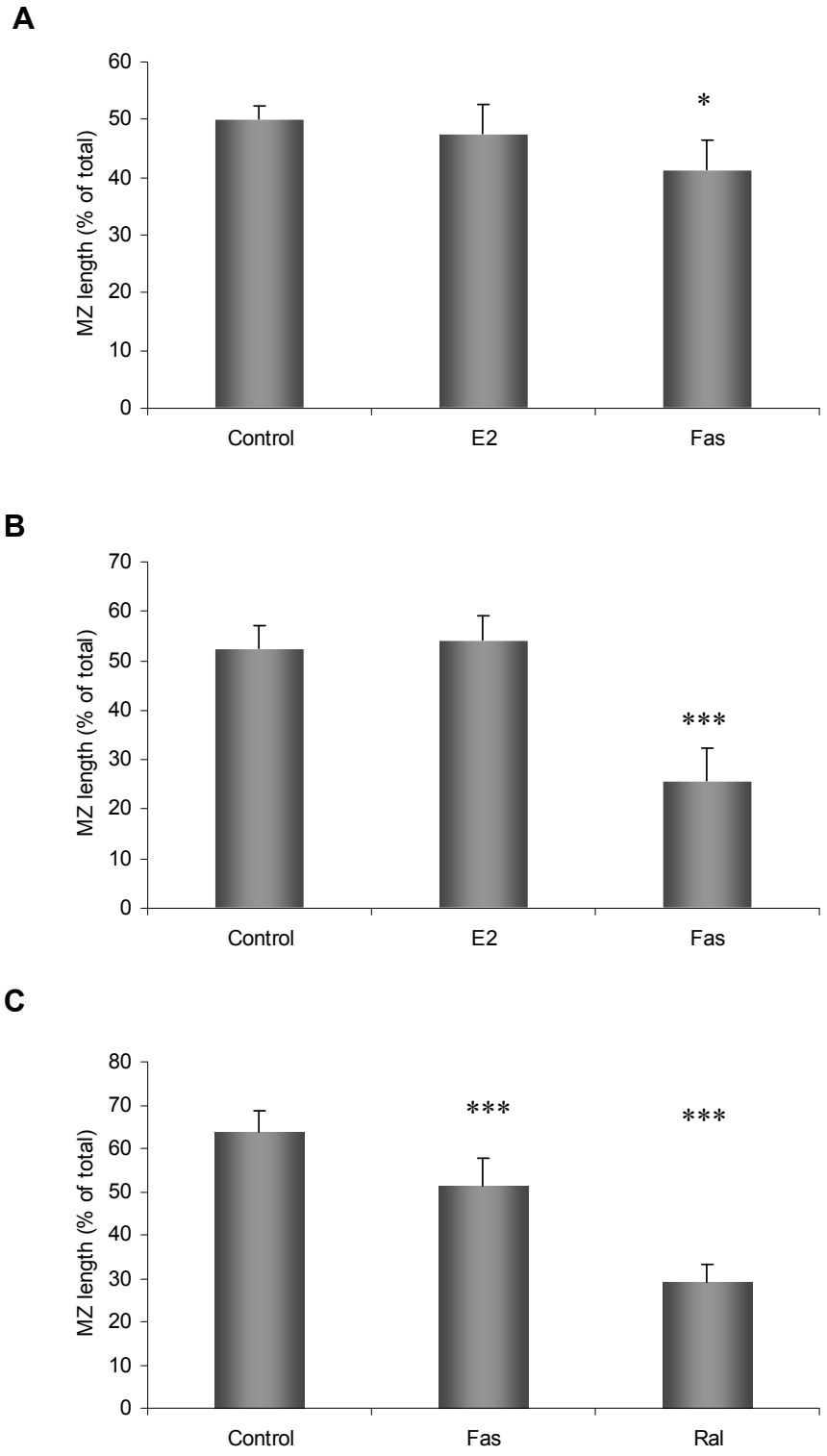
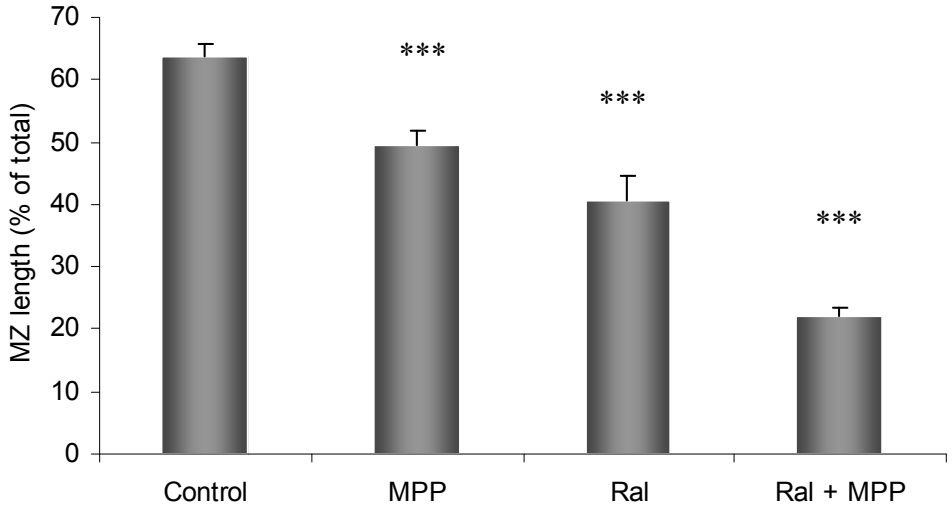


Figure 4.7 – Effect of E2, Faslodex or Raloxifene on mineralising zone growth of metatarsals cultured over 14 days. Data are expressed as means ± SEM (n = 6). (A) Effect of E2 or Faslodex at 10^{-6} M **P*<0.05 compared with controls. (B) Effect of E2 or Faslodex at 10^{-5} M ****P*<0.001 compared with controls. (C) Effect of Faslodex or Raloxifene at 10^{-5} M ****P*<0.001 compared with controls.

A



B

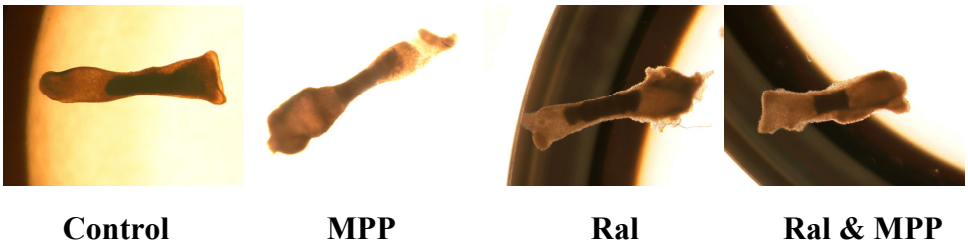
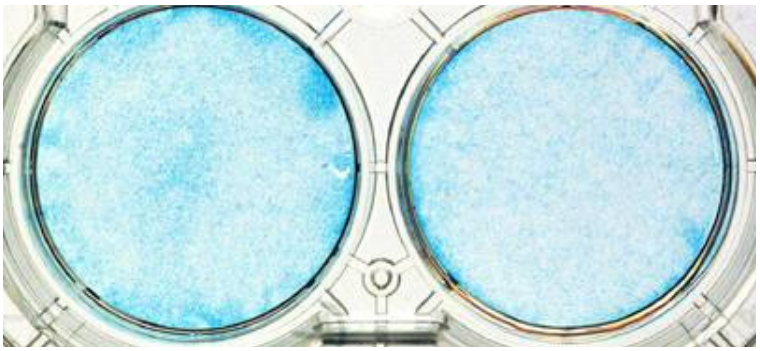


Figure 4.8 – Effect of MPP, Raloxifene or Raloxifene & MPP on mineralising zone growth of metatarsals at 10^{-5} M from 18-day old foetal Swiss mice cultured over 14 days. (A) Data expressed as means ± SEM (n = 6). ***P<0.001 compared with controls. (B) Digital image of a control metatarsal (control) and metatarsals treated with Raloxifene (Ral), MPP and the combination of Raloxifene & MPP (Ral & MPP) after 14 days exposure.

4.5.3 Primary murine chondrocytes

Primary murine chondrocytes are reported to retain their differentiated phenotype in culture for several days. This is demonstrated in the primary murine chondrocytes used in these experiments as they show positive staining with Alcian Blue on day 10 of culture reflecting the deposition of cartilage matrix (Figure 4.9).

A



B

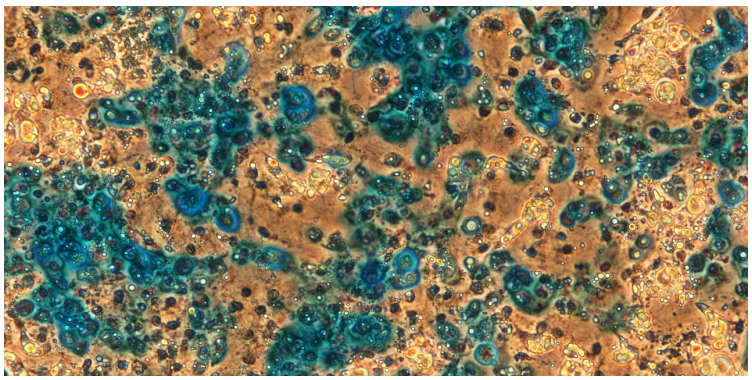


Figure 4.9 – Primary murine chondrocytes after Alcian Blue staining (A) Naked eye view on day 10 after 18 hours exposure to Alcian Blue (B) Magnified view

E2 exposure for 24 hours at a final concentration of 10^{-10} M, 10^{-8} M and 10^{-6} M had no effect on primary murine chondrocyte proliferation as analysed with [^{3}H]thymidine incorporation on day 3 of culture (Figure 4.10).

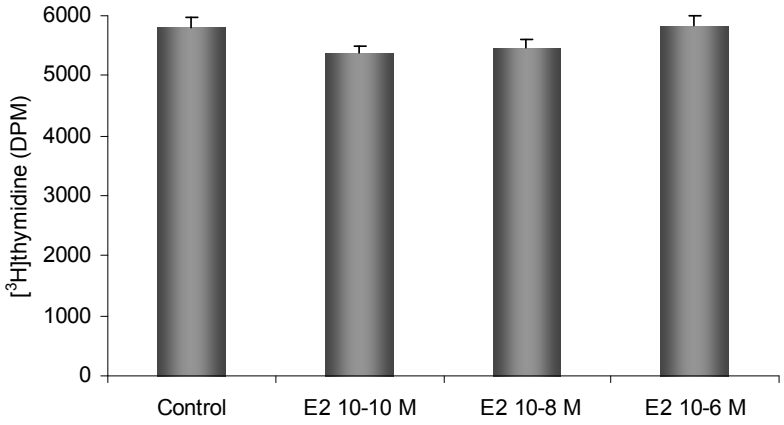


Figure 4.10 – Effect of E2 on primary murine chondrocyte proliferation as assessed by [^{3}H]thymidine uptake after 24 hour exposure. Data are expressed as means ± SEM (n=6).

Exposure of the primary chondrocytes to Faslodex, the non-specific ER antagonist, at a final concentration of 10^{-6} M for 24 hours resulted in a decrease in proliferation (10% reduction vs. control, $P<0.001$; Figure 4.11). A similar reduction in cell proliferation was observed after 24 hour exposure to raloxifene and MPP in combination at a final concentration of 10^{-6} M (11% reduction vs. control, $P<0.001$; Figure 4.11).

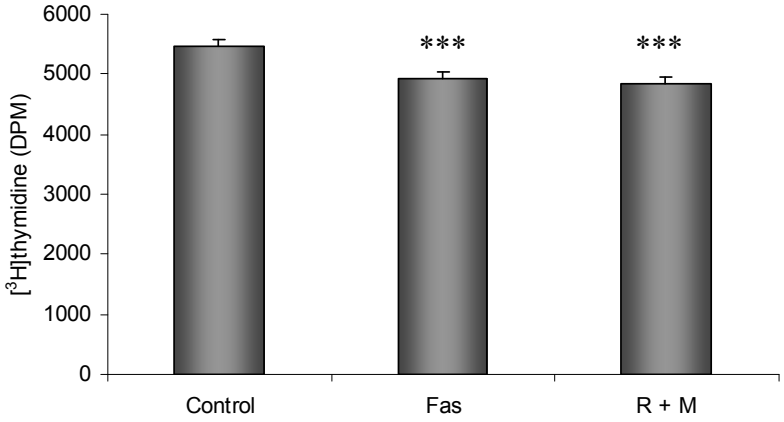


Figure 4.11 – Effect of Faslodex or Raloxifene & MPP on primary murine chondrocyte proliferation as assessed by [^{3}H]thymidine uptake after 24 hour exposure at 10^{-6} M. Data are expressed as means ± SEM (n = 6). ***$P<0.001$ compared with control cells.

4.6 Discussion

Exogenous E2, at a final concentration of 10^{-6} M or 10^{-5} M, had no effect on longitudinal growth in the foetal murine metatarsal culture model. Similarly, blocking the effect of endogenous E2 with Faslodex, the non-specific ER antagonist, also did not affect linear growth. A similar pattern of growth was observed in metatarsals obtained from female and male embryos. Chagin *et al* have also investigated the effect of E2 and Faslodex on the growth of the foetal rat metatarsal culture system and their findings were published during the completion of this project.[129] Their experiments were performed in a very similar fashion: the three middle metatarsals were dissected from the hind legs of 20-day old rat embryos and cultured for 19 days in serum-free medium. They found no effect of E2 on longitudinal growth at a final concentration of 10^{-8} M or 10^{-6} M. Metatarsals derived from both male and female embryos showed no response to E2. After 7 days of culture ERα and ERβ were widely expressed within the metatarsals and associated mainly with resting and proliferative chondrocytes. Whereas, aromatase P450 (key enzyme in oestrogen synthesis) was mainly expressed in hypertrophic chondrocytes. In contrast to our findings they found that Faslodex, at a final concentration of 10^{-5} M, reduced linear growth significantly after 7 days of culture. This response was concentration-dependent as exposure to Faslodex at a final concentration of 10^{-6} M had no effect. After exposure to Faslodex they found a relative decrease of intensity of IGF-1, IGF-2 and IGF-1Rβ immunostaining. The response to Faslodex in the foetal rat metatarsal culture system therefore differed to that observed in the mouse system. As the methodology was remarkably similar between the two studies it suggests that the discordant responses are likely due to species differences. It is the

only published report of the effect of E2 on metatarsal organ culture. Other groups have described the effect of E2 on culture of the mandibular condyle, as an alternative model of endochondral ossification. Talwar *et al* described the effects of E2 on the postnatal rat mandibular condyle culture system.[205] Mandibular condyles, obtained from 8-week old female rats, were exposed to E2 at 10^{-8} M in the presence of 10% FBS and cultured for 4 days. E2-treated condyles had a significant decrease in total cartilage thickness and mitotic index. There was an associated increase in type X collagen deposition into the extracellular matrix in the hypertrophic zone in the E2-supplemented group after 2 days. Similar results in the rat mandibular condyle culture system were described by Ng *et al.*[206] Female-derived rat condyles exposed to E2 at 10^{-8} and 10^{-6} M for 4 days had a qualitative decrease in hypertrophic chondroblasts, thickness of condylar cartilage and significant decrease in proteoglycan production. The effects of E2 on the mouse mandibular condyle culture system have also been reported.[124] Condyles obtained from 3.5 to 5.5 week old male and female mice were cultured in media supplemented with 2% FBS and exposed to E2, testosterone or DHT at a final concentration of 10^{-8} M for 3 days. The sex steroids had a direct stimulatory effect on DNA synthesis (thymidine uptake) which was sex-specific with male-derived condyles responding maximally to testosterone and female-derived condyles to E2. The E2-induced stimulation of proliferation in female-derived condyles was dependent on age with an increase of 80% observed at 4 weeks of age compared to only 40% at 5 weeks of age. In spite of the E2-induced stimulation of proliferation E2-treated male-derived condyles were 25% smaller than controls on morphometric analysis (data not shown for female-derived condyles). Thus, the effect of E2 on the proliferation of the mandibular condyle culture system

differed between the two species. This could be due to differences in species, age of the animals (3.5-5.5 week old mice compared to 8 week old rats) or experimental conditions. Further, the mandibular condyle may not be the most suitable model for investigating the effect of E2 on linear growth as there are differences reported between the process of endochondral ossification in condyles and in long bones in mice.[207]

We found no effect of raloxifene on the linear growth of the foetal murine metatarsal model. There are no other reports of the effect of raloxifene on GP chondrocytes *in vitro*. The only published data of the effects of raloxifene on longitudinal bone growth are two studies which describe the effects of raloxifene *in vivo* on OVX rabbits[208] and rats.[209] Nilsson *et al* found that raloxifene had E2-like effects on the GP of OVX rabbits. Both E2 and raloxifene inhibited GP function and led to a decrease in chondrocyte proliferation and rate of longitudinal bone growth. Raloxifene had E2-like effects on the structure of the GP with a decrease in GP height, decrease in the number of proliferative and hypertrophic cells per column and decrease in terminal hypertrophic cell height. Both raloxifene and E2 hastened epiphyseal fusion.[208] Evans *et al* also found an E2-like reduction in longitudinal bone growth compared to sham-operated control rats.[209] Raloxifene acts as an E2-agonist at the growth plate and as E2 had no effect on linear growth it is perhaps to be expected that raloxifene would similarly have no effect on the murine foetal metatarsal culture system. When raloxifene, a SERM with higher ERβ binding affinity, was co-incubated with a specific ERα antagonist (MPP) there was a marked reduction in linear growth suggesting that ERβ agonists inhibit linear growth in mice.

This inhibitory effect of ERβ on longitudinal growth is supported by the findings of Chagin *et al* in female knock-out mice models (see Table 1.1).[147] Young adult BERKO mice showed an increase in axial and appendicular skeletal growth suggesting that ERβ inhibits skeletal growth in young adult female mice. Interestingly, the growth plates were consistently fused in the appendicular skeleton of 18-month-old ERKO mice. The growth plate fusion that occurred in αERKO mice must be mediated through ERβ because old DERKO ($ER\alpha^{-/-}\beta^{-/-}$) mice displayed unchanged, unfused growth plates. Both of these knockouts have supra-physiological serum levels of E2 due to disturbed feedback regulation. In addition, exposure of the foetal murine metatarsals to MPP alone led to a reduction in longitudinal growth. As MPP is a specific ERα antagonist[75], it suggests that ERα agonists stimulate growth in mice. This is in accordance with female ER knock-out mice models where ERKO mice (ERβ present) have reduced femur length compared to BERKO mice (ERα present).[147] There are no previous reports of the effect of raloxifene and MPP on longitudinal bone growth either *in vivo* or *in vitro*.

Exogenous E2, at a final concentration of 10^{-6} M and 10^{-5} M, did not alter the growth of the mineralising zone of the metatarsals. However, blocking the effect of endogenous E2 with Faslodex led to a reduction in the growth of the mineralising zone. This effect was concentration-dependent with a greater reduction observed in metatarsals exposed to Faslodex at a final concentration of 10^{-5} M. Interestingly, both raloxifene and MPP alone, at a final concentration of 10^{-5} M, led to a reduction in mineralising zone length with a more marked reduction when used in combination. As raloxifene is principally an ERβ agonist and MPP an ERα antagonist this suggests

that ERα stimulates mineralisation whereas ERβ inhibits it. As the total metatarsal length was unchanged, this suggests that one or more of the zones must be increased in size to compensate for the reduction in the length of the mineralising zone. There may be an increase in the size of the hypertrophic zone as fewer chondrocytes are undergoing terminal differentiation. Endogenous E2 may play an important role in the terminal differentiation of chondrocytes.

In primary murine chondrocytes exposure to E2 had no effect on cell proliferation. This is in agreement with the findings in the murine ATDC5 chondrocyte cell line in chapter 2. Whereas, a reduction in cell proliferation was observed after exposure to Faslodex, the non-specific ER antagonist, suggesting that endogenous E2 may be important in the maintenance of murine chondrocyte proliferation in monolayer culture. Exposure to raloxifene and MPP in combination also led to a reduction in primary murine cell proliferation.

4.7 Conclusions

Exposure to exogenous E2 had no effect on either physiological model of murine growth. However, endogenous E2 appears to be important for maintaining cell proliferation in monolayer culture of primary murine chondrocytes but is not a prerequisite for normal longitudinal growth in the murine foetal metatarsal culture system. However, Faslodex did significantly reduce the length of the mineralising zone in the metatarsal culture system suggesting that endogenous E2 plays a role in mineralisation which may involve the terminal differentiation of hypertrophic chondrocytes within the growth plate. The mineralising zone was also reduced after

exposure to the selective ERα antagonist, MPP, suggesting that E2 may mediate its effect on mineralisation via ERα. The classical ERs appear to have opposing roles in the control of linear growth and mineralisation of the foetal murine metatarsal culture system. The data suggest that ERα stimulates growth and mineralisation whereas ERβ inhibits them.

Chapter 5

Investigation of growth inhibitory effect of Raloxifene & MPP

Chapter Contents

5.1 Introduction

5.2 Hypothesis

5.3 Aims

5.4 Materials and Methods

5.4.1 ATDC5 cell culture

5.4.2 Western blotting analysis

5.5 Results

5.6 Discussion

5.7 Conclusions

5.1 Introduction

In chapter 4 it was observed that treatment with raloxifene and MPP in combination led to retardation of longitudinal growth in the foetal murine metatarsal culture system. In the absence of readily available primary chondrocytes the ATDC5 cell line is an excellent model to investigate the molecular mechanisms causing growth restriction in metatarsals exposed to raloxifene and MPP. It has previously been established that the ATDC5 cell line undergoes the temporal sequence of events that occur during longitudinal bone growth *in vivo*.

It has been described previously that different growth plates within the same animal grow at different rates.[210] These differences in growth velocity are mainly due to the duration of the G_1 phase in proliferating chondrocytes[211] which suggests that cell cycle genes regulating G_1 progression are of crucial importance in regulating endochondral bone growth. Progression through the cell cycle is controlled by cyclin-dependent kinases (CDKs) and changes in their activity can alter chondrocyte proliferation at three different points.[212] Firstly, increased CDK activity is required to accelerate proliferation when cells enter the proliferative zone of the growth plate; secondly, changes in CDK activity can affect the speed of cell cycle proliferation and thus proliferation in the proliferative zone and thirdly inhibition of CDK activity is necessary for exit of the cell cycle upon onset of hypertrophic differentiation. The regulators of the G_1 phase, which include cyclin D1 and p21, have been shown to be targets of both extracellular and intracellular signalling pathways during cartilage development. Within the growth plate, cyclin D1 is specific for the proliferative zone at the mRNA[213] and protein levels[214] and multiple mitogenic stimuli seem to

converge on cyclin D1 gene. The cyclin E gene controls the G1/S transition in a complex with CDK2 and is also a target of mitogenic signalling in chondrocytes. p21 is one of seven CDK inhibitors. p21 gene expression is controlled by several different pathways which include: FGF signalling through STAT-1[215-217] thyroid hormone[218], bone morphogenetic protein (BMP)-2[219], SOX-9[220] and signalling through the c-Raf/MEK/ERK MAP kinase cascade.[221;222]

E2 is a potent mitogen in a number of target tissues including the mammary gland where it plays a critical role in the development and progression of breast carcinoma. This E2-induced mitogenesis has been shown to be associated with the recruitment of non-cycling, G_0 cells into the cell cycle and an increased rate of progression through G_1 phase. Further studies have elucidated that E2 regulates the expression and function of the cell cycle proteins, c-Myc and cyclin D1, and activates cyclin E-CDK2 complexes, all of which are rate limiting for progression from G_1 to S phase.[223;224]

5.2 Hypothesis

It was hypothesised that raloxifene and MPP in combination may have an inhibitory effect on cell cycle progression and reduce G_1/S phase transition.

5.3 Aims

To assess the effect of raloxifene and MPP alone and in combination on the cell cycle proteins: cyclin D1, cyclin E, c-Myc and p53 in ATDC5 chondrocytes using Western blotting analysis.

5.4 Materials and methods

5.4.1 ATDC5 cell culture

ATDC5 cell culture was performed as described in chapter 2 (2.4.2). On the second day of culture, ATDC5 cells were treated with raloxifene, MPP or raloxifene & MPP at a final concentration of 10^{-6} M in 0.01% DMSO. Control cells received 0.01% DMSO. The cells were then cultured for a further 48 hours.

5.4.2 Western blotting analysis

ATDC5 cells were lysed on day 4 in PhosphoSafe extraction buffer (Merck Chemicals Limited, Nottingham, UK) and protein concentration measured as previously described (2.4.4). Lysates (corresponding to 25 µg protein) were run on 10% Bis–Tris gels (Invitrogen) and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBST; 50 mM Tris–HCl, 300 mM NaCl, 0.1% Tween-20, pH 7.6) at 4°C overnight with gentle shaking. The membranes were probed for 1 hour at room temperature with primary antibodies raised in mouse against cyclin D1, c-Myc, and p53 (1:500 dilution in 5% milk) and raised in rabbit against cyclin E (1:500 dilution). β-actin expression was also measured as a loading control (1:5000 dilution in 5% milk). The blots were washed four times in TBST, each wash lasted 15 minutes. The membranes

were then incubated with goat anti-mouse or anti-rabbit secondary antibody for 1 hour (1:5000 dilution in 5% milk). The antibodies used are listed in Tables 5.1 and 5.2. The membranes were washed, as described above, and developed using the electrogenerated chemiluminescent labelling (ECL)-plus Western Blotting Detection System (Amersham Biosciences).

Table 5.1 – Primary antibodies used for western blotting analysis

Protein	Antibody	Source	Dilution
Cyclin E	Rabbit polyclonal	AbCam	1:100
Cyclin D1	Mouse monoclonal	AbCam	1:200
C-Myc	Mouse monoclonal	AbCam	1:500
p53	Mouse monoclonal	AbCam	1:500
β-actin	Mouse monoclonal	Sigma	1:5000

Table 5.2 – Secondary antibodies used for western blotting analysis

Protein	Antibody	Source	Dilution
Cyclin E	Goat anti-rabbit IgG peroxidase	Cell Signalling Technology	1:5000
Cyclin D1	Goat anti-mouse IgG peroxidase	Sigma	1:5000
C-Myc	Goat anti-mouse IgG peroxidase	Sigma	1:5000
p53	Goat anti-mouse IgG peroxidase	Sigma	1:5000
β-actin	Goat anti-mouse IgG peroxidase	Sigma	1:5000

5.5 Results

Expression levels of the cell cycle proteins, cyclin E, cyclin D1, C-Myc and p-53, were determined by Western blotting analysis using polyclonal or monoclonal antibodies. A control housekeeping protein, β-actin, was used to indicate the relative levels of the protein loaded on to the gel. Exposure of ATDC5 cells to raloxifene and MPP in isolation, for 48 hours at a final concentration of 10^{-6} M, had no effect on the cell cycle proteins: cyclin E, cyclin D1, C-Myc and p-53 compared to control cells (Figure 5.1). Whereas exposure of the ATDC5 cells to raloxifene and MPP in combination, for 48 hours at a final concentration of 10^{-6} M, led to a reduction in both cyclin E and p-53 protein levels. Cyclin D1 and c-Myc were unaffected by raloxifene and MPP treatment (Figure 5.1).

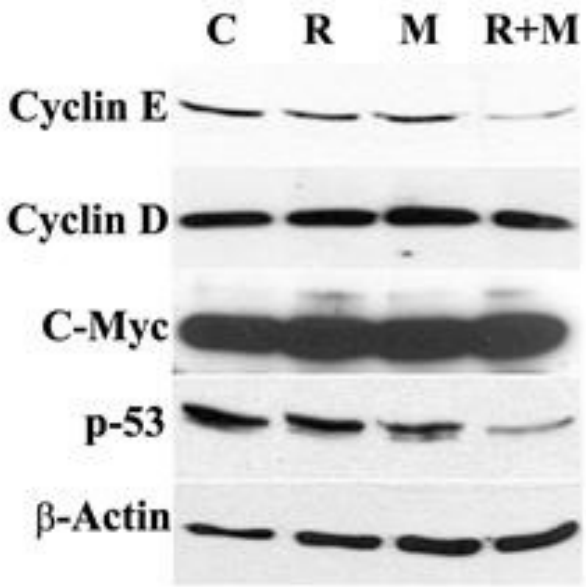


Figure 5.1 – Western blot: Effect of Raloxifene, MPP or Raloxifene and MPP on cell cycle proteins compared to control ATDC5 cells (C) after 48 hours exposure at 10^{-6} M.

5.6 Discussion

The levels of the cell cycle proteins were unchanged in the ATDC5 cells treated with raloxifene or MPP alone. However, when the cells were exposed to the same compounds in combination a decrease in cyclin E and p53 protein levels were observed. Cyclin E specifically regulates the G_1/S phase transition of the cell cycle. A high level of cyclin E accelerates the transition of the cell through the G_1 phase and is of prognostic value in breast cancer, with high levels of cyclin E in the tumour correlating with a poorer outcome and low levels with a good outcome.[225] The G_1/S phase cell cycle check point controls the commitment of cells to transition through the "gap" phase (G_1) and enter into the DNA synthesis phase (S). Two cell cycle kinase-complexes, CDK4/6-cyclin D and CDK2-cyclin E, work in collaboration to relieve inhibition of a dynamic transcription complex that contains the retinoblastoma protein, Rb, and the transcription factor, E2F. Several different stimuli exert control over the checkpoint acting through transcription factors to induce specific members of the INK4 or KIP/CIP families of CDK inhibitors (Figure 5.2). p15, p16, p18 and p19 are members of the INK4 tumour suppressor family and exert control over the CDK4/6-cyclin D complex whereas p21 and p27 influence the CDK2-cyclin E complex. As exposure of ATDC5 cells to raloxifene and MPP in combination led to a decrease in cyclin E protein levels but did not alter cyclin D levels it suggests that the effects of Raloxifene and MPP may be mediated by the CDK inhibitors p21 or p27. These findings suggest a possible mechanism for the growth retardation observed in the foetal metatarsal culture system exposed to raloxifene and MPP. As MPP is a specific ERα antagonist and raloxifene is mainly an ERβ agonist it is likely that the effects are mediated by ERβ. Similar findings

have been reported in the HCT8 human colon cancer cell line where over-expression of ERβ inhibited cell proliferation through regulation of cell cycle components. Investigation of the cell cycle showed a decrease in cyclin E and an increase in the CDK inhibitor p21 which was similar to our findings. Flow cytometry analysis provided evidence for blocking of the G_1/S phase progression which was induced by ERβ over-expression. Further, the magnitude of this effect was affected by the level of ERβ over-expression.[202] Therefore ERβ may regulate cell proliferation through the control of key cell cycle modulators and arrest in G_1/S phase transition in chondrocytes as well as in colonic cells.

5.7 Conclusions

Exposure of ATDC5 chondrocyte cells to raloxifene or MPP in combination led to a decrease in the cell cycle proteins cyclin E and p53. The decrease in cyclin E could reflect a decrease in progression of the G_1/S phase in the cell cycle. Therefore, ERβ may regulate cell proliferation in chondrocytes through the control of key cell cycle modulators.

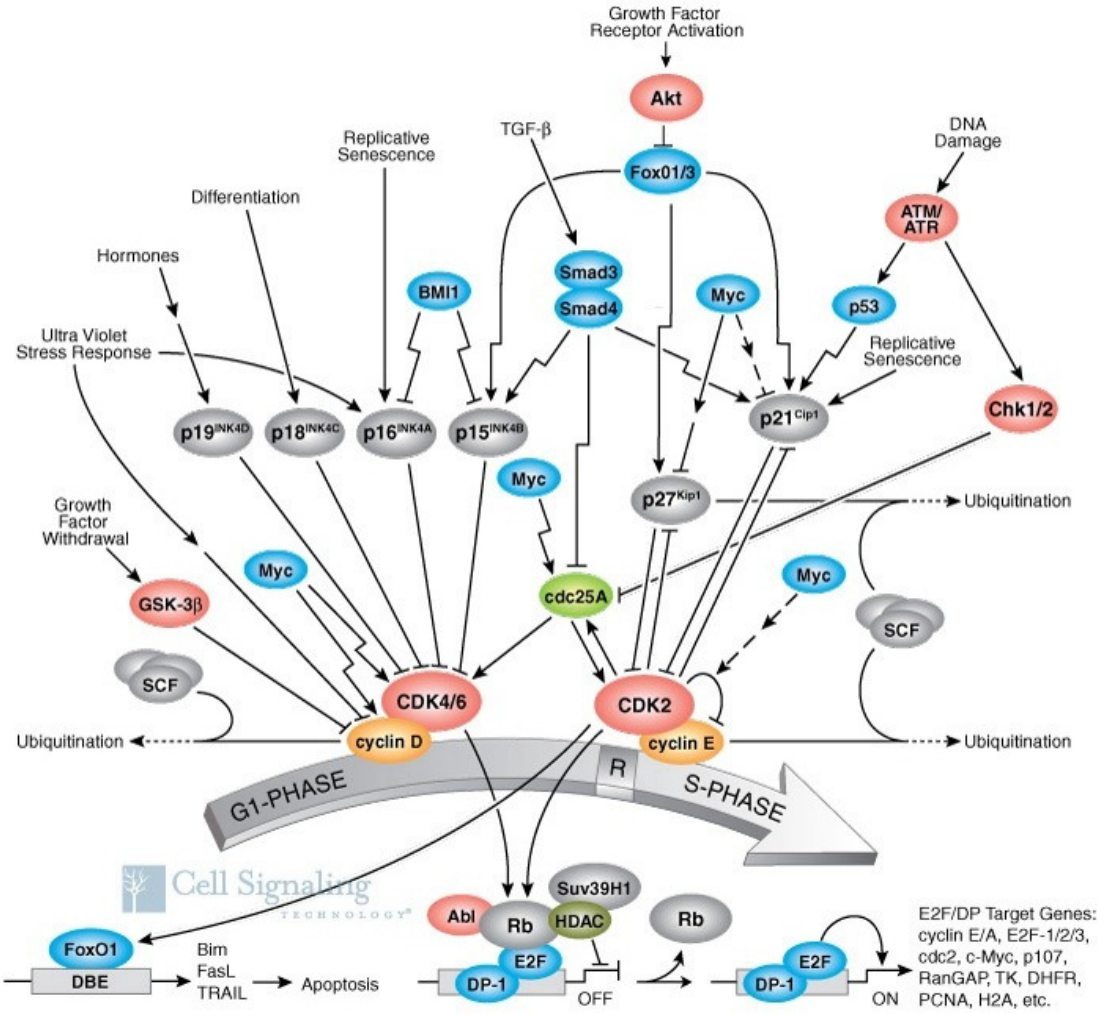


Figure 5.2 – Control of the G_1/S cell cycle checkpoint
Pathway diagram reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com)

Chapter 6

Effect of oestradiol on mineralisation

Chapter Contents

6.1 Introduction

6.2 Aims

6.3 Materials and Methods

6.3.1 Foetal metatarsal organ culture

6.3.2 Non-radioactive calcium phosphate deposition assay

6.3.3 ALP activity in ATDC5 chondrocytes

6.3.4 Statistical analysis

6.4 Results

6.4.1 Calcium content of metatarsals treated with Faslodex or E2

6.4.2 ALP activity in ATDC5 cells treated with Faslodex or Raloxifene

6.5 Discussion

6.6 Conclusions

6.1 Introduction

In chapter 4 it was observed that E2 treatment had no effect on the mineralising zone length of the foetal murine metatarsal model whereas Faslodex led to a reduction in the mineralising zone length but with no effect on overall longitudinal growth. As the calcium concentration of bone is a reflection of the amount of mineralisation that has occurred a measure of the calcium concentration of metatarsals treated with E2 and Faslodex would be invaluable. A reduction in the calcium concentration of metatarsals treated with Faslodex in support of the findings from the murine metatarsal culture system would be expected. Anderson *et al* describe a highly sensitive and simple assay which is an alternative to the routinely used radiometric biomineralisation assay which employs radioactive $^{4.5}Ca$.[226] In addition, as the well established ATDC5 chondrocyte cell line has been shown to undergo the temporal sequence of events that occur during longitudinal bone growth *in vivo* it provides an excellent model to investigate the possible enzymatic mechanisms leading to the reduction in mineralising zone length observed in metatarsals treated with Faslodex.

6.2 Aims

I. Analyse the calcium content of metatarsals treated with E2 and the non-specific ER antagonist Faslodex

II. Investigate the effect of the E2-related chemicals, Faslodex and raloxifene on ALP activity of ATDC5 cells during terminal differentiation

6.3 Materials and methods

6.3.1 Foetal metatarsal organ culture

The metatarsals were treated with Faslodex or E2, at a final concentration of 10^{-5} M, in 0.1% ethanol for 14 days as described in chapter 4 (4.4.1). Control metatarsal bones received 0.1% ethanol only. At the end of the culture period the metatarsals were washed with sterile PBS and stored at -20°C until analysis.

6.3.2 Non-radioactive calcium phosphate deposition assay

Each metatarsal was placed in a glass vial with 1 ml of 0.6N HCl and solubilised for 24 hours. The calcium content of the HCl supernatant was then determined colorimetrically by the *O*-cresolpthalein complexone method (Calcium Kit, Sigma).[226] Five standards of calcium were prepared, ranging from 0.6125 mM to 10 mM, from a calcium standard (10mg/dl, Calcium kit). 2 µl of calcium standard or sample (acidified supernatant) was incubated with 200 µl of calcium working reagent (Colour Reagent and Base Reagent Mix). The 96-well plate was then incubated at room temperature for 1 minute and the absorbance read at 570 nm using a microplate reader. The absorbencies of the samples were compared to a standard curve generated from the absorbencies from the standards.

6.3.3 ALP activity in ATDC5 chondrocytes

ATDC5 cell culture was performed as described in chapter 2 (2.2.2). Faslodex or raloxifene were added to ATDC5 cells at day 13, at a final concentration of 10^{-6} M, and the cells were then incubated for 7 days. For analysis of ALP activity, cells were rinsed with PBS and lysed with 0.9% NaCl and 0.2% Triton-X 100 and centrifuged

at 12000g for 15 minutes at 4°C. The supernatant was assayed for protein content and ALP activity as a measure of cell number and chondrocyte differentiation, respectively. The protein content of the supernatant was measured as described in chapter 2 (2.4.3). Enzyme activity was determined by measuring the cleavage of 10mM p-nitrophenyl phosphate (pNPP) at 410nm. Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein.[176]

6.3.4 Statistical analysis

Data were analysed by ANOVA. All data are expressed as the mean ± SEM of at least six replicates within each experiment, and statistical analysis was performed using SPSS (version 15). Significance was determined as $P < 0.05$.

6.4 Results

6.4.1 Calcium content of metatarsals treated with Faslodex or E2

Exposure to E2 for 14 days, at a final concentration of 10^{-5} M, had no effect on the calcium concentration in the metatarsal bones whereas Faslodex, at a final concentration of 10^{-5} M, caused a reduction in the calcium concentration (Figure 6.1). After the calcium assay was performed the mineralising zones were no longer present in the metatarsal bones demonstrating that the calcium mineral has been leached (Figure 6.2).

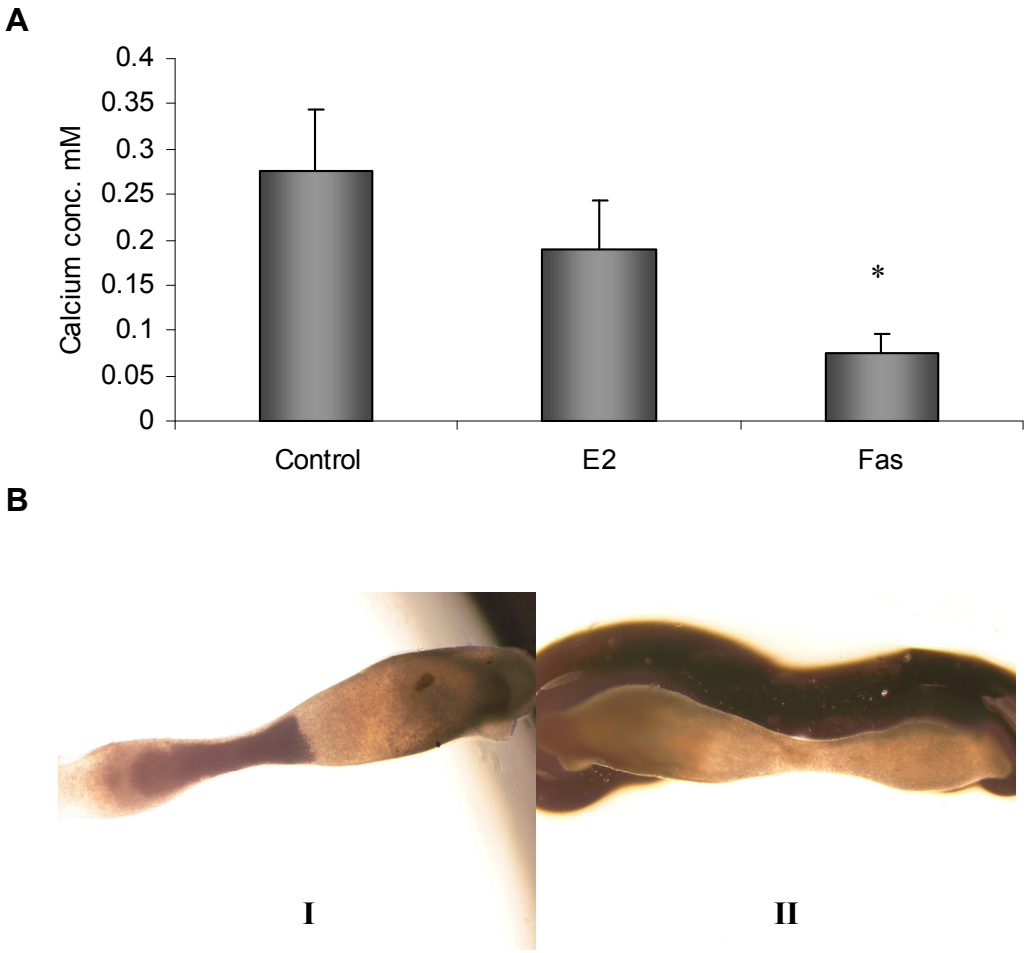


Figure 6.1 – Effect of E2 or Faslodex on calcium concentration of metatarsals from 18-day old foetal Swiss mice cultured over 14 days at 10^{-5} M as assessed by calcium assay. (A) Data are expressed as means ± SEM (n = 6). *P<0.05 compared with control metatarsals. (B) Digital image of a control metatarsal on day 14 of culture (I) and after the calcium assay was performed (II).

6.4.2 ALP activity in ATDC5 cells treated with Faslodex or Raloxifene

After 7 days of exposure both Faslodex and Raloxifene, at a final concentration of 10^{-6} M, reduced ALP activity of ATDC5 chondrocytes during terminal differentiation compared to control cells (Figure 6.2).

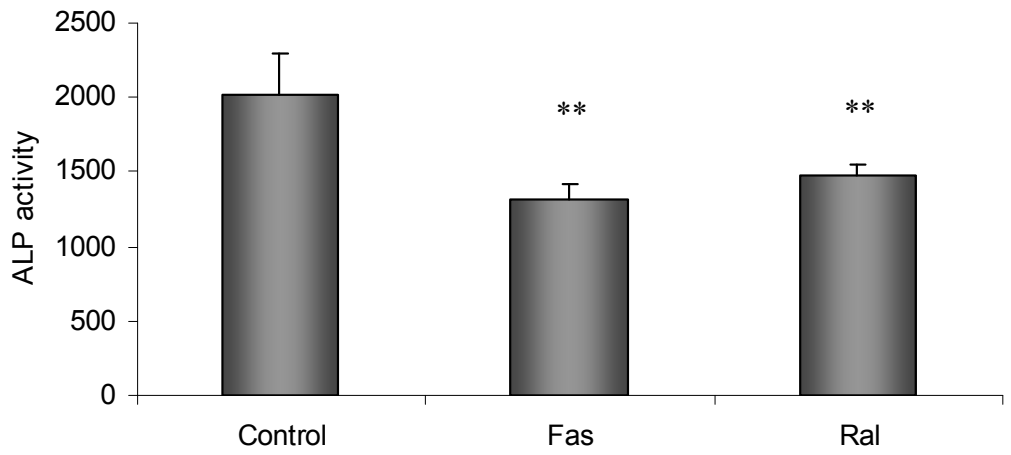


Figure 6.2 – Effect of Faslodex or Raloxifene on ALP activity in ATDC5 cells (nmoles pNPP hydrol/min/mg protein) after 7 days exposure at 10^{-6} M. Data are expressed as means ± SEM (n = 6). ***P*<0.01 compared with control cells.

6.5 Discussion

The foetal murine metatarsals exposed to Faslodex showed a reduction in calcium concentration when compared with control bones after 14 days in culture. These findings are in accordance with the data obtained from the foetal metatarsal culture system where Faslodex treatment led to a reduction of the mineralising zone length. Exogenous E2 had no effect on the mineralisation of foetal metatarsals with an unchanged mineralising zone length and calcium concentration after 14 days of

culture. In contrast, a reduction in mineralisation was observed when endogenous E2 action was blocked with the non-specific ER antagonist, Faslodex suggesting that endogenous E2 is important for mineralisation. As the overall length of metatarsals treated with Faslodex was not affected it suggests that the proliferative and/or the hypertrophic zones of the metatarsals are increased following the withdrawal of E2 action by Faslodex. There may be an increase in non-mineralised hypertrophic chondrocytes. Exposure of the ATDC5 chondrocyte cell line to Faslodex or raloxifene led to a reduction in ALP activity during terminal differentiation as compared to control cells. Whereas, exposure to exogenous E2 did not alter the ALP activity of ATDC5 chondrocytes as previously described in chapter 2. The process by which chondrocytes mineralise their extracellular matrix is complex and tightly controlled both temporally and spatially and is limited to a few layers of hypertrophic chondrocytes at the chondro-osseous border. Terminally differentiated hypertrophic chondrocytes are characterised by an increase in intracellular calcium concentration which is essential for the production of matrix vesicles. These small membrane-enclosed particles are released from chondrocytes[227;228] and allow calcium phosphate precipitation into mineral crystals. However, the mechanisms regulating matrix mineralisation are not completely understood and it is still unclear whether it is an active process effected by the presence of promoters of mineralisation or a passive process occurring in the absence of mineralisation inhibitors.[229] However, it is clear that mineralisation of the matrix is a biphasic process with the first phase occurring completely within the matrix vesicle and is dependent on the attainment of sufficiently high concentrations of inorganic phosphate for the formation of de novo hydroxyapatite crystals. The generation of the inorganic phosphate was previously

attributed to the actions of ALP[230] whereas it is now evident that other phosphatases also play a role, such as pyrophosphatase, 5'AMPase, and ATPase and the recently described PHOSPHO1, which are known to be present in matrix vesicles.[230-233] In addition, ALP has been localised to the outer surface of the matrix vesicle and has been shown to act as a pyrophosphatase *in vivo*. During the second phase, the mineral crystals pierce the matrix vesicle membrane and enlarge with the addition of calcium and phosphate ions present in the extravesicular space. These mineral sphericules associate closely to collagen fibrils. The process of mineralisation, along with the low oxygen tension, attracts blood vessels from the underlying primary spongiosa.[234] Thereafter, the remaining hypertrophic chondrocytes undergo apoptosis, which leaves a scaffold for new bone formation. ALP is therefore a matrix vesicle associated phosphatase which is involved in mineralisation along with other phosphatases. Thus, the reduction observed in the mineralisation of foetal murine metatarsals treated with Faslodex or raloxifene could be due to a direct effect on ALP activity or alternatively the decrease in ALP levels could represent a decrease in differentiation of the chondrocytes. A reduction in the mineralisation of foetal murine metatarsals is also reported after exposure to the glucocorticoid dexamethasone however a reduction in overall growth is also seen with a non-significant increase in hypertrophic zones. Whereas, metatarsals exposed to IGF-1 show a reduction in mineralisation along with an increase in overall length and size of hypertrophic zones.

6.6 Conclusions

Foetal murine metatarsals exposed to Faslodex, the non-specific ER antagonist, showed a reduction in calcium concentration after 14 days exposure. Faslodex-treated metatarsals had been shown to have normal longitudinal growth but a reduced mineralising zone growth in chapter 4. In the ATDC5 cell line exposure to Faslodex or raloxifene during terminal differentiation led to a reduction in ALP activity. Thus, the decrease in mineralisation observed in murine metatarsals treated with Faslodex or raloxifene could be due to a direct effect on ALP activity or it may reflect a decrease in chondrocyte differentiation.

Chapter 7

Effect of oral oestrogen on longitudinal growth in girls with primary ovarian insufficiency

Chapter Contents

7.1 Introduction

7.2 Aims

7.3 Subjects and Methods

7.3.1 Subjects

7.3.2 Methods

7.3.3 Ethical approval

7.3.4 Statistical analysis

7.4 Results

7.4.1 POI associated with Turner syndrome

7.4.2 Non-Turner syndrome associated POI

7.5 Discussion

7.6 Conclusions

7.1 Introduction

During the first ten weeks of development, the primordial germ cells undergo multiple mitotic divisions and differentiate into primordial follicles. The primordial follicle consists of a primary oocyte surrounded by a layer of spindle-shaped granulosa cells. A primary oocyte is an oocyte that has not yet undergone its first meiotic division. Primordial follicles continue to multiply by mitosis and girls are endowed with their maximum number of 6-7 million at approximately 20 weeks of gestation.[235] Before birth, the primary oocytes enter a final round of DNA synthesis and are then arrested in prophase of the first meiotic division until shortly before ovulation. Thereafter, there is a gradual decrease in the number of follicles due to atresia, which is independent of ovulation. At birth, the number of follicles has decreased to 700 000 and fallen further to 400 000 at the time of puberty. Each month the antral or Graafian follicle, a completely new secretory structure, develops within the ovary from a primordial follicle. The persistent atresia of oocytes together with monthly ovulation leaves few follicles remaining at menopause.[236] Menopause, defined as the permanent cessation of menses, is due to the depletion of potentially functional primordial follicles and usually occurs at a mean age of 50 years.[237] If menopause occurs before the age of 40 years it is considered to be premature. In view of the constant oocyte atresia, early menopause could occur in any female who begins life with a decreased number of follicles, or experiences accelerated follicle apoptosis or destruction. This condition is now referred to as primary ovarian insufficiency (POI) in preference to previous nomenclature (premature menopause, premature or primary ovarian failure, hypergonadotrophic hypogonadism).[238;239] POI is said to exist when a woman who is less than 40 years

old has amenorrhea for 4 months or more, with two serum FSH levels obtained at least 1 month apart within the menopausal range.[240;241] POI differs from menopause in that the ovarian function is variable and unpredictable in 50% of cases, and approximately 5 to 10% of women conceive and deliver a child after the diagnosis has been made.[240;242-245] Thus, POI includes a continuum of impaired ovarian function rather than a dichotomous state.[238] In the majority of women diagnosed with spontaneous POI the cause remains unclear. However, spontaneous POI in 46,XX individuals can occur as part of a syndrome while many single genes (bone morphogenetic protein 15, diaphanous homolog 2, and inhibin alpha subunit) have been associated with non-syndromic POI. Structural abnormalities in the X chromosome apart from specific gene mutations are also implicated. POI occurs through two principal mechanisms: follicle depletion and follicle dysfunction.[241] Follicle depletion signifies that no primordial follicles remain in the ovary. This may be due to an inadequate initial pool of primordial follicles *in utero*, accelerated expenditure of follicles or destruction of follicles (see Table 7.1). By contrast, the term follicle dysfunction indicates that follicles remain in the ovary but a pathological process is preventing their normal function (see Table 7.2).

The most common genetic condition associated with POI is Turner syndrome (TS) and is the result of follicle depletion. Girls with TS have a normal quantity of oocytes until the third month of gestation, but apoptosis is then accelerated[246-248] usually resulting in the depletion of oocytes by the first decade of life. Therefore, only 10% of TS women reach menarche. However, patients with 45,X/46,XX mosaicism comprising a normal second X chromosome in the 46,XX cell line are more likely to

menstruate (40%) and may not develop overt POI for many years. Undetected X chromosome mosaicism could explain some cases of spontaneous POI.[249] The cause of the ovarian dysfunction remains unclear despite the identification of a critical region of the X chromosome for ovarian development and function (Xq13 to Xq26 with the exception of Xq22).[250]

Table 7.1 – Causes of ovarian follicular depletion
Adapted from Nelson *et al*, 2009[239]

Cause	Findings	Gene Identification (location)
Insufficient follicle number		
Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES)	Familial POI; Pervasive block in primordial follicle development in knockout mouse	*FOXL2* Forkhead transcription factor (3q23)
Spontaneous accelerated follicle loss		
Turner syndrome	Normal pool of primordial follicles established in foetal ovary but accelerated apoptosis of follicles usually depletes stores before puberty; in oocytes, both X chromosomes must be present and active to prevent accelerated follicular atresia; individual genes responsible not identified	
Metabolic-toxin-induced follicle loss		
Galactosaemia	Abundant, normal appearing follicles in 5-day-old infant but rare follicles, fibrous-streak stroma, and immature-hyalinised follicles in post-pubertal females	*GALT* Galactose-1-phosphate Uridylyltransferase (9p13)
Iatrogenic		
Surgery	After unilateral oophorectomy women have higher FSH levels and poorer response to ovarian stimulation and an earlier menopause than women with two ovaries	
Chemotherapy	8% of childhood cancer survivors at 18y develop POI; associated factors include: Hodgkin's lymphoma, alkylating agents (cyclophosphamide/procarbazine), and older age at diagnosis (>12y)	
Radiotherapy	30% develop POI when treated with radiation and alkylating agents in combination. POI is > 50% in women >21y treated with alkylating agents and almost 100%, regardless of age, in women undergoing preparation for bone marrow transplantation	
Environmental-toxin–induced follicle loss		
Industrial exposure to 2-bromopropane	POI in 16 Korean women exposed to cleaning solvent	

Table 7.2 – Causes of ovarian follicular dysfunction
Adapted from Nelson *et al*, 2009[239]

Cause	Findings	Gene Identification (location)
Signal defect		
FSH-receptor mutation	Primary amenorrhoea, small ovaries and elevated FSH levels; presence of ovarian follicles on biopsy; rare except in Finland (founder effect)	*FSHR* FSH receptor (2p21-p16)
LH-receptor mutation	Primary amenorrhoea, low E2, elevated LH levels and cystic ovaries; ovarian follicles on ultrasound	*LHCGR* LH/choriogonadotrophin receptor (2p21)
G-protein mutation	Secondary amenorrhea, high LH/FSH and low E2 levels that responded to gonadotrophin in pseudohypoparathyroidism	
Enzyme deficiency		
Isolated 17,20-lyase deficiency	Primary amenorrhea, high LH/FSH enlarged cystic ovaries due to block in E2 synthesis	*CYP17A1* 17-α hydroxylase/17,20-lyase enzyme (10q24-3)
Aromatase deficiency	Primary amenorrhea; ovarian enlargement or hyperstimulation as ovary unable to aromatise androstenedione to E2	*CYP19A1* Cytochrome P450, family 19, subfamily A, polypeptide 1
Lipoid congenital adrenal hyperplasia	Severe adrenal insufficiency; progress through puberty but cycles anovulatory and develop high LH levels and polycystic ovaries; lipoid deposition in theca cells disrupts StAR-independent steroidogenesis	*StAR* Steroidogenic acute regulatory protein (8p11-2)
Autoimmunity		
Autoimmune lymphocytic oophoritis	Lmphocytic infiltration of antral follicles into theca, but sparing of primordial follicles, multifollicular ovaries	
- Polyglandular failure type 1 or APECED (Autoimmune PolyEndocrinopathy-Candidiasis-Ectodermal Dystrophy)	Mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency accompanied by POI in 60% of cases	*AIRE* Autoimmune regulator (21q22-3)
- Polyglandular failure type 2	Adrenal insufficiency and hypothyroidism associated with POI in 10% of cases	HLA association
Insufficient follicle number		
Luteinized Graafian follicles	40% of idiopathic spontaneous 46,XX POI have antral follicles on ultrasound, on histology >60% are luteinised, major mechanism of follicular dysfunction	

POI affects approximately 1 in 100 women by the time they reach 40 years of age[251] and an underlying cause is only identified in the minority of cases. In a recent series of 357 consecutive women presenting with POI a genetic cause was only determined in 7%.[252] The aetiology and prevalence of POI in girls below 18 years of age has not previously been described.

7.2 Aims

I. To identify the incidence and prevalence of primary ovarian insufficiency within children in the West of Scotland

II. To investigate the aetiology of primary ovarian insufficiency in a tertiary referral centre

III. To describe the oestrogen treatment prescribed and its effect on height velocity, bone maturation and final height

7.3 Subjects and Methods

7.3.1 Subjects

All girls who attended the endocrinology clinic at the Royal Hospital for Sick Children (RHSC), Glasgow with a diagnosis of POI from January 1989 to January 2000 and aged from 0 to 18 years were included. They were identified using the database established by the endocrinology service in 1989 after the appointment of Dr Malcolm Donaldson, Senior Lecturer in Child Health. The diagnostic categories

retained were as follows: primary ovarian failure/insufficiency, TS, gonadal or ovarian dysgenesis, galactosaemia, ovarian failure associated with cancer related therapies and autoimmune ovarian failure.

7.3.2 Methods

To determine the prevalence of POI the number of affected girls aged from 0 to 16 years attending the endocrine clinic each year from 1989 to 2000, with a West of Scotland postcode, were ascertained and the mean number calculated for the 11-year period. The denominator or female population aged between 0 and 16 years in the West of Scotland was obtained from the statistics section of the General Register Office for Scotland (www.gro-scotland.gov.uk). The areas included were Ayrshire, Argyll & Bute, City of Glasgow, Dunbartonshire, Dunbartonshire, Lanarkshire and Renfrewshire. To determine the incidence of POI the number of new cases of POI diagnosed each year from 1989 to 2000 was determined by examining the endocrine database at the Royal Hospital for Sick Children (the tertiary centre for these postcodes), and the mean number calculated for the 11-year period. The denominator used was the same for calculating prevalence. It was assumed that the endocrine team would be consulted on all cases of POI in the West of Scotland. POI is a rare event, because the absolute numbers measured for incidence and prevalence per year were less than 100, Poisson distribution was assumed. 95% Confidence Intervals were obtained by multiplying the number of events by the appropriate confidence level factors from a table.[253] The 95% Confidence Interval for each event was then divided by the denominator to give the 95% Confidence Interval for prevalence or incidence. A retrospective review of case notes of girls identified by the database,

who were born before January 1991 and would have now reached FH, was performed to investigate the effects of oestrogen therapy on pubertal growth and FH. The following data were recorded: age at diagnosis, aetiology, karyotype, clinical presentation, parental heights and family history. At each outpatient clinic visit during oestrogen treatment the following information was collected: height, weight, pubertal staging, blood pressure, bone age, pelvic ultrasound, serum gonadotrophins (LH, FSH) and treatment regime. Bone age was determined according to the Tanner and Whitehouse radius, ulna, short bones score (TW2 method).[254] At the end of pubertal induction the age of bony fusion (bone age scored as 16 years), uterine development on pelvic ultrasound and final height was recorded.

7.3.3 Ethical approval

This work was acknowledged by our local Research & Development department to constitute audit therefore ethical approval was not required.

7.3.4 Statistical analysis

Unpaired T-tests were used to investigate any differences, for continuous normally distributed data, between TS girls with spontaneous menarche and those who required pubertal induction with oral oestrogen using SPSS version 15. Significance was determined as $P<0.05$. For girls with non-TS associated POI descriptive analysis was only performed in view of the small number of patients. All results are described as mean (SD) unless otherwise indicated.

7.4 Results

One hundred and forty-one girls attended the endocrinology clinic with a diagnosis of POI over the 11 year period. The majority of girls either had TS (83.7%) or acquired POI associated with total body irradiation (TBI) and bone marrow transplantation (12.8%). The aetiology of POI is shown in Figure 7.1.

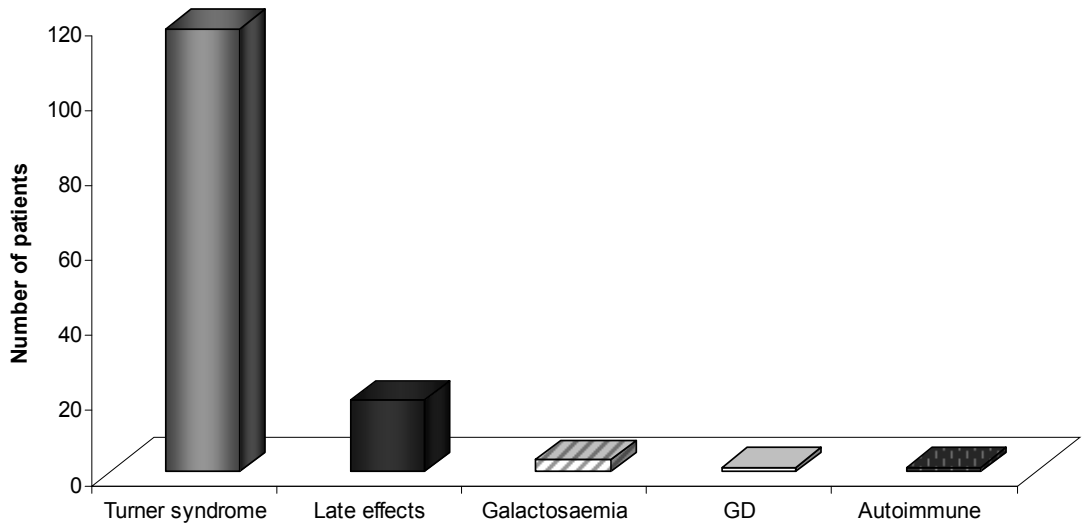


Figure 7.1 – Aetiology of POI in patients who attended RHSC from 1989 to 2000 (n = 141): GD; gonadal dysgenesis.

The prevalence of all cases of POI in the West of Scotland during 1989 and 2000 was 32 (95% Confidence Interval 25 to 40) per 100 000 girls aged between 0 and 16 years. The incidence of all cases of POI was 4 (95% Confidence Interval 1 to 7) per 100 000 girls per year. The prevalence of TS in the West of Scotland paediatric population was 30 (95% Confidence Interval 23 to 38) per 100 000 girls and the incidence was 3 (95% Confidence Interval 1 to 7) per 100 000 girls per year. The prevalence of non-TS associated POI was 2 (95% Confidence Interval 0.4 to 5) per 100 000 girls; and the incidence 0.5 (95% Confidence Interval 0.01 to 3) per 100 000 girls per year.

7.4.1 POI associated with Turner syndrome

One hundred and eighteen girls attended the endocrine clinic with TS confirmed by karyotype and their characteristics are shown in Table 7.3.

Table 7.3 - Characteristics of all girls with TS who attended the endocrine clinic in RHSC between 1989 and 2000

	TS girls (n = 118)
Age at diagnosis (y)	5 (0 - 17.3)*
Target height (cm)	160.6 (4.9)
Birth weight (kg)	2.8 (0.6)
Gestation (wk)	38.8 (2.5)

*Median (range)

Ninety-three girls are now 18 years or over and have reached final height. However, eleven girls have moved out with the West of Scotland so follow up data are not available. The characteristics of the remaining 82 girls who are now 18 years or over and have reached final height are depicted in Table 7.4. Sixteen girls (17.2%) had spontaneous puberty (SP) including menarche and therefore did not require pubertal induction. Sixty-six girls required induction of puberty and were treated with incremental doses of oral oestrogen; 64 girls received oral ethinylestradiol (EE2) and 2 girls received oestradiol valerate (Progynova) initially and then oral EE2. The oral EE2 regimes used to induce puberty are described in Table 7.5 and the uterine findings on pelvic ultrasonography are shown in Table 7.6. The girls' most recent bone ages along with their corresponding daily EE2 dose are shown in Figure 7.2.

Table 7.4 – Characteristics of TS girls who attended the endocrine clinic between 1989 and 2000 who have reached final height (n = 82)

	Induction with oral EE2	**Induction with oral Progynova**	**Spontaneous puberty (SP)**
	n=64	**n=2**	**n=16**
At start of oestrogen (or B1 if SP)			
Age at diagnosis (y)*	5.9 (5.8)	8 (4)	8.9 (4.1)*
Age (y)	13.6 (1.7)	11.7 (0.7)	11.1 (0.8)
Height (cm)	137.4 (11)	119.5 (1.1)	129.8 (5.3)
Height SD score	-2.8 (1.1)	-3.9 (0.2)	-2.1 (0.5)
Target height (cm)	161.2 (4.7)	156.1 (4.5)	159.8 (4.5)
Sitting height (cm)	76 (5.2)	-	72 (2.3)
Sitting height SD score	-2.5 (1.1)	-	-1.9 (0.6)
At end of induction (or B5 if SP)			
Age (y)	17.9 (1)	14.7 (0.1)	15.9 (0.7)
Height (cm)	150.2 (7)	133.4 (1.1)	149.3 (5)
Height SD score	-2.2 (1.2)	-4.4 (0.2)	-2.2 (0.8)
Sitting height (cm)	83.2 (3.1)	74.1 (3.4)	83 (2.5)
SH SD score	-2.7 (1.1)	- 4.2 (1.1)	-2.2 (0.8)
Δ Height SD score	0.6 (0.6)	-0.5 (0.1)	0.1 (0.6)
Δ SH SD score	-0.3 (0.8)	-	-0.3 (0.6)
Peak height velocity	Not observed	Not observed	8.1 (0.9)

*Excluding antenatal diagnosis in 2 girls

Table 7.5 - Oral ethinylestradiol regimes employed to induce puberty in girls with POI associated with TS (n = 64)

Dose of EE2 (µg/day)	**Duration of EE2 (y)**	**Number of patients (max 64)**
0.5	0.8 (0.2)	2
1	1.1 (0.5)	5
2	1.4 (1.1)	62
4	1.3 (0.9)	61
5	0.7 (0.4)	3
6	0.5 (0.6)	51
8	0.4 (0.3)	49
10	0.6 (0.3)	52
14	0.2	1
15	0.3 (0.2)	9
16	0.2	1
Total duration of EE2	4.3 (1.4)	49
EE2 duration if incomplete induction at transfer	3.6 (1.8)	16

Table 7.6 – Uterine development of girls with TS at beginning and end of puberty as assessed by pelvic ultrasonography

	Induction with oral oestrogen (n=66)	**Spontaneous puberty (n=16)**
At start of oestrogen (or B1 if SP)		
Age (y)	13.5 (1.7)	11.1 (0.8)
Uterine length (cm)	3.8 (1)^{NS}	3.3 (0.7)^{NS}
Fundal-cervical ratio	0.8 (0.3)^{NS}	0.9 (0.2)^{NS}
At end of induction (or B5 if SP)		
Age (y)	17.8 (1)	15.9 (0.7)
Uterine length (cm)	6 (1.2)*	6.9 (1.2)*
Fundal-cervical ratio	1.2 (0.3)*	1.5 (0.3)*

Difference between groups: *P<0.05; NS: not significant.

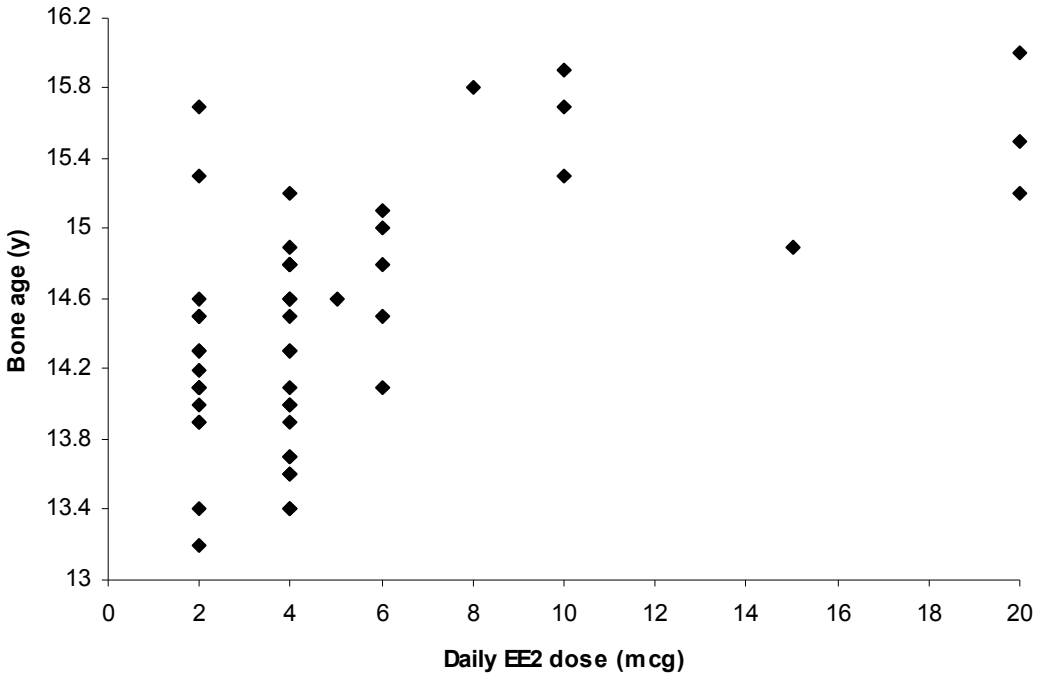


Figure 7.2 – Latest bone age performed in TS girls who required pubertal induction with corresponding daily oral EE2 dose

7.4.2 Non-Turner syndrome associated POI

Twenty-three girls had POI that was not associated with TS and their mean age (SD) at diagnosis was 14.1 (2.6) years. The majority of these girls (78.3%) had POI associated with total body irradiation (TBI) for bone marrow transplantation (BMT); 6 for solid malignancy, 11 for leukaemia and 1 for β-Thalassaemia major. Their underlying conditions are shown in Figure 7.3.

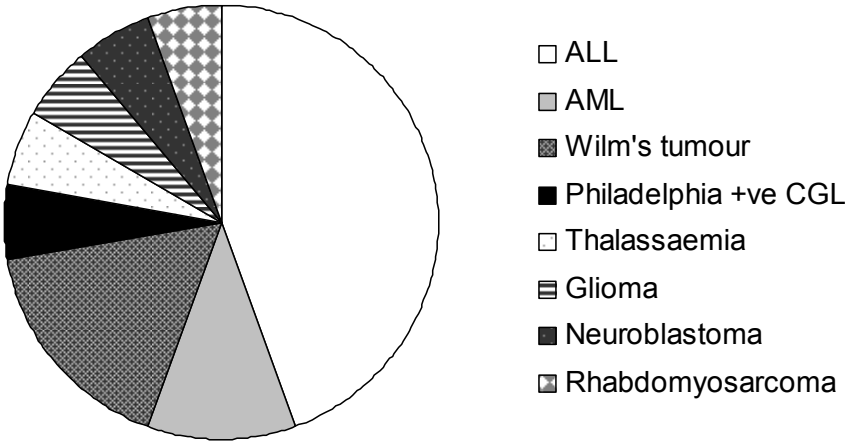


Figure 7.3 – Initial diagnosis of patients with POI associated with late effects of cancer-related treatment including TBI (n=18): ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia.

The mean age (SD) at diagnosis in girls with POI associated with TBI and bone marrow transplantation was 13.7 (2.6) years. Nine girls presented with absent puberty and the remaining nine girls presented with secondary amenorrhea or oligomenorrhea. So far, sixteen girls have commenced oral EE2 and their characteristics are depicted in Table 7.7. No further growth data are available for three of the girls who presented with absent puberty (1 died; 1 moved, 1 followed up in local tertiary centre). The oral EE2 regimes used to induce puberty are shown in Table 7.8.

Table 7.7 – Characteristics of girls with POI associated with TBI and BMT treated with oral EE2 (n=16)

	Absent puberty		**Amenorrhea**
Number of patients	6	3	7
At start of EE2			
Age (y)	13.2 (1.3)	12.7 (0.3)	16.7 (1.9)
Height (cm)	138.8 (6.1)	150.4 (4.4)	161.2 (4.1)
Height SD score	-2.6 (1.3)	-1 (0.6)	-0.2 (0.8)
Sitting height (cm)	68.7 (1.7)	76.6	83.2 (2.2)
Sitting height SD score	-4.7 (1.9)	-1.6	-2.1 (1.4)
Uterine length (cm)	3 (0.9)	3	3.8 (0.2)
Fundal-cervical ratio	0.8 (0.2)	-	1 (0)
At end of induction (daily EE2 dose reached 20mcg)			
Age (y)	16.5 (1.4)		17 (1.7)
Height (cm)	152.3 (8.7)		161.2 (4.1)
Height SD score	-1.8 (1.5)		-0.2 (0.8)
Sitting height (cm)	80.7 (4.7)	No data	83.2 (2.2)
Sitting height SD score	-3.4 (1.5)	available	-2.2 (1.3)
Δ Height SD score	1 (1.1)		0
Uterine length (cm)	4.4 (1.1)		5.7 (1)
Fundal-cervical ratio	1.1 (0.4)		1.6 (0.4)

Table 7.8 – Oral ethinylestradiol regimes used in girls with POI associated with TBI and BMT

Dose of oral EE2 (µg/day)	**Duration of EE2 (y)**	
	Absent puberty (n=6)	**Amenorrhea (n=7)**
2	1.2 (0.7)	0.1 (0)
4	1.3 (0.7)	0.1 (0)
5	1.3	-
6	0.3 (0.2)	0.1 (0)
8	0.2 (0)	0.1 (0)
10	0.9 (0.5)	0.6 (0.3)
14	2.3	0.3
15	0.5	-
Total	3.3 (1.6)	0.3 (0.5)

In the five remaining girls, POI was associated with galactosaemia (n=3), APECED (Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy; n=1) and gonadal dysgenesis (n=1). The mean (SD) age at diagnosis was 16.2 (1.6) years. Four girls presented with amenorrhea or oligomenorrhea and had already reached their FH when EE2 was commenced. The remaining girl presented with absent

puberty associated with galactosaemia and subsequently moved to Aberdeen to live in a residential school and no further data are available. Their characteristics are therefore not shown.

7.5 Discussion

The commonest cause of POI in this cohort of girls is, not surprisingly, Turner syndrome. As the reported frequency of TS is 1 in 2500 live female births the number of girls in the West of Scotland diagnosed with TS would be expected to be approximately 40 per 100 000 girls. The prevalence of TS in this cohort of girls from the West of Scotland was 30 per 100 000 with 95% Confidence Intervals of 23 to 38. There would, therefore, appear to be a shortfall in the numbers of girls diagnosed with TS in the West of Scotland between 1989 and 2000. It is possible that girls with TS were diagnosed and seen out-with the endocrinology clinic at RHSC particularly during the early part of the study period before Dr Donaldson's endocrinology service was fully established. During the first 5 years of the study the prevalence of TS in the West of Scotland was 27 per 100 000 girls compared with 32 per 100 000 girls for the last 5 years of the review period. In addition, some girls with TS whose presenting features were solely pubertal delay or pubertal arrest may have been referred to the adult sector. Therefore the numerator used for the prevalence and incidence calculations may be an underestimate.

In this cohort of TS girls the median age at diagnosis was 5 years which compares favourably with other centres.[255] The mean birth weight was 2.8 kg (-1.1 SD score) at 38.8 weeks gestation. The frequency of spontaneous menarche of 17.2% in this

cohort is very similar to that reported in Italian TS girls by Pasquino *et al* in 1997.[56] Spontaneous puberty was most frequently associated with the mosaic karyotypes: 45,X/47,XXX (n=5) and 45,X/46,XX (n=4). Only 1 girl had a classic 45,X karyotype. They demonstrated acceleration of growth during puberty with a mean peak height velocity of 8.1 cm per year. In girls who required an artificial induction of puberty the effect of exogenous oestrogen on growth depended on the dose employed. In two girls who initially received a relatively high dose of oral oestrogen, in the form of oestradiol valerate (Progynova), subsequent linear growth was reduced with a mean FH of 133.4 centimetres and a mean delta height SD score of -0.5 SD score. Sixty-four girls received incremental doses of oral ethinylestradiol from a mean age of 13.6 years. To begin with, very small daily doses of 0.5 to 2 μg were used. Linear growth continued and a mean FH of 150.2 centimetres was reached at a mean age of 17.9 years with a mean delta height SD score of +0.6 SD score. Their FH was similar to girls with spontaneous puberty who reached a mean FH of 149.3 centimetres. However, the actual oral EE2 regime followed in individual girls was highly variable with no consistency in the dose or duration of each increment. It was therefore not possible to assess the effect of EE2 dose on HV in such a disparate group. Pre-pubertal pelvic ultrasound findings were similar in TS girls with spontaneous and induced puberty with a mean uterine length of 3.3 centimetres at 11 years and 3.8 centimetres at 13.6 years respectively (P=0.151). These uterine lengths are on the 10th and 3-10th centile, respectively, on reference charts derived from normal unaffected girls. These smoothed centile curves for uterine length and fundal-cervical ratio (FCR) were devised by Griffin *et al* (see Appendix 2 and 3).[256] On completion of puberty, girls with a spontaneous onset had a mean uterine length of

6.9 centimetres (25th centile) which was larger than those who required pubertal induction who had a mean of 6 centimetres (3-10th centile; *P*=0.029). Similarly, the FCR in girls with spontaneous and induced puberty were initially 0.9 (25-50th centile) and 0.8 (3-10th centile; *P*=0.527) and after puberty were 1.5 (25th centile) and 1.2 (3-10th centile; *P*=0.033) respectively. TS girls with spontaneous puberty therefore achieved the normal post-pubertal heart shaped configuration of the uterus (FCR > 1) whereas this only occurred in 48% of girls who required pubertal induction. The poor uterine development in girls with TS who have required artificial induction of puberty with oral EE2 therapy has previously been described by Paterson *et al*.[257] Reduced uterine dimensions after pubertal induction with oral oestrogen has also been described by other groups in which a variety of different preparations of oestrogen were employed.[258-260] The underdevelopment of the uterus observed in TS girls treated with oral EE2 may be a reflection that the oestrogen regime currently employed is suboptimal. EE2 is a synthetic oestrogen usually administered orally and extensively metabolised by the liver before it reaches the systemic circulation it is therefore possible that a natural transdermal oestrogen formulation may improve uterine development. Normal uterine development has been claimed in TS girls treated with either natural conjugated oral E2 (oestradiol valerate) or topical E2 gel in early to mid-adolescence however FCR were not described.[261;262] There may nevertheless be an underlying abnormality of the uterus, in association with TS, which makes it less responsive to exogenous oestrogen therapy. In TS women who undergo assisted conception with donor oocytes there is a higher incidence of first trimester miscarriage of 50-60% compared to other groups with POI.[263-265] In TS women difficulties in embryo transfer have been attributed to

reduced endometrial thickness[265], hypoplastic uteri[265], suboptimal endometrial response[264] and uterine malformation.[265] Achievement of normal uterine dimensions may reduce the number of pregnancy-related problems faced by TS women who undergo assisted conception.

Non-TS associated POI was most commonly due to TBI in combination with BMT. Fifty percent of the girls had reached menarche prior to developing their principal diagnosis. During the initial phase of treatment for their underlying condition troublesome vaginal bleeding was common and usually controlled with oral norethisterone or subcutaneous LHRH analogue therapy (Zoladex). The effect of oral oestrogen on growth could only be assessed in six patients of whom four also had GH deficiency with or without hypothyroidism. They started EE2 at a mean age of 13.2 years and on average completed their induction in 3.3 years. Their mean FH was 152.3 centimetres (-1.8 SD score) with a mean delta height SD score of +1.0. Their mean final sitting height SD score was -3.4 reflecting a marked reduction in spinal height due to previous spinal irradiation. A similar effect of radiotherapy was observed on uterine growth. The mean uterine length and FCR, after completing pubertal induction with oral EE2, were 4.4 centimetres (3-10th centile) and 1.1 (3rd centile) respectively. Uterine development after EE2 therapy was better in the girls who had full pubertal development prior to developing their illness. Their mean uterine length and FCR, after completing a more rapid incremental regime of oral EE2 with an average duration of 0.3 years, was 5.7 centimetres (25th centile) and 1.6 (25th centile) respectively. Radiotherapy is reported to have a greater effect on uterine development when administered at an early age.[266] The effect of EE2 on

bone maturation and in particular the dose of EE2 associated with bony fusion was difficult to assess as only six bone ages were scored as 15.5 years or greater. The corresponding daily EE2 dose ranged from 2 to 20 µg.

POI is a continuum and this is emphasised by a girl with galactosaemia who conceived spontaneously 6 years after she presented with secondary amenorrhea associated with elevated gonadotrophins and reduced uterine length on ultrasound. She had been taking oestrogen replacement, in the form of Prempak C, when she conceived and subsequently went on to deliver a healthy baby boy at term.

As non-TS associated POI is an uncommon condition even in a tertiary referral centre the introduction of evidence-based guidelines would improve patient care. It is most commonly observed in girls who have received cancer-related therapies in particular TBI and BMT. After the diagnosis has been made it is important that a delay in starting oestrogen therapy is avoided as these girls often have other risk factors for developing osteoporosis for example previous prolonged corticosteroid therapy, extended periods of reduced mobility and acquired GH deficiency. Although long term outcome data is not available two girls have reduced bone mineral density on DXA scanning at 18-19 years and a further 2 girls have values within the osteoporotic range. Non-compliance with oestrogen treatment is also an issue as many girls on standard replacement doses, after pubertal induction has been completed, continue to have elevated serum gonadotrophins. Adherence to therapy may be increased by improving patient education for example with an information

booklet explaining the rationale of treatment and in particular describing the possible consequences of poor compliance with therapy.

7.6 Conclusions

Primary ovarian insufficiency is an uncommon condition with low prevalence rates in the West of Scotland in girls aged 16 years and under. The commonest cause of POI is not surprisingly associated with Turner syndrome. The next leading cause is iatrogenic POI secondary to the effects of cancer related therapy in particular total body irradiation associated with bone marrow transplantation. Girls with POI associated with TS were treated with oral oestrogen at a mean age of 13.6 years to induce puberty. Initially low doses of oestrogen (mostly EE2) were used to allow linear growth to continue. TS girls with a spontaneous onset of puberty showed a different growth pattern during puberty with evidence of a growth spurt and better uterine development at the end of puberty on pelvic ultrasound compared to those who required artificial induction with oral oestrogen. The actual oestrogen regime followed in individual patients for pubertal induction was highly variable so it was not possible to meaningfully analyse the effects of the oestrogen dose on height velocity or bone maturation in this retrospective audit. An accurate analysis of the effect of oestrogen on linear growth and bone maturation in Turner syndrome is therefore only likely to be possible within the context of a prospective clinical trial where ethically agreed protocols, including a standardised regime of oral oestrogen for pubertal induction, are strictly adhered to and is the focus of the next chapter.

Chapter 8

Effect of oral oestrogen on longitudinal growth in girls with Turner syndrome

Chapter Contents

8.1 Introduction

8.2 Hypothesis

8.3 Aims

8.4 Subjects and Methods

8.4.1 Subjects

8.4.2 Study design

8.4.3 Statistical analysis

8.5 Results

8.5.1 Effect of Oxandrolone on final height

8.5.2 Effect of age of Ethinylestradiol initiation on final height

8.5.3 Effect of Oxandrolone and Age of Ethinylestradiol initiation on FH

8.5.4 Effect of GH duration prior to Ethinylestradiol on final height

8.5.5 Effect of Ethinylestradiol on pubertal progression

8.5.6 Effect of Ethinylestradiol on height velocity

8.5.7 Effect of Ethinylestradiol on bone maturation

8.6 Discussion

8.7 Conclusions

8.1 Introduction

Short stature in girls with TS is almost universal. Although there is no evidence of classical GH deficiency[52;53] biosynthetic GH in supra-physiological doses has been shown to accelerate growth[267;268] and its efficacy in improving FH outcome has been established.[269;270] The mean "height gain" (mean FH – projected height) in the Canadian randomised controlled trial of GH therapy in TS was 7.3 cm.[270] A target height of 150 centimetres is now a realistic goal in most girls with TS.[268;271] However all studies have shown marked variation in individual outcome with some girls doing badly despite GH treatment.[269] At least some of the response to GH is explained by an X-linked imprinting effect as TS girls who retain a maternal X chromosome are reported to have a greater mean height gain with GH than those with a paternal X chromosome.[272] Although some studies have reported an improved FH outcome with early GH treatment[273-275] a number of other studies have failed to support this association.[276;277] Recent reports suggest that the number of years of GH therapy prior to pubertal induction may be predictive of outcome.[273;278] It has been suggested that a minimum of four oestrogen-free years of GH treatment should be the goal.[279]

Most girls with TS have primary ovarian insufficiency due to gonadal dysgenesis and require oestrogen treatment for pubertal induction. The optimal age at which to start oestrogen replacement remains controversial. Delaying puberty for as long as possible has previously been advocated in an attempt to prolong the growth period before epiphyseal fusion.[280] However, there may be psychological and social consequences of late pubertal induction. In addition, a recent study has indicated that

delayed puberty does not affect FH outcome.[277] Furthermore, data from the US and the Netherlands suggest that if GH is started early enough, puberty can be induced at an age-appropriate time of 11-12 years without a negative effect on FH.[273;281] In addition to oestrogen deficiency, adolescents and adults with TS and gonadal dysgenesis also have reduced levels of serum androgens as the ovary usually produces 50% of circulating androgens.[282;283] As androgens also promote longitudinal growth the addition of oxandrolone at the age that adrenarche usually occurs has been advocated to promote growth in TS. The use of this weak synthetic non-aromatisable anabolic steroid in the promotion of growth in TS remains contentious. Oxandrolone has been shown to increase HV and improve short term growth when used in combination with GH,[268;271] while a favourable effect on FH has now been reported when oxandrolone was used in combination with GH. However these studies did not include a comparison group[271;284] and other studies have failed to show this benefit.[280;285] More recently Stahnke *et al* have reported an increase in mean FH of 3.4 centimetres in girls treated with combination therapy compared to GH alone.[286] However, after initial randomisation to either GH alone or in combination with oxandrolone, 0.1 mg/kg/day for the first year followed by 0.05 mg/kg/day, some of the participants underwent a change in treatment regime. This was either due to the development of side effects in the combination arm, in the form of virilisation, or a waning HV in the GH-only arm. In addition, the GH dose was not standardised throughout the study period between the 2 groups. The virilisation was observed during the first year of the study when the higher dose of oxandrolone was used (0.1 mg/kg/day).[286]

8.2 Hypothesis

It was hypothesised that pubertal induction with oestrogen would have a dose-dependent effect on HV with an increase of HV at low doses of oestrogen and a decrease in HV at higher doses. Secondly, the age of pubertal induction (at either 12 or 14 years) would not affect FH as girls who started oestrogen earlier would show a greater response to oestrogen and grow faster but for a shorter period so that total height gained would be the same between the two groups.

8.3 Aims

I. To assess the effect of a standardised oestrogen regime on height velocity, bone maturation and final height in girls with TS treated with a standard dose of growth hormone

II. To identify what dose of oestrogen is associated with 1) peak height velocity and 2) bony fusion

8.4 Subjects and Methods

The United Kingdom Turner study is a randomised, double-blind placebo-controlled study of growth promoting treatment in TS organised by the British Society for Paediatric Endocrinology and Diabetes (BSPED) and running from 1999 to 2009. The study objectives are to examine the influence on FH of a) oxandrolone versus placebo from 9 years of age and b) the introduction of oral oestrogen therapy at either 12 or 14 years. The population studied were girls with TS who were receiving a standard dose of GH (10 mg/m^2/week by daily injection). The effect of oestrogen

on height velocity in these girls is not part of the UK study protocol and is the subject of this chapter.

8.4.1 Subjects

One hundred and six girls with TS were enrolled from 36 hospitals in the United Kingdom during the period of recruitment (November 1999 to December 2003). TS, defined as a loss or abnormality of the second X chromosome in a major cell line, was confirmed by karyotype. Inclusion criteria were girls with genetically confirmed TS and primary ovarian insufficiency who participated in the UK Turner study and therefore received a standard dose of GH therapy (10 mg/m^2/week) and who both required and completed the pubertal induction protocol with oral oestrogen. Girls were excluded from the study if they had any associated conditions which may interfere with growth e.g. inflammatory bowel disease. Girls with spontaneous puberty and normal FSH levels from 11 years of age (<10 IU/L, performed annually), usually in association with either 45,X/46,XX or 45,X/47,XXX mosaicism, and who did not therefore require pubertal induction were included but analysed separately. Written informed consent was obtained from the girls and their parents or guardians. The original study protocol was approved by Multi-Centre Research Ethics Committee (MREC). Additional approval was obtained from MREC in September 2008 to allow me to analyse the anonymised raw data (Appendix 1). The Dysmorphology Scoring system was devised by Dr Malcolm Donaldson and Emma Jane Gault (Turner Research Assistant) for the UK Turner Study to quantify the presence and severity of certain dysmorphic features which commonly occur in TS and is shown in Table 8.1. The maximum possible score is 28.

Table 8.1 – Dysmorphology scoring system

DYSMORPHIC FEATURES			
0 = Feature not present; 1 = Mild-moderately affected; 2 = Severely affected. If in doubt select lower category	**0**	**1**	**2**
• Hyperconvex nails +/- nail-fold oedema			
• Short fourth/fifth metacarpal			
• Cubitus valgus			
• Naevi			
• Ptosis			
• Epicanthic folds			
• Oblique palpebral fissures			
• Low set/rotated ears			
• High palate			
• Dental overcrowding			
• Micrognathia			
• Neck webbing/low hairline			
• Broad chest			
• Lymphoedema hands/feet/limbs			

8.4.2 Study design

Recruits were randomised to oxandrolone (0.05 mg/kg/day; maximum 2.5 mg) or placebo from 9 years and further randomised to oestrogen or placebo at 12 years with the placebo group starting oestrogen at 14 years. Girls enrolling after 9 years of age were immediately randomised to oxandrolone or placebo. Oestrogen was administered as EE2 tablets: 2 µg/day in year one, 4 µg/day in year two, for the third year 6 µg/day for 4 months followed by 8 µg/day for 4 months and then 10 µg/day for the remaining 4 months. Thereafter, 20 µg/day was given as an adult replacement dose. Norethisterone 5 mg tablets for the first 5 days of each calendar month were commenced when either the daily EE2 dose reached 10 µg or when breakthrough bleeding occurred. Girls recruited after 12 years were not randomised and automatically assigned to start EE2 at 14 years and formed the "late group". At enrolment participants were commenced on GH or had their existing GH dose adjusted to 10 mg/m^2/week. GH was then administered by daily subcutaneous

injections at bedtime until FH. The GH dose was adjusted to calculated body surface area every 4 to 6 months to maintain a fixed dose. FH was defined as HV less than 1 centimetre per year and a BA score of 15.5 years or greater. Height, sitting height and weight were measured at enrolment and at subsequent visits until FH. HV was calculated using the formula: $height_{current} - height_{previous}/age_{current} - age_{previous}$ and expressed as centimetre/year. Where possible the interval between heights was 1 year with a minimum of 0.5 year and a maximum of 1.3 years. Peak height velocity (PHV), defined as the maximum HV observed during the adolescent growth spurt, was determined by measuring sequential HV three years before and after the onset of breast development. If a peak consistent with PHV was identified, and in particular if this coincided with documentation of puberty, the age and magnitude were recorded. The height standard deviation (SD) score was calculated using reference values for healthy UK girls.[287] Mid-parental height was calculated using the formula: ½ x ($height_{mother} + height_{father} - 12.5$) centimetres. During GH pubertal stages were assessed according to Tanner[288]. BA was determined by a single observer (WP) according to the Tanner and Whitehouse radius, ulna, short bones score (TW2 method).[254]

8.4.3 Statistical analysis

Analysis of the effect of oxandrolone, late pubertal induction and interaction of the 2 independent variables on FH was performed by multiple regression using DataDesk software package (Data Description Incorporated, version 6.2.1) by Professor Tim Cole. I performed all subsequent statistical analysis using SPSS version 15: unpaired T-tests to investigate the effect of oxandrolone or onset of EE2 on continuous

normally distributed data; general linear model (GLM) with repeated measures to investigate effect of oxandrolone, onset of EE2 or GH duration prior to EE2 on HV; ANOVA to assess effect of pubertal onset, either spontaneous or artificial at 12 or 14 years, on height gained during puberty. Significance was determined as $P<0.05$. Results are expressed as mean (SD) unless otherwise indicated.

8.5 Results

One hundred and six girls were recruited at a median age of 10 years (7.3-13.6). Their progress through the study is shown in Figure 8.1. Fifty-one girls were randomised to oxandrolone treatment and 55 to placebo. Fourteen girls have withdrawn leaving a study population of 92 (43/49 in oxandrolone/placebo arm respectively). Four participants were withdrawn in the initial stages due to organisational errors which resulted in significant protocol violations. The remaining 10 girls have withdrawn due to significant non-adherence, poor attendance or personal choice. Seven girls withdrew before the second randomisation. Sixty girls were randomised to start EE2: 29 girls at 12 years (12 previously randomised to oxandrolone), 31 girls at 14 years (14 previously randomised to oxandrolone). Twenty-one girls were recruited after EE2 randomisation would have occurred and commenced EE2 at 14 years; these were designated as the “late group”. Ten of these girls were randomised to oxandrolone treatment. 18 participants had SP with normal FSH levels (<10 IU/L) and did not require EE2 therapy. 11 girls had previously been randomised to oxandrolone and 7 to placebo. Girls with SP had the following karyotypes: 45, X/46, XX n= 12; 45, X/46, XrX n=1; 45, X/46XiXq n=1; 45, X/47,XXX n=2; 46, XX with deletions n=2. To date, 75 girls have reached FH.

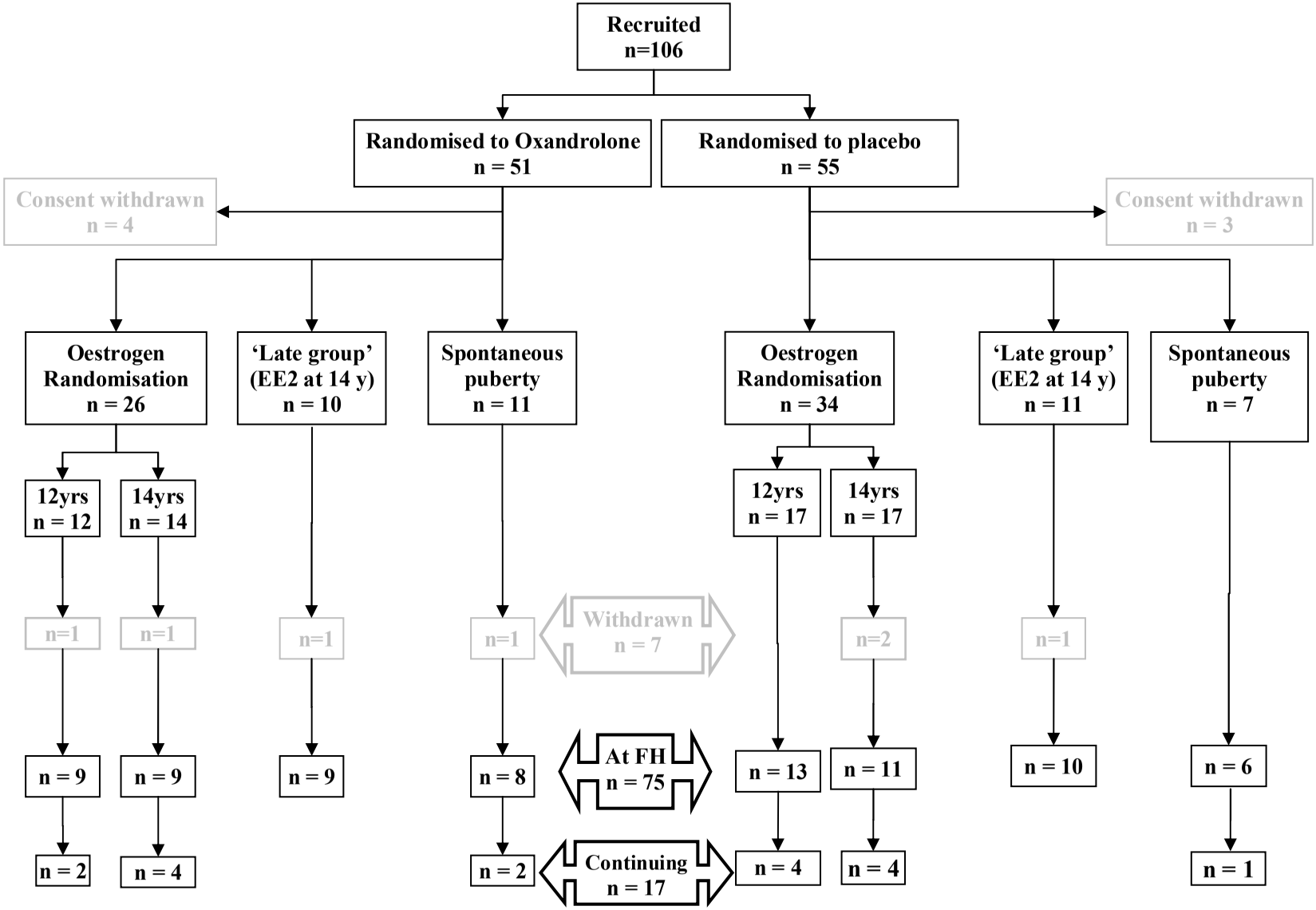


Figure 8.1 – Flow diagram of progress through UK Turner study

8.5.1 Effect of Oxandrolone on final height

The baseline and FH status of the participants of the UK Turner study according to initial randomisation to oxandrolone or placebo are shown in Table 8.2. Oxandrolone was shown by multiple regression to increase FH by 5.0 centimetres (p=0.0002, n=75). There were no significant adverse events reported such as voice deepening or clitoromegaly.

Table 8.2 – Participant characteristics according to Oxandrolone randomisation

	Oxandrolone	Placebo
At enrolment	**n = 51**	**n = 55**
Age (y)	10.3 (1.6)	10.3 (1.6)
Height (cm)	126.7 (8.7)	125.6 (7.9)
Height SDS	-2.1 (0.8)	-2.2 (0.8)
Age at GH (y)	7 (2.5)	6.9 (3)
At final height	**n = 35**	**n = 40**
Age (y)	16.4 (1.3)	16.7 (1.3)
Height (cm)	153.9 (4.8)	148.9 (6.2)
Height SDS	-1.4 (0.8)	-2.3 (1)
Δ Height SD score	0.6 (0.8)	0 (0.7)

8.5.2 Effect of age of Ethinylestradiol initiation on final height

The characteristics of participants according to EE2 randomisation at baseline and FH are shown in Table 8.3. Neonatal data were similar between the four groups with a mean birth weight of 2.8 kilograms (-1.1 SD score) at 38.9 weeks gestation. Girls were enrolled at a similar age apart from the "late group" who by definition were assigned to EE2 at 14 years of age because they had enrolled later than 12.25 years. The height SD scores and target heights were similar between all the groups at enrolment. The girls who had a spontaneous onset of puberty, and did not require EE2, were diagnosed later at a median age of 7.2 years. None of the girls with spontaneous puberty had a classical 45,X karyotype whereas in the EE2 at 12 years,

EE2 at 14 years, and the "late group" this karyotype was found in 46.4%, 48.1% and 37.1% of girls, respectively. Using multiple regression, the effect of the timing of EE2 initiation on FH was shown to be significant. Delaying the onset of EE2 to 14 years was shown to increase FH by 3.7 centimetres (p = 0.03, n = 42).

Table 8.3 – Participant characteristics according to EE2 randomisation

	Randomised to EE2 at 12y	Randomised to EE2 at 14y	"Late group" EE2 at 14y	No EE2 required
At enrolment	**n = 29**	**n = 31**	**n = 21**	**n = 18**
Age (y)	9.6 (1)	9.7 (1.2)	12.6 (0.4)	9.9 (1.2)
Bone age (y)	9.4 (1.4)	9.5 (1.7)	11.8 (1.3)	10.2 (1.9)
Height (cm)	122.8 (6.9)	123.9 (7)	135.5 (5.7)	124.6 (8)
Height SD score	-2.2 (0.8)	-2 (0.9)	-2.5 (0.8)	-2 (0.8)
Target height (cm)	162.8 (3.8)	162.4 (4.9)	163.1 (3.2)	162.9 (4.5)
Age at diagnosis (y)*	0.6 (0-9.8)	1.6 (0-9.8)	3.1 (0-13.4)	7.2 (0.5-10.4)
Dysmorphology score*	6 (0-18)	5 (1-16)	6 (0-15)	4 (0-8)
Age at GH (y)*	6.4 (2.4-10.2)	5 (2.2-10.5)	8.9 (4-13.8)	8.2 (1.7-10.9)
At final height	**n = 22**	**n = 20**	**n = 19**	**n = 14**
Age (y)	16.2 (1)	17 (0.8)	17.6 (1)	15.1 (0.9)
Height (cm)	149.2 (6.9)	153.2 (4.4)	151.9 (6)	150.8 (6.5)
Height SD score	-2.2 (1.2)	-1.7 (0.7)	-1.9 (1)	-1.7 (1.1)
Δ Height SD score	0 (0.6)	0.3 (0.9)	0.6 (0.8)	0.2 (0.9)

*Median (range)

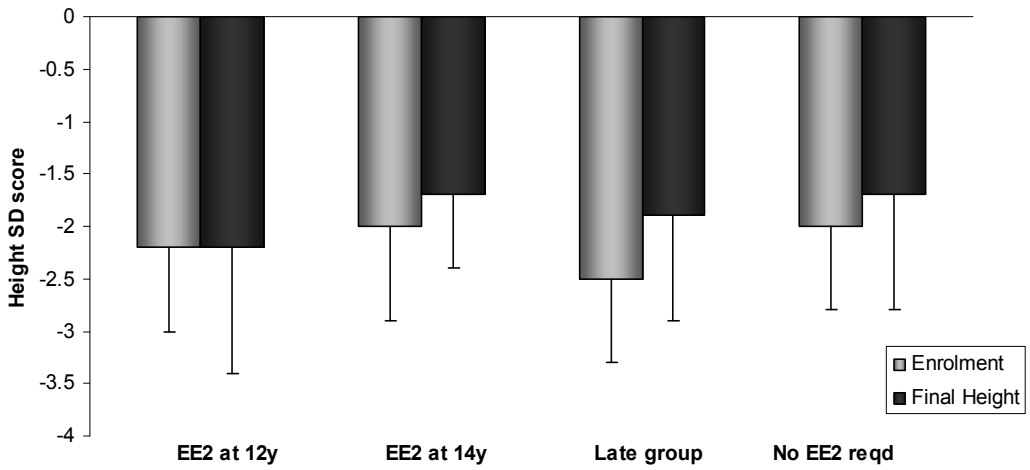


Figure 8.2 – Height SD score for chronological age at enrolment (■) and final height (■) according to EE2 randomisation

8.5.3 Effect of Oxandrolone and Age of Ethinylestradiol initiation on FH

The baseline and final height characteristics of participants according to both oxandrolone and EE2 randomisations are demonstrated in Table 8.4. The effects of the double randomisation are depicted in Figure 8.3. The change in height SD score from enrolment to FH is shown for all groups in Figure 8.4. The effect of oxandrolone versus placebo on FH in girls who commenced EE2 at 12 years was 8.2 centimetres. The effect of delaying EE2 from 12 years of age until 14 years on FH in the placebo group was 6.4 centimetres. Whereas, the effect of oxandrolone and delaying EE2 until 14 years of age on FH compared to the placebo group with EE2 at 12 years of age was 8.4 centimetres. Therefore the interaction between oxandrolone and delaying pubertal induction to 14 years was negative, as an effect closer to 14 centimetres would have been observed if the two interventions were synergistic, and close to significance (p=0.06, n = 42). The effect of oxandrolone or EE2 at 14 years versus placebo with EE2 at 12 years was 7.6 centimetres.

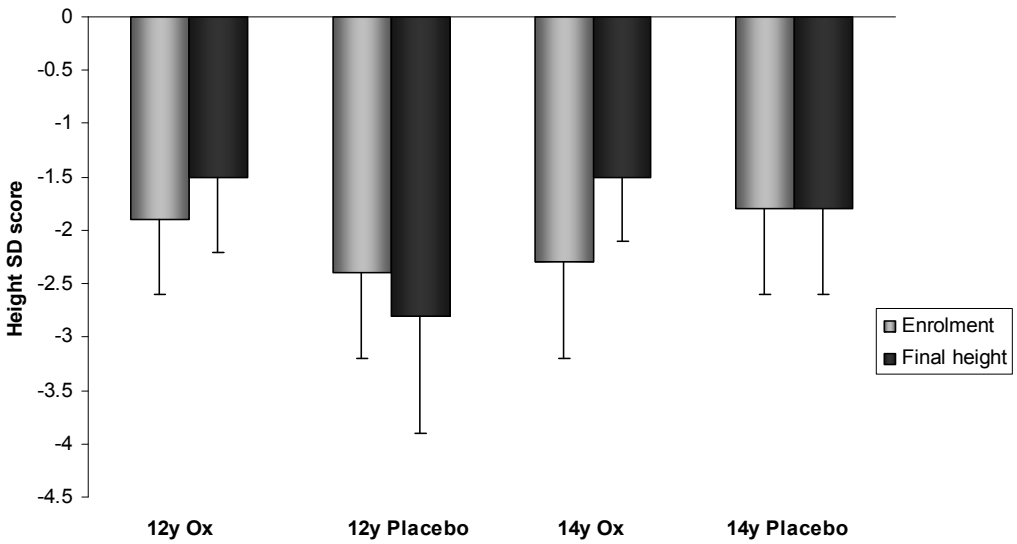


Figure 8.3 – Height SD score for chronological age at enrolment (■) and final height (■) according to Oxandrolone and EE2 randomisation

Table 8.4 – Participant characteristics according to Oxandrolone and EE2 randomisation

	Randomised to EE2 at 12y		Randomised to EE2 at 14y		"Late group" EE2 at 14y		No EE2 required	
	Oxandrolone	**Placebo**	**Oxandrolone**	**Placebo**	**Oxandrolone**	**Placebo**	**Oxandrolone**	**Placebo**
At enrolment	**n =12**	**n = 17**	**n = 14**	**n = 17**	**n = 10**	**n = 11**	**n = 11**	**n = 7**
Age at Diagnosis (y)*	0.1 (0-9.8)	4.6 (0-9.2)**	4.5 (0-9.8)	1.5 (0-9.8	2.8 (0-12.7)	3.1 (0-13.4)	6.6 (0.7-10.4)	8.3 (0.5-10.1)
Dysmorphology Score*	8 (0-18)	6 (1-18)	5.5 (1-16)	5 (2-15)	6 (0-12)	7 (2-15)	5 (0-8)	3.5 (0-7)
Age (y)	10.1 (1.3)	9.4 (0.8)	9.6 (1.3)	9.7 (1.2)	12.5 (0.4)	12.8 (0.4)	9.9 (1.2)	9.8 (1.4)
Height (cm)	126.4 (6.9)	120.3 (6)	121.6 (6.7)	125.8 (6.9)	137.2 (6.8)	133.9 (4.2)	124.8 (7.6)	125 (9.2)
Height SD score	-1.9 (0.7)	-2.4 (0.8)	-2.3 (0.9)	-1.8 (0.8)	-2.1 (0.9)	-2.9 (0.6)	-2 (0.9)	-2 (0.8)
Target Height (cm)	163.8 (3.4)	162 (4)	161.5 (4.1)	163.1 (5.5)	165.1 (2.6)	161.6 (2.9)	163 (4.7)	162.6(4.5)
Age at GH (y)*	5.4 (4-10.2)	7.3 (2.4-10.1)	6.1 (2.2-10.5)	4.2 (2.4-9.6)	8.3 (4.5-12.8)	10.3 (4-13.8)	6 (3.2-10.9)	8.7 (1.7-10.3)
GH before EE2 (y)*	6.6 (1.8-8)	4.7 (1.9-9.6)	7.9 (3.5-11.9)	9.8 (4.4-11.6)	5.7 (1.2-9.5)	4.5 (0.2-10)	-	-
At final height	**n = 9**	**n = 13**	**n = 9**	**n = 11**	**n = 9**	**n = 10**	**n = 8**	**n = 6**
Age (y)	16.4 (1.2)	16.1 (1)	16.9 (0.9)	17.1 (0.8)	17.1 (0.9)	18.1 (0.8)	15 (0.9)	15.2 (0.9)
Height (cm)	154 (4.2)	145.9 (6.6)	154.3 (3.7)	152.3 (4.8)	155.3 (4.3)	148.9 (5.9)	151.9 (6.8)	149.4 (6.4)
Height SD score	-1.5 (0.7)	-2.8 (1.1)	-1.5 (0.6)	-1.8 (0.8)	-1.3 (0.7)	-2.4 (1)	-1.5 (1.2)	-2 (1)
Δ Height SD score	0.4 (0.5)	-0.3 (0.5)	0.9 (1)	-0.1 (0.5)	0.7 (0.8)	0.4 (0.8)	0.2 (1)	0.1 (0.9)

*Median (range) **Excluding 2 antenatal diagnoses

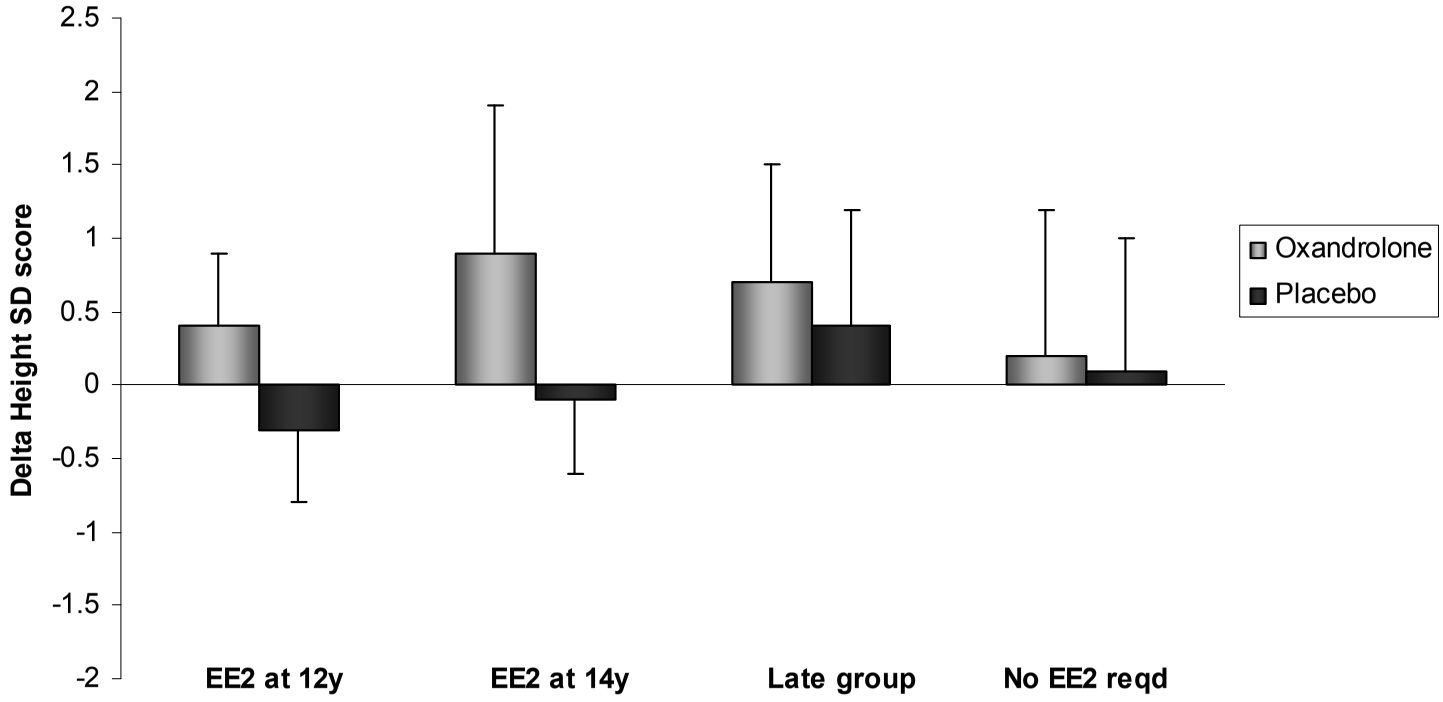


Figure 8.4 – Difference in height SD score from enrolment to final height according to randomisation to Oxandrolone (■) or placebo (■)

8.5.4 Effect of GH duration prior to Ethinylestradiol initiation on FH

The characteristics of participants according to EE2 randomisation and duration of GH therapy prior to initiation of EE2 are depicted in Table 8.5. As it has been suggested that the duration of GH prior to pubertal induction may be predictive of outcome[273;278] and a minimum of four oestrogen-free years should be the goal[279] this interval was used to evaluate the participants' outcome in terms of FH attained. Surprisingly, a shorter duration of GH therapy before EE2 initiation was associated with a larger increase in delta height SD score as illustrated in Table 8.5. However, this is probably a reflection of the increase in height status (improvement in height SD score) that usually occurs during the first two years of GH therapy. With the moderate GH doses employed in this study, namely 10 mg/m^2/week, an increase in height SD score usually occurs within the first two years of therapy but no further increase is observed thereafter with prolonged GH usage.[281] However, when doses are increased from 10 mg/m^2/week after the first year of therapy to 14 mg/m^2/week subsequent increases in height SD score are observed after both four years and seven years of GH therapy.[281] Thus, participants who had received GH treatment for longer than four years prior to initiation of EE2 therapy would already have improved their height SD score prior to enrolling in the study whereas participants who had only recently commenced GH therapy would show an improvement in height status during the study period. As the actual height at the time of commencing GH therapy was not recorded as part of the study protocol the "total height gained" from growth promoting therapies could not be estimated.

Table 8.5 – Participant characteristics according to EE2 randomisation and GH duration before EE2

	Randomised to EE2 at 12y		Randomised to EE2 at 14y		"Late group" (EE2 at 14y)	
	GH < 4y	GH >4y	GH < 4y	GH >4y	GH < 4y	GH >4y
At enrolment	**n = 9**	**n = 20**	**n = 2**	**n = 29**	**n = 8**	**n = 13**
Age (y)	9.6 (0.7)	9.7 (1.2)	10.7 (0)	9.6 (1.2)	12.8 (0.4)	12.6 (0.4)
Ht (cm)	121 (4.9)	123.6 (7.7)	122.1 (3.7)	124 (7.2)	133.1 (3.4)	137 (6.4)
Ht SD score	-2.4 (0.6)	-2.1 (0.8)	-3 (0.5)	-1.9 (0.8)	-3 (0.7)	-2.2 (0.8)
Target Ht (cm)	162.5 (3.6)	163 (4)	166.3 (5.2)	162.1 (4.8)	161.8 (2.2)	163.9 (3.6)
Age at GH (y)	9.2 (0.7)	5.6 (1.4)	10.3 (0.2)	5.2 (2)	12.2 (1.2)	7.2 (1.9)
GH before E2	2.8 (0.7)	6.4 (1.4)	3.7 (0.2)	8.8 (2)	1.8 (1.2)	6.8 (1.9)
At final height	**n = 7**	**n = 15**	**n = 2**	**n = 18**	**n = 7**	**n = 12**
Age (y)	16.1 (0.6)	16.3 (1.1)	16.8 (1.6)	17 (0.8)	18.1 (0.7)	17.3 (1)
Ht (cm)	150.7 (6.1)	148.5 (7.3)	150.9 (0.6)	153.5 (4.5)	151.9 (6.9)	152 (5.8)
Ht SD score	-2 (1)	-2.4 (1.2)	-2 (0.2)	-1.6 (0.7)	-2 (1.1)	-1.9 (1)
Δ Ht SD-score	0.4 (0.7)	-0.2 (0.5)	0.9 (0.3)	0.3 (0.9)	1 (0.8)	0.3 (0.7)

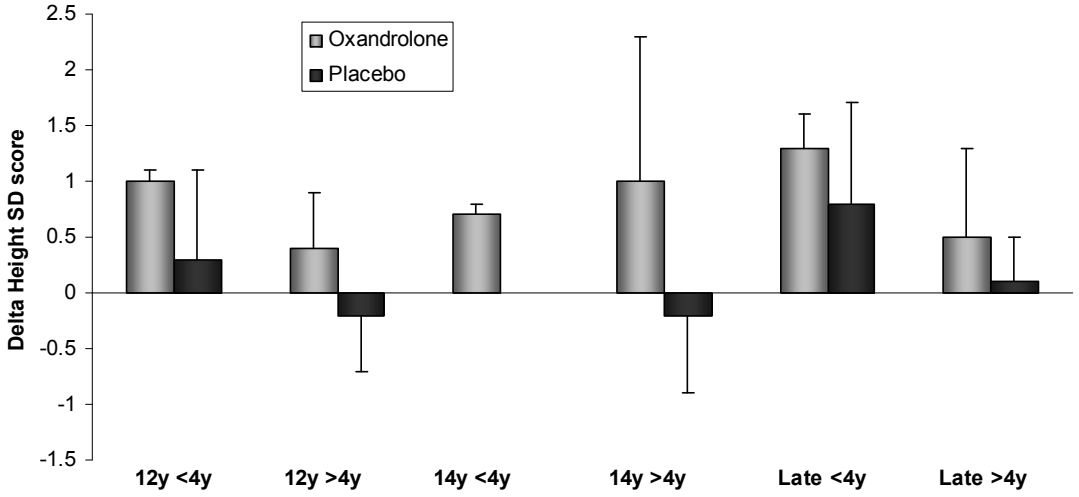


Figure 8.5 – Delta height SD score in girls treated with EE2: according to randomisation to Oxandrolone (■) or placebo (■); Age at EE2 (12y or 14y) and GH duration pre-EE2 (>4y or <4y)

8.5.5 Effect of Ethinylestradiol on pubertal progression

Girls who commenced oral EE2 at 12 years of age reached Tanner breast stage B2 at a mean age of 12.9 years and reached breast stage B5 at 15 years. The process of pubertal induction therefore lasted three years (Table 8.6). This "early" group therefore achieved breast stage B2 and B5 on average two years later than normal unaffected girls as the median age of menarche in a contemporary cohort of UK girls is 12.9 years.[289] Age at menarche has changed little over the last 50 years since Tanner *et al* reported a median age of 13 years in a cohort of UK girls born in the 1950's.[290] Girls, who started EE2 at 14 years of age, showed a similar rate of progression and reached breast stage B5 after 3 years of pubertal induction. They were therefore 4 years behind their peers in terms of pubertal development. By contrast, girls with spontaneous puberty, who did not require oestrogen therapy, demonstrated a normal pattern of pubertal development reaching breast stage B2 at 11.4 years and menarche at 12.9 years. Some of the girls who received pubertal induction did show evidence of spontaneous breast development however this either did not progress or regressed before EE2 therapy was commenced. Unpaired T-tests were used to examine any effect of oxandrolone on pubertal progression in the three groups. No effect was observed with oxandrolone treatment, P values > 0.05.

Table 8.6 - Pubertal progression with age at each Tanner breast stage achieved

	EE2 at 12y (n = 28)	EE2 at 14y (n = 47)	No EE2 required (SP) (n = 17)
Tanner breast stage 2	12.9 (1.2)	14.1 (1.3)	11.4 (1.2)
Tanner breast stage 3	13.9 (1)	15.1 (1.4)	12.2 (1.6)
Tanner breast stage 4	14.8 (1)	16.1 (1.4)	13.1 (1.2)
Tanner breast stage 5	15 (0.5)	17.1 (1.4)	13.4 (0.5)
Spontaneous Menarche	-	-	12.9 (1)

8.5.6 Effect of Ethinylestradiol on height velocity

The HV of girls who commenced oral EE2 at 12 years of age is demonstrated in Figure 8.6. During the first year of EE2 treatment the HV was similar to the rate observed in the year prior to the onset of EE2. Thereafter a deceleration in HV was observed. Girls who were randomised to oxandrolone treatment had a higher HV than those who received placebo until the age of 13 years (Figure 8.7A; *P*=0.008). Thereafter, there was no difference in HV between the two groups (*P*=0.935). Similarly, participants who had commenced GH less than 4 years before the initiation of EE2 were shown to have a higher HV until the age of 12 years (before the onset of EE2 therapy) than those girls who had been established on GH therapy for longer than 4 years (Figure 8.7B; *P*=0.015). However, all girls showed a similar HV after commencing EE2 therapy (Figure 8.7). The effects of oxandrolone randomisation and duration of GH before commencing E2 at 12 years of age are shown in Figure 8.7C.

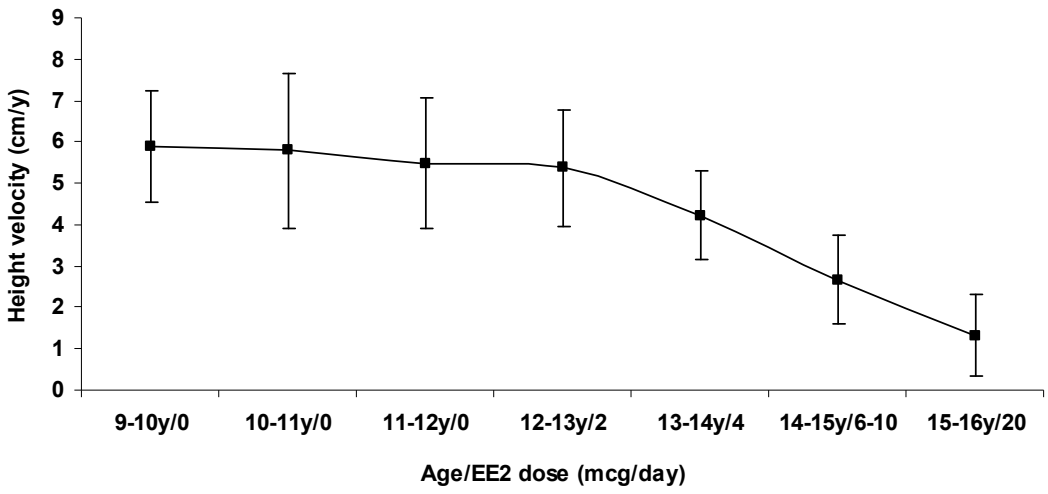


Figure 8.6 – Height velocity of girls treated with EE2 at 12 years (n = 28)

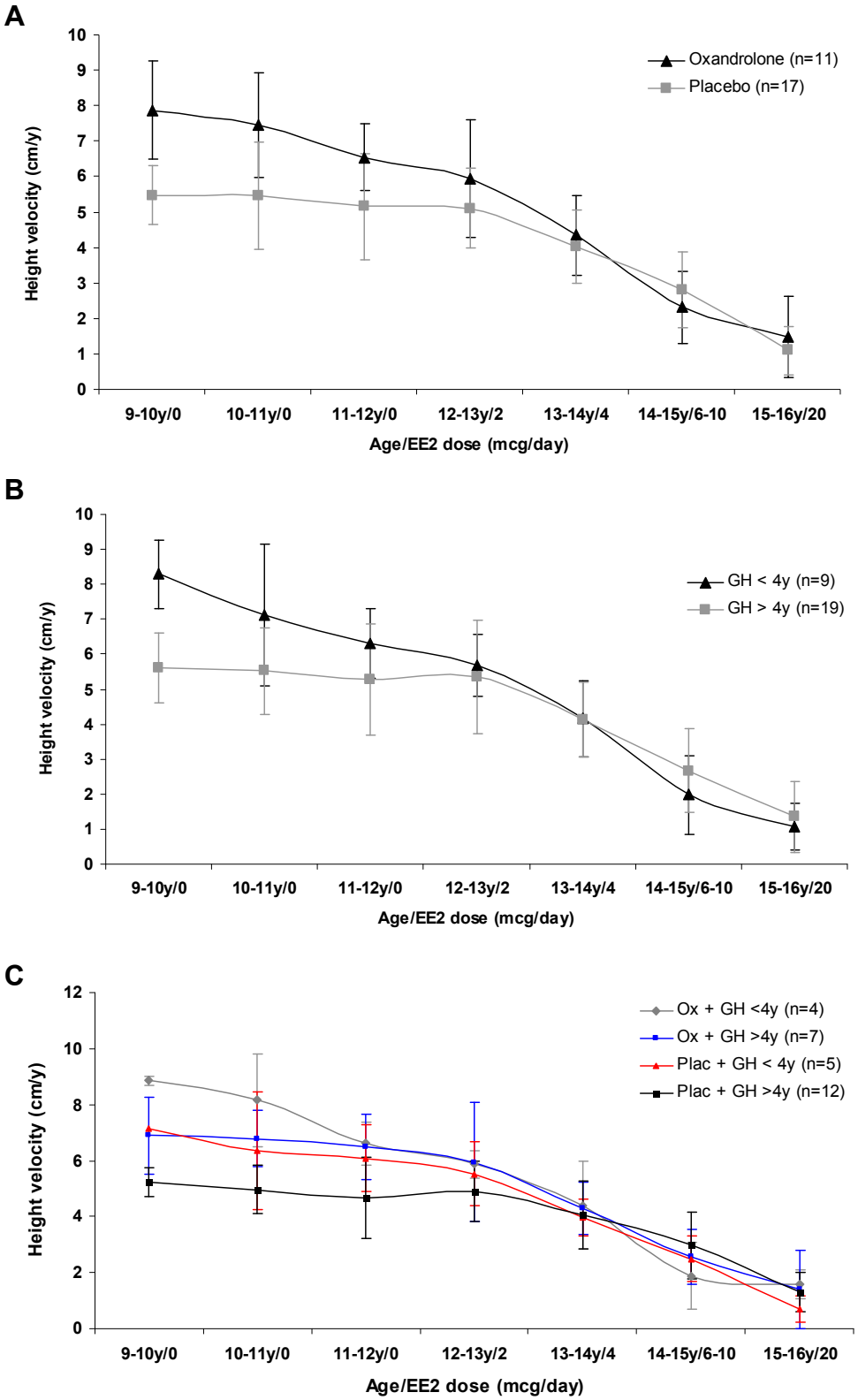


Figure 8.7 – Height velocity of girls treated with EE2 at 12 years according to: A) Oxandrolone randomisation B) GH duration before EE2 C) Oxandrolone randomisation and GH duration before EE2.

The growth rate of girls who commenced EE2 therapy at 14 years of age is depicted in Figure 8.8. A deceleration in HV was observed from 11-12 years of age which increased after the onset of 2 µg per day of EE2 at 14 years of age. A further decrease in HV was observed when the daily dose was increased to 4 µg at 15 years of age. Subsequent increases of the EE2 dose led to further deceleration in growth. Girls who were randomised to oxandrolone treatment had a higher HV than those who received placebo until the age of 15 years (Figure 8.9A; $P<0.001$). Thereafter there was no significant difference between the groups ($P=0.388$). Participants who had commenced GH therapy less than 4 years before the initiation of EE2 were shown to have a higher HV than those who had been established on GH for longer than 4 years until the age of 15 years (Figure 8.9B; $P=0.018$) thereafter there was no significant difference ($P=0.292$). The effects of oxandrolone randomisation and GH duration before commencing EE2 at 14 years of age are shown in Figure 8.9C.

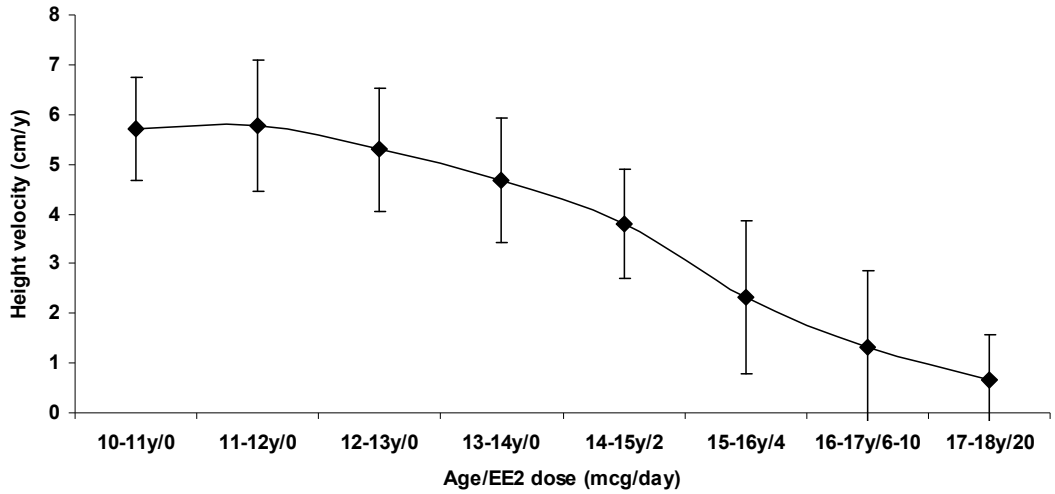


Figure 8.8 – Height velocity of girls treated with EE2 at 14 years (n = 47)

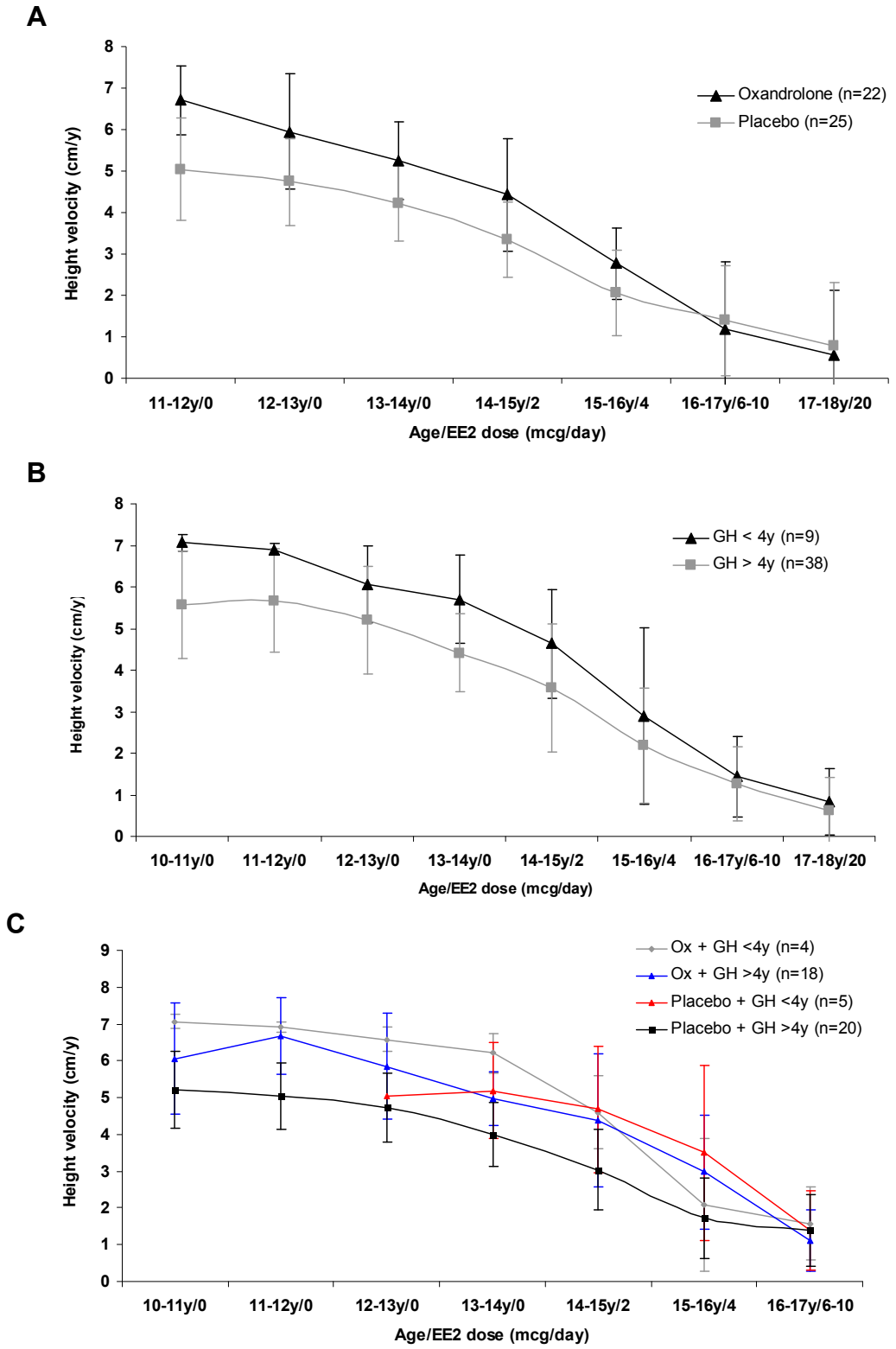


Figure 8.9 – Height velocity of girls treated with EE2 at 14 years according to: A) Oxandrolone randomisation B) GH duration before EE2 C) Oxandrolone randomisation and GH duration before EE2.

The HV according to age of pubertal induction or daily EE2 dose are demonstrated in Figures 8.10 and 8.11 respectively. The growth rate of an untreated cohort of TS girls described by Ranke *et al* shows the constant deceleration in HV that occurs in TS in the absence of treatment.[291] The higher HV observed in the girls in the UK Turner study prior to EE2 is attributable to GH therapy. When EE2 therapy was commenced at 12 years of age, HV appeared to be maintained until the daily EE2 dose was increased to 4 µg (Figures 8.10, 8.11). However, when EE2 therapy started at 14 years of age the natural deceleration in growth was enhanced by the daily EE2 dose of 2 µg. The height velocities of TS girls who started EE2 at 12 years of age differed from those girls who commenced EE2 at 14 years between the ages of 12 to 17 years (GLM repeated measures P=0.006).

The growth rate of girls with SP who did not require pubertal induction is depicted in Figure 8.12. They showed acceleration in HV associated with breast development with a mean PHV of 8.5 cm per year. The HV of girls with a spontaneous onset of puberty is compared with the HV of girls who required an artificial induction of puberty with oral EE2 in Figure 8.13. The height velocities of girls with a spontaneous onset of puberty differed from TS girls who commenced E2 at 12 years of age (GLM repeated measures P <0.001).

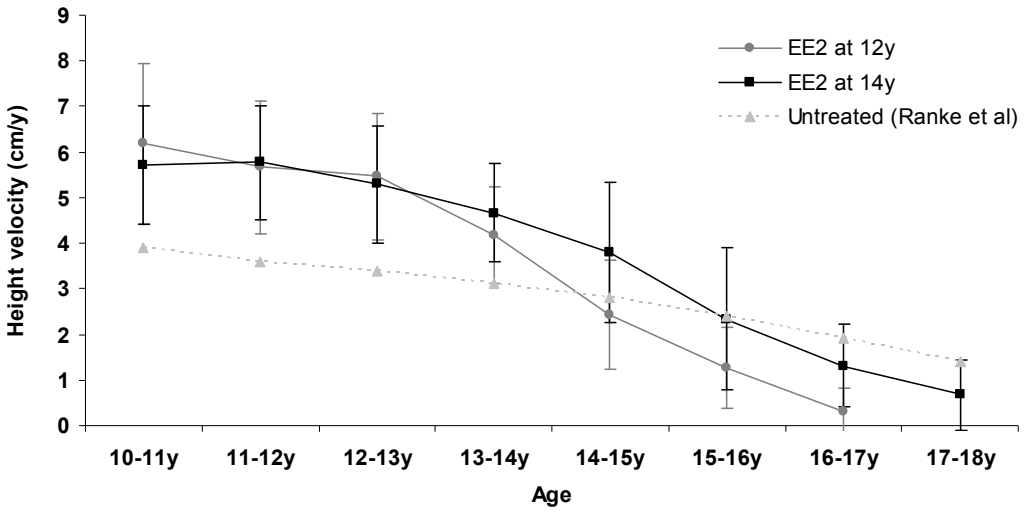


Figure 8.10 – Height velocity according to age of pubertal induction compared to an untreated historical cohort described by Ranke *et al*, 1983.

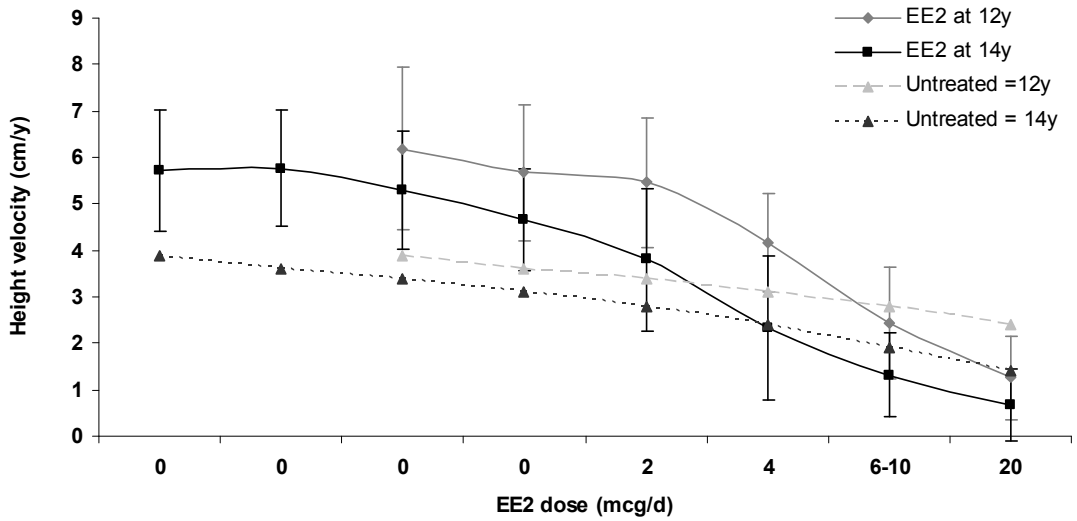


Figure 8.11 – Height velocity according to EE2 dose (patient age different between the two groups) compared to an untreated historical cohort at a similar age described by Ranke *et al*, 1983.

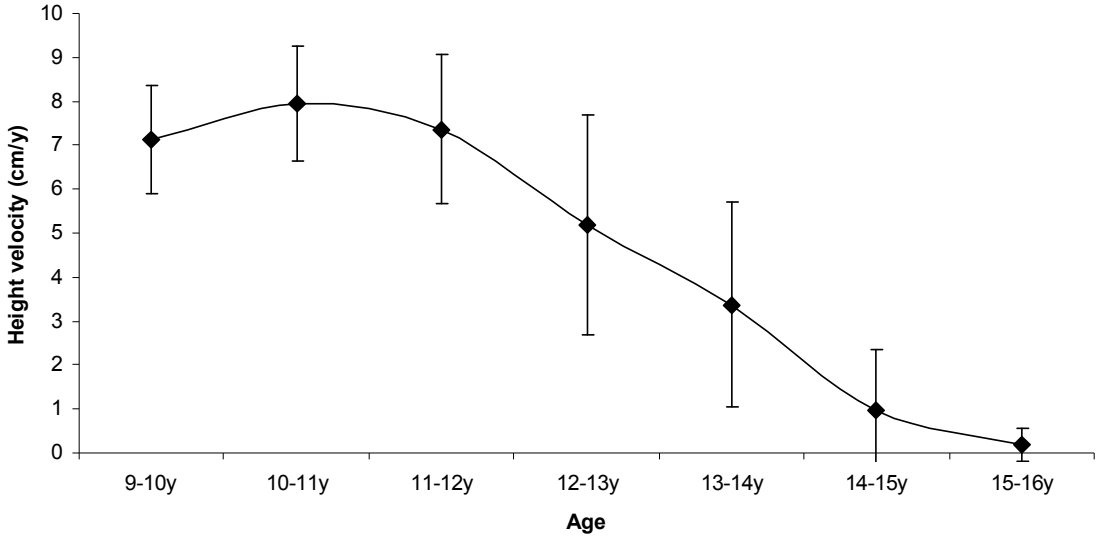


Figure 8.12 – Height velocity of girls with spontaneous puberty (n = 17)

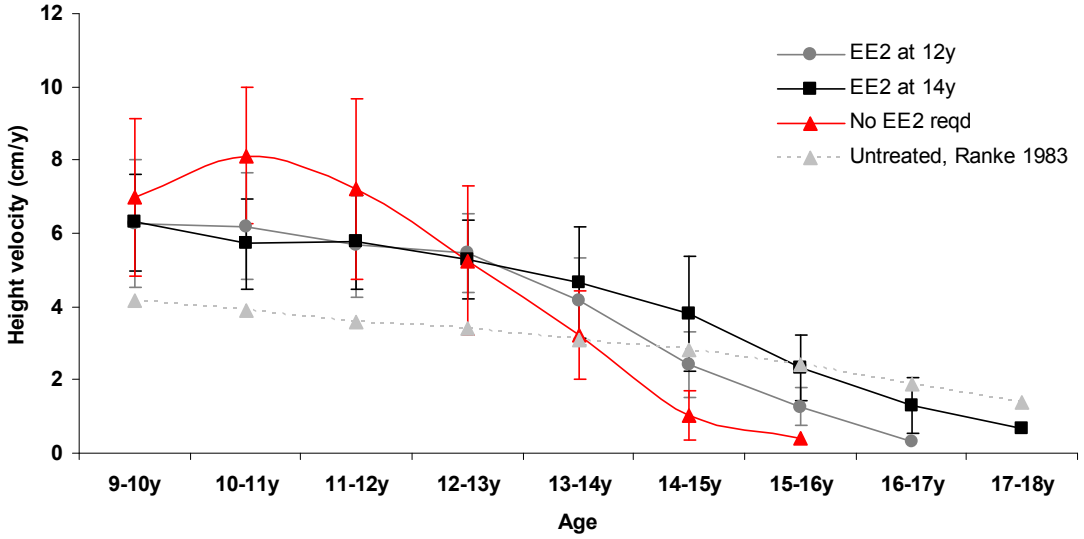


Figure 8.13 – Height velocity of girls with spontaneous puberty compared to girls who required artificial induction with oral EE2

The gain in height during puberty according to its onset, either spontaneous or induced at 12 or 14 years, is demonstrated in Figure 8.14. The age at onset of puberty was determined as the last age at which Tanner breast stage was recorded as B1 if puberty had a spontaneous onset i.e. age at the clinic visit immediately preceding the first documentation of pubertal development (Tanner breast stage 2). If puberty was artificially induced the age at onset was determined by the age at which oral EE2 was commenced which was either 12 or 14 years of age. The amount of height gained during puberty was higher in girls who had a spontaneous onset of puberty compared to girls who required an artificial induction of puberty at either 12 or 14 years (ANOVA: $P<0.001$, $P<0.001$ respectively). In addition, girls who commenced oral EE2 at 12 years of age grew more during puberty than girls who received oral EE2 from 14 years (ANOVA: $P<0.001$).

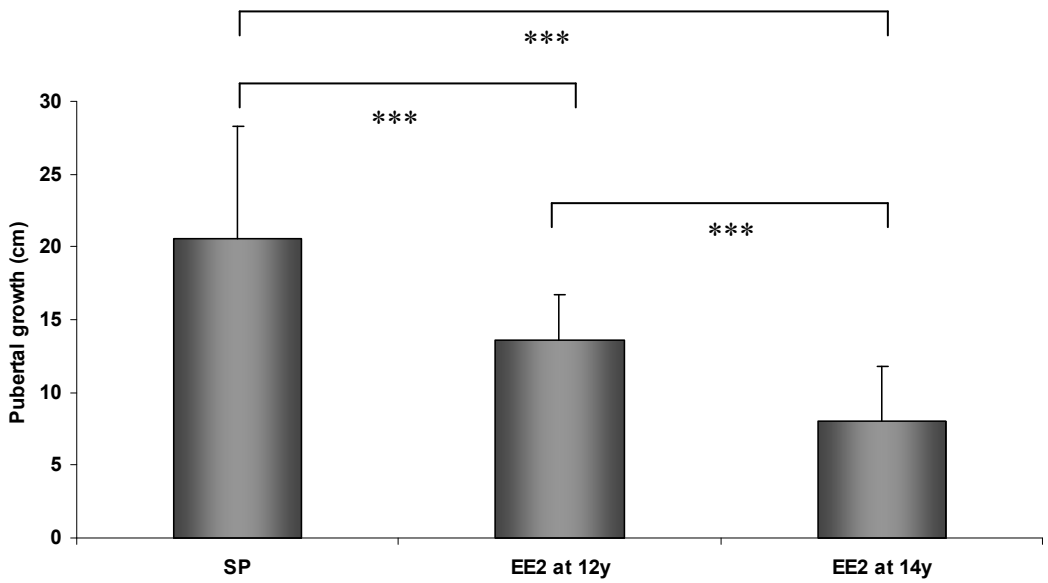


Figure 8.14 – Height gain during puberty of girls with spontaneous puberty compared to girls who required artificial induction with oral EE2 at 12 or 14 years. Post hoc comparisons between groups (Bonferroni corrected) with relative significance levels are shown: ***$P<0.001$

8.5.7 Effect of Ethinylestradiol on Bone Maturation

The bone maturation (change in BA per chronological year) of participants who commenced EE2 at 12 or 14 years of age is demonstrated from the age of 11 to 17 years in Figure 8.15. Using unpaired T-tests a difference in bone maturation was observed between the two groups from the age of 14 years onwards, with girls who commenced EE2 at 12 years showing faster bone age maturation and earlier growth plate fusion (14-15y: P=0.014; 15-16y: P=0.011; 16-17y: P=0.002). It is noteworthy that bone maturation in girls who commenced EE2 at 14 years remained less than 1 per chronological year from 12 to 17 years (Figure 8.15). The bone maturation over the 3 year period, from the age of 12 to 15 years, for girls who commenced EE2 at 12 or 14 years of age is shown in Figure 8.16. There was an increase in bone maturation in girls who commenced EE2 at 12 years of age compared to those who started EE2 at 14 years of age (Figure 8.16; P=0.006). Oxandrolone had no effect on bone maturation in either girls who commenced EE2 at 12 years (P=0.33) or at 14 years (P=0.36) see Figure 8.17.

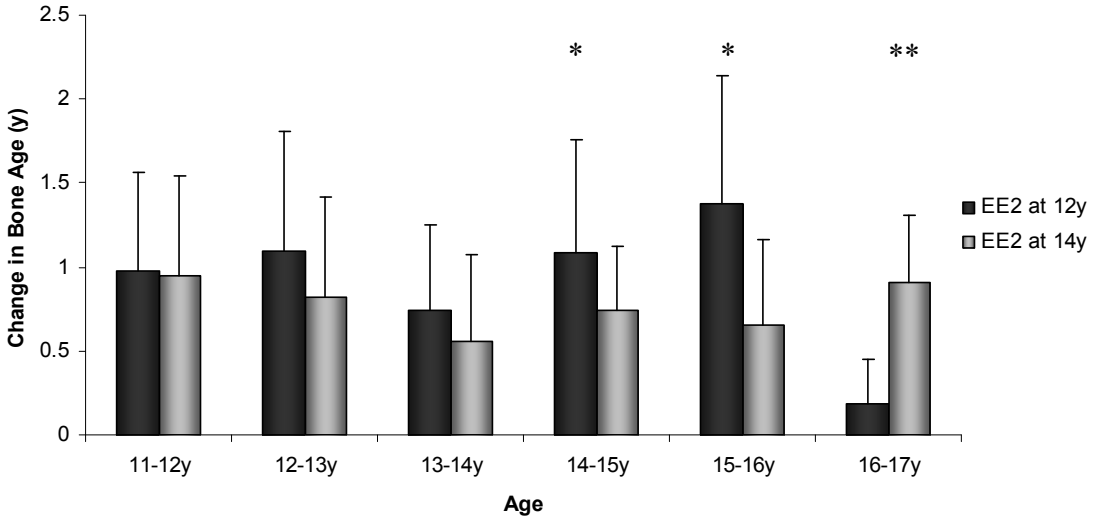


Figure 8.15 – Bone maturation of girls who started EE2 at 12y (■) or 14y (■) from 11-17 years of age: *P <0.05, **P<0.01

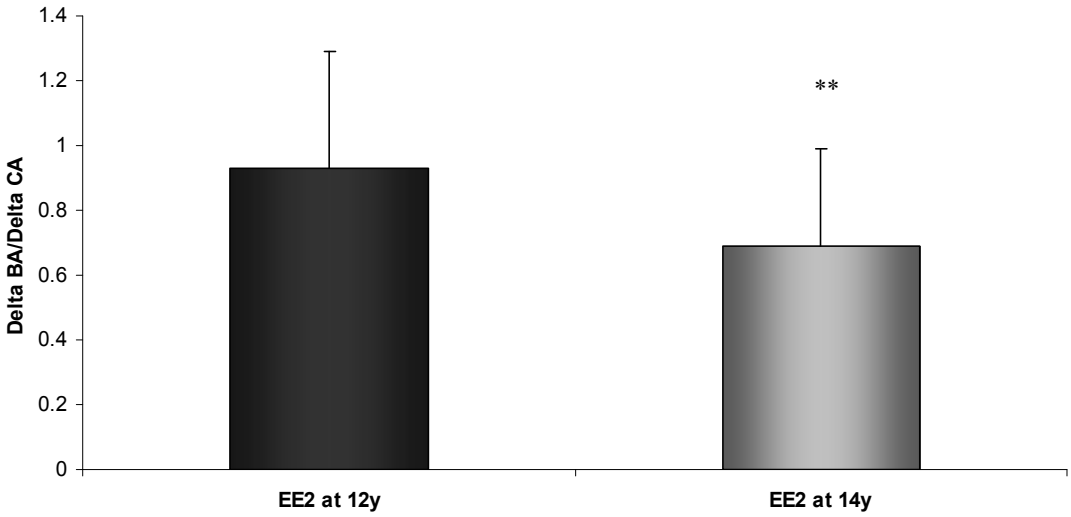


Figure 8.16 – Bone maturation of girls who started EE2 at 12y (■) or 14y (■) from 12-15 years of age: ***P*<0.01

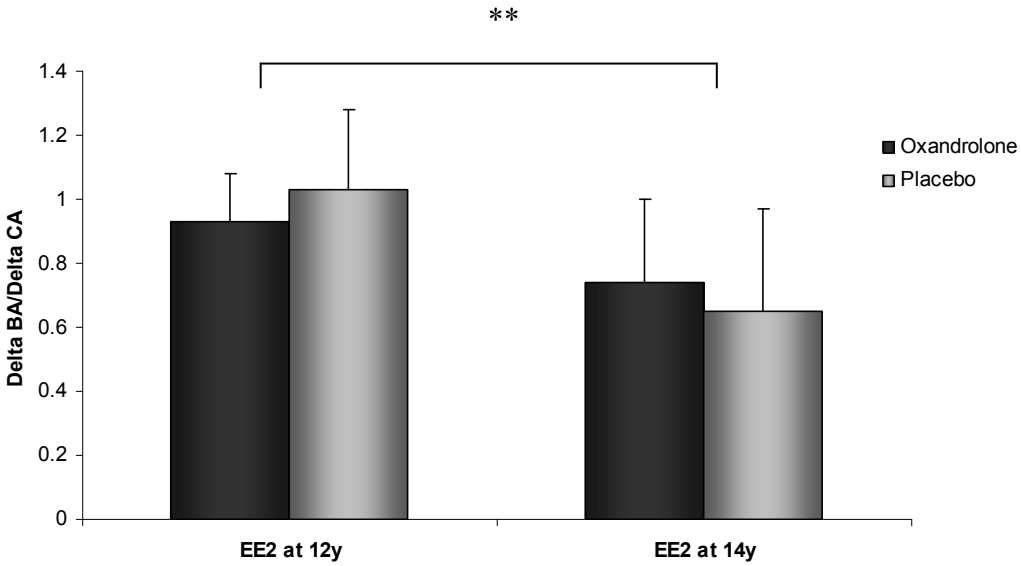


Figure 8.17 – Bone maturation of girls who started EE2 at 12y (■) or 14y (■) from 12-15 years of age according to Oxandrolone randomisation: ***P*<0.01

8.6 Discussion

Oxandrolone, the weak anabolic steroid, had a positive effect on FH. Importantly, no significant side effects were reported during the study and in particular there were no reports of virilisation with the oxandrolone doses used. The growth promotion observed with oxandrolone therapy could be due to a systemic effect via the GH-IGF1 system or a direct action on the androgen receptor in growth plate cartilage. As higher GH doses, than that employed in this study, are known to further improve FH status[281] it is possible that a further increase in GH could have occurred with oxandrolone therapy and account for the height gain. However, oxandrolone treatment has been shown to consistently improve HV without an effect on GH secretion in girls with TS[292] and in boys with CDGA.[89;90;293] In spite of this, an increase in IGF1 concentrations have been observed with oxandrolone in some[90;292] but not all of these studies[89;293]. Moreover, other studies have reported that oxandrolone does increase GH secretion in boys with CDGA.[87;294;295] Yet, hexarelin-induced GH release in short normal boys was not affected by oxandrolone.[296] Recently, the effects of oxandrolone at the level of the growth plate have been investigated by Chagin *et al* in the rat metatarsal organ culture model.[297] Oxandrolone and testosterone were both shown to have no effect on linear growth in this system even in the presence of charcoal treated serum. It is noteworthy that oxandrolone had the least effect on FH in girls with spontaneous puberty who had significant residual ovarian function (Figure 8.4; oxandrolone versus placebo delta height SD score was 0.2 versus 0.1). The TS girls who required pubertal induction may have shown a better response to oxandrolone as they have POI which is also associated with a 50% reduction in circulating androgen levels.

Delaying pubertal induction with oral EE2 until the age of 14 years was shown to increase FH by 3.7 centimetres. A similar increase in FH of 3.4 centimetres was reported by Chernausek *et al* when pubertal induction was delayed from 12 to 15 years of age.[278] However, this was probably an underestimate as FH was defined by the last height performed after the age of 13.5 years. The interaction between oxandrolone and delaying EE2 until 14 years of age was negative and close to significance suggesting that the their effects on FH are not synergistic but antagonistic. It would seem, therefore, that in girls with POI associated with TS treatment with oxandrolone would allow the introduction of oestrogen therapy at 12 years of age without having a negative impact on FH. An earlier pubertal induction would be desirable for psychological, social and physical reasons including uterine development, bone and cardiovascular health.

The rate of pubertal progression observed in this cohort of TS girls, according to age at Tanner breast stage achieved, was comparable to that of normal unaffected girls. The mean age at Tanner breast stage 2, 3 and 4 was similar to that described by van Pareren *et al* who used an alternative low dose oral oestrogen regime to induce puberty in TS girls from 12 years of age.[281] The regime consisted of 17β-oestradiol (E2): 5 μg/kg/day during the first 2 years, 7.5 μg/kg/day in the third year and 10 μg/kg/day thereafter until GH was discontinued at which point the E2 dose was increased to a dose of 1 mg/day and subsequently to 2 mg/day. As 1 mg of E2 is equivalent to 10 μg ethinylestradiol[298] a 40 kg TS girl would have received the equivalent of 2 μg EE2 per day in the first 2 years followed by 3 μg per day in the third year and 4 μg per day thereafter. In the cohort described by van Pareren *et al*

sixty girls received oral E2 at a mean age of 12.7 years and reached Tanner breast stage 2 and 4 at a mean age of 12.9 years and 14.7 years respectively. The pubertal development of this cohort was recently described in more detail by Bannink *et al*.[260] A significant delay in achieving Tanner breast stage 5 was observed in these girls which may be attributable to the oestrogen regime as the gradual increase in E2 was sustained up to the fourth year of pubertal induction. At the age of 19 years only 50% of the TS women had reached breast stage 5 compared to 90% of normal Dutch women.[260] In contrast, Chernausek *et al* employed an oral oestrogen regime using conjugated equine oestrogens in the form of Premarin.[278] The daily oestrogen dose for the first six months was 0.3 mg and thereafter 0.625 mg was given which is the equivalent of 4.8 µg and 10 µg ethinylestradiol respectively.[298] The dose of oestrogen was therefore relatively high and increased fairly rapidly. Not surprisingly the pubertal progression was swift with approximately 80% of girls achieving breast stage two or greater after 6 months of oestrogen therapy and 90% of girls achieving breast stage three or greater after 18 months of which 35% had reached breast stage four or five.

Girls with TS who have not received any hormonal treatment show a gradual deceleration in HV.[291] The introduction of 2 µg of oral ethinylestradiol at 12 years of age led to stabilisation of the downward trend in HV for one year after EE2 introduction. This is similar to the findings of Chernausek *et al* where TS girls treated with oral 0.3 mg Premarin (equivalent to 4.8 µg EE2) at 12 years of age maintained their HV for 1 year after commencing oestrogen and then showed a rapid deceleration in HV.[278] By the age of 15-16 years their HV was less than 1 cm per

year. van Pareren *et al* also reported a stabilisation of HV in TS girls who commenced 5 µg/kg/day E2 (equivalent to 0.05 µg/kg/day EE2 ≈ 2 µg in 40kg girl) at 12 years of age.[281] No further HV data was reported from this cohort. In contrast, when pubertal induction was delayed until 14 years of age in the UK Turner study the gradual decline in HV continued after EE2 was introduced with no stabilisation of HV during the first year of treatment. Whereas, Chernausek *et al* report a stabilisation of HV after oestrogen is introduced at 15 years of age. However, no further HV data was provided after 16 years of age from this smaller cohort of TS girls (n=16-29).[278] Massa *et al* reported a deceleration in HV in TS girls established on GH therapy when oral ethinylestradiol (0.1 µg/kg/day ≈ 4µg in 40kg girl) was introduced.

The sub-group of TS girls with significant residual ovarian function, as evidenced by a spontaneous onset of puberty and normal FSH levels, showed a different growth pattern during puberty. They had a growth spurt that was of normal timing and amplitude compared to unaffected girls. This group of girls all had a mosaic karyotype, and so did not include any girls with the classical 45,X karyotype, which suggests that the presence of a pubertal growth spurt may be a reflection of a less severe underlying skeletal dysplasia. However, at enrolment girls with SP had a similar degree of short stature to girls with POI who required EE2 treatment and dysmorphology scores were not statistically different between the groups. It is therefore more likely that the growth patterns differ because spontaneous puberty is more growth enhancing than one which has been artificially induced with oral EE2. As it is recognised that the administration of oral EE2 may not be the most

physiological method of inducing puberty it is possible that induction with a transdermal formulation of a natural oestrogen may improve growth during puberty in girls with TS and primary ovarian insufficiency. Although girls with SP did show growth acceleration and a greater height gain in puberty a positive effect on FH compared to girls who required oral EE2 was not demonstrated.

The introduction of oral EE2 at 12 years of age did not affect bone maturation, as compared to those treated at 14 years, during the first two years of EE2 therapy. However, after 14 years of age bone maturation was significantly greater in girls who commenced EE2 at 12 rather than 14 years of age. Similarly, van Pareren *et al* reported no change in bone maturation in girls treated with low dose E2 during the year after E2 initiation as compared to their bone maturation the year before E2.[281] Whereas, Chernausek *et al* observed a significant increase in bone maturation during the year that oestrogen therapy was initiated.[278] These differences observed in bone maturation are likely to be related to the dose of oestrogen used.

8.7 Conclusions

Oxandrolone treatment has an unequivocal positive effect on FH in girls with TS when commenced at 9 years of age. Similarly, delaying the onset of EE2 therapy to induce puberty until 14 years significantly improves FH outcome in TS girls with POI. There is a negative interaction suggesting that there would be little to gain from implementing both interventions. It would seem, therefore, that the use of oxandrolone would obviate the need to delay pubertal induction until 14 years of age in girls with POI associated with TS. The negative interaction does however suggest

that there is a finite amount of growth which can be obtained from growth promoting therapies in TS and that commencing GH at too early an age for example during infancy may not confer an advantage to FH.

The standardised low dose oral EE2 regime induced puberty over a 3 year period which corresponds to that observed in normal unaffected girls. Low dose oral EE2 maintained HV for 1 year when introduced at 12 years of age but led to an immediate deceleration at 14 years of age. Thereafter, the natural deceleration in TS girls was augmented with subsequent increases in EE2. Girls who received an artificial induction of puberty with oral EE2 therefore showed no acceleration of growth during puberty. Whereas, TS girls with a spontaneous onset of puberty had a growth spurt that was of normal timing and amplitude. The difference in pubertal growth pattern may be explained by a less severe underlying skeletal dysplasia or more likely, it may be a reflection that puberty with a spontaneous onset is more growth enhancing than an artificial puberty with exogenous oestrogen. These findings underline the need to examine the effects of more physiological methods of oestrogen administration, such as a transdermal formulation, on height velocity as well as other markers of pubertal growth and development and would form the basis of future studies.

Chapter 9

General discussion and future work

9.1 General discussion

Longitudinal growth in children, which is mainly due to chondrocytic activity at the epiphyseal growth plate, is influenced by many hormones and growth factors. Their influence is even more complex during the accelerated period of pubertal growth. Sex steroids play an essential role in pubertal growth both at the systemic level via the GH/IGF1 axis and at the local level of the growth plate. It is now widely accepted that oestrogen is the key hormone in controlling acceleration of growth during puberty and growth plate fusion. Although abnormalities of growth during puberty are very common the mechanisms that control the onset and cessation of pubertal growth are not clear. The work within this thesis has endeavoured to increase our knowledge of the effects of oestrogen on longitudinal growth through a variety of *in vitro* and *in vivo* studies involving bench and clinical work.

At the outset it was demonstrated that both of the classical oestrogen receptors, ERα and ERβ, were expressed in human and murine chondrocyte cell lines. Disappointingly, in the absence of foetal bovine serum, oestradiol had no effect on proliferation, differentiation or apoptosis of chondrocyte cells in monolayer culture or on linear growth in the foetal murine metatarsal culture system. However, this is in accordance with the recent work by Chagin *et al*.[129] In addition, oestradiol did not convey a protective effect to chondrocytes exposed to the pro-inflammatory cytokines, TNF-α and IL-1β in monolayer culture. However, endogenous oestrogen appears to play an important role in maintaining chondrocyte proliferation in monolayer culture of human and murine cells but is not a prerequisite for normal linear growth in the foetal murine metatarsals as demonstrated by their respective

responses to the non-specific oestrogen antagonist Faslodex. In contrast, Chagin *et al* described a reduction in linear growth in foetal rat metatarsals exposed to Faslodex.[129] As the methods and concentrations of Faslodex studied were remarkably similar between the experiments the observed difference in metatarsal growth after exposure to Faslodex is likely to be due to species differences. However, Faslodex did significantly reduce the length of the mineralising zone in murine foetal metatarsals suggesting that endogenous oestrogen is important for mineralisation. This was supported by the finding that Faslodex-treated metatarsals showed a reduction in calcium concentration compared to controls after 14 days exposure. As the overall length of the metatarsals was not affected following exposure to Faslodex, it suggests that one or more of the other growth plate maturational zones must have increased. One possible explanation is that oestrogen's role in mineralisation involves the terminal differentiation of hypertrophic chondrocytes and in the absence of endogenous oestrogen the hypertrophic zone increases due to a reduction in terminal differentiation. The observed reduction of ALP activity in murine ATDC5 chondrocytes after exposure to Faslodex could reflect a reduction in chondrocyte differentiation, or alternatively, it could be due to a direct effect of Faslodex on ALP activity. Interestingly, the mineralising zone of the metatarsals was also reduced after exposure to the selective ERα antagonist, MPP suggesting that oestrogen may mediate its effect on mineralisation via ERα.

An interesting finding in both human and murine chondrocytes was the reduction of chondrocyte proliferation in the presence of MPP, a selective ERα antagonist, and raloxifene, a prototypical selective oestrogen receptor modulator with higher ERβ

binding affinity. This was accompanied by an increase in apoptosis of chondrocytes. A similar effect was observed in foetal murine metatarsals treated with MPP and raloxifene in combination with a marked reduction in linear growth observed after five days in culture. A less marked restriction in growth was seen in metatarsals treated with MPP in isolation. As MPP is a specific ERα antagonist and raloxifene acts mainly as an ERβ agonist it is likely that these effects are mediated by ERβ. These findings suggest that the oestrogen receptors in the growth plate may have opposing actions with ERβ acting as a brake on chondrocyte proliferation and growth and ERα promoting growth. The proliferation and differentiation of growth plate chondrocytes are ultimately under the control of cell cycle modulators such as cyclins, cyclin-dependent kinases (CDK) and their inhibitors. The levels of some key cell cycle proteins from ATDC5 chondrocytes were therefore analysed after exposure to MPP and raloxifene in combination. A reduction was found in the cell cycle proteins cyclin E and p53 but cyclin D1 and c-Myc were unaltered on Western Blot analysis. Cyclin E specifically regulates the G_1/S phase transition of the cell cycle. As exposure of ATDC5 cells to MPP and raloxifene in combination led to a decrease in cyclin E but did not alter cyclin D1 levels it suggests that their effects may be mediated by the CDK inhibitors p21 or p27. In the human HCT8 colon cancer cell line over-expression of ERβ inhibited cell proliferation through regulation of cell cycle components with decreased levels of cyclin E and an increase in the CDK inhibitor p21. Flow cytometry analysis provided evidence for blocking of the G1/S phase progression which was induced by ERβ over-expression.[200] Thus, ER may regulate cell proliferation through control of cell cycle modulators and arrest in G1/S phase transition in chondrocytes as well as in colonic cells.

In the second part of the thesis the results from the clinical studies investigating the effect of oral oestrogen on longitudinal growth in girls with POI are presented. POI is an uncommon condition in childhood with low prevalence rates in girls aged 16 years and under. The commonest cause in this cohort of girls is POI associated with TS (83.7%). Non-TS associated POI is rare and the leading cause is iatrogenic secondary to the effects of total body irradiation for bone marrow transplantation (12.8%). A significant proportion of these girls developed POI after full pubertal development so few cases were available to investigate the effect of oestrogen on linear growth. In addition, previous radiotherapy affected their spinal growth and other acquired endocrinopathies such as GH deficiency or hypothyroidism were common. The majority of girls with TS had POI but 17.2% had significant ovarian reserve and underwent a spontaneous puberty. The actual regime of oral oestrogen followed in individual patients with TS was highly variable so it was not possible to assess the effects of the oestrogen dose on HV or bone maturation in the context of a retrospective study. However, in the second study it was possible to analyse the effect of oral oestrogen on HV, bone maturation and FH in girls with TS as a standardised regime of oral ethinylestradiol was employed as part of the prospective multi-centre UK Turner study. The aims of this randomised, double-blind, placebo-controlled study of growth promoting treatment in TS was to assess the impact of oxandrolone and the age of onset of EE2 at either 12 or 14 years on FH. The effect of EE2 on HV was not part of the UK study protocol and additional ethical approval was therefore obtained to analyse the raw data. Oxandrolone treatment and delaying pubertal induction to 14 years of age both significantly improved FH in girls with TS but there was a negative interaction suggesting that the two interventions are likely to

be antagonistic. However, oxandrolone therapy would allow the introduction of oestrogen at a younger age. The standardised oral EE2 regime employed in the study induced puberty over a three year period which corresponds to that observed in normal unaffected girls. Low dose EE2 (2 μg/day) maintained HV for one year when it was introduced at 12 years of age. Thereafter, the natural deceleration recorded in a historical cohort of TS girls[291] was augmented with subsequent increases in EE2. In contrast, the same low dose EE2 commenced at 14 years of age led to a reduction in HV which increased further with subsequent EE2 dose increases. TS girls who received oral E2 to induce pubertal development therefore did not show any growth acceleration and peak height velocities were not observed. Girls with preserved ovarian function exhibited a different growth pattern during puberty with a mean peak HV of 8.5 centimetres/year. This may be explained by a less severe underlying skeletal dysplasia or it may be a reflection that puberty with a spontaneous onset is more growth enhancing.

During puberty in normal girls, oestradiol levels increase[299] and are significantly associated with HV.[300] However, after reaching peak HV the growth rate slows despite high circulating levels of E2. Therefore the effect of oestrogen on longitudinal growth changes at this point. The laboratory effects of oestradiol found in this thesis suggest that ERα may stimulate or maintain growth, and ERβ may inhibit growth. The obvious question is how these observations might be involved in the complex relationship between puberty, oestrogen and height velocity. This could be due to changes of agonist (oestrogen) affinity for the oestrogen receptor, differences in receptor expression (absolute number or relative numbers of subtypes),

or receptor coupling with second messengers.

Affinity studies show that the half maximal effective concentration (EC_{50}) of oestradiol at ERα is achieved at slightly lower concentrations than ERβ.[301] Therefore it is conceivable that the ERα effect could predominate at lower systemic concentrations of oestradiol and that ERβ could become more important at higher concentrations. It is also possible that the expression of ERα reduces or ERβ increases in the growth plate after reaching peak HV. Analysis of the ER expression in the human growth plate during puberty has not been done in a sequential fashion due to the limited availability of growth plate specimens from healthy children. Nilsson *et al* have confirmed the presence of both ERα and ERβ along with the androgen receptor in all zones of the growth plate at each pubertal stage. However, this was a cross sectional study of children, mainly with leg length inequality.[115] Since the completion of the laboratory component of this thesis the new GPR30 oestrogen receptor has been reported to be expressed in the human growth plate.[132] The expression of this receptor declines with pubertal staging. However, its effect on the growth plate has yet to be characterised. Obviously if selective agonists and antagonists of the oestrogen receptor GPR30 became available it would be of great interest to repeat the experiments performed in chapters 3 and 4.

The observations from the Turner study component of this thesis show that the introduction of oral EE2 is associated with pubertal development but no acceleration in growth in contrast to that claimed by workers using parenteral administration.[302;303] These studies which were both performed by Rosenfield *et al*

showed acceleration in growth in TS with the use of systemic E2 in the form of low dose monthly depots of oestradiol cypionate.[302;303] However, study numbers were small and did not include a control group. This difference observed in growth pattern with oral EE2 in contrast to parenteral E2 may reflect its incomplete bioavailability due to pre-systemic metabolism and subsequent lower circulating oestrogen levels. Also oestrogen levels are more likely to fluctuate with oral administration than absorption from a parenteral depot. It is possible that the plasma levels achieved with oral EE2 are not consistently high enough to improve HV but still increase bone maturation. In addition, direct effects of oral oestrogen on hepatic IGF-1 secretion are probably important as several studies have shown decreased IGF-1 concentrations after administration of oral oestrogen[304-306] compared with unchanged IGF-1 levels after transdermal oestrogen.[304] It is difficult to make any hypotheses about the role of oestrogen in growth in TS without knowing what plasma concentrations were actually achieved with the different treatment routes. However, the parental dose employed seems to be higher at 134 µg active oestradiol per month (bioavailability of 1) versus 60 µg per month (bioavailability of 0.4-0.45).[307]

Furthermore, in TS there could be a difference in expression of ERα and ERβ compared to normal girls. It is quite conceivable the oestrogen deficiency associated with POI in early life could influence the expression of ERs.

9.2 Future work

The results presented within the first part of this thesis describe the effect, or rather lack of effect, of exogenous E2 at the level of the growth plate and suggest potential different roles of the classical oestrogen receptors in the growth plate. However, further work is required to clarify the precise mechanisms of action of each ER in the growth plate. The growth retardation observed in metatarsals exposed to raloxifene and MPP in combination was an interesting finding and suggests that ERβ may have an inhibitory effect on linear growth. As growth retardation was also present, but not as marked, after exposure to the specific ERα antagonist it suggests that ERα has an opposing action to ERβ and promotes growth. The mechanisms leading to growth inhibition in murine metatarsals could be examined further by studying the expression of the oestrogen receptors along with BMP and VGF. Histology of the metatarsals with measurement of zones would be useful, in particular to compare the effects of MPP alone and MPP in combination with raloxifene. The reduction of the cell cycle regulating proteins cyclin E and p53 in ATDC5 cells exposed to raloxifene and MPP in combination suggests that ERβ regulates proliferation through cell cycle components. This could occur through the action of CDK inhibitors p21 or p27 and further experiments examining these cell cycle regulators could shed further light on the precise pathways/molecular mechanisms of action of raloxifene and MPP. Further work in primary chondrocytes (murine and/or human) as opposed to a transformed cell line would be preferable as this would be more representative of the human situation. The role of ERβ in regulating chondrocyte growth could be examined further by stable transfection techniques to over-express ERβ or alternatively small interfering RNA (siRNA) technology could be utilised to knock

down ERβ expression. Alternatively, this could be studied in tissue or cells obtained from ERβ knock out mice.

Endogenous oestrogen appears to be important for mineralisation of metatarsals as reflected by their response to the non-specific ER antagonist, Faslodex. The role that oestrogen plays in mineralisation of the metatarsals could be clarified by further experiments incorporating histology and immunohistochemistry/in-situ hybridisation to detect zone specific protein and gene expression e.g. collagen type X to assist in measurement of the different zones to confirm if there is an increase in the hypertrophic zone in metatarsals exposed to Faslodex. A potential further experiment would be to compare the effects of Faslodex alone with Faslodex in combination with bisphosphonates to investigate if bisphosphonates can ameliorate the effects of withdrawal of endogenous oestrogen on mineralisation in this model.

In the clinical component of this thesis it was observed that girls with POI associated with TS did not show growth acceleration during pubertal induction with oral oestrogen and had sub-optimal uterine development at the end of induction. Rosenfield *et al* showed acceleration in growth in TS with the use of monthly depots of oestradiol.[303] Less invasive forms of parenteral oestrogen have been investigated and recent studies employing transdermal oestrogen patches or gel to induce puberty in hypogonadal girls have shown encouraging results.[308;309] A study comparing a transdermal regime of natural oestrogen with the standard oral EE2 regime would be a logical next step. In order to recruit a suitably large enough cohort of girls with TS it would best be performed as a multicentre study. It would be interesting to know if

transdermal oestrogen does improve the amplitude of growth during puberty and would therefore allow the introduction of oestrogen at a more appropriate age without having a negative impact on FH. The circulating levels of oestrogen obtained in the plasma would be useful in comparing the different formulations but may not be feasible within a multi-centre setting, but could be performed in a sub-group. Analysis of data from the UK Turner study, in which the interaction between oxandrolone and late pubertal induction is negative rather than additive,
points towards there being a finite amount of potential growth that TS girls can achieve with growth promoting therapy. It follows from this conclusion that starting growth hormone therapy at a particularly early age in TS for example in infancy may not confer an advantage in terms of final height, and that the use of oxandrolone from 9 years may allow the clinician to begin pubertal induction at a more physiological age without having a negative impact on final height.

Much work remains to be done in the field of growth and pubertal induction in Turner syndrome. Apart from testing the effect of different protocols of pubertal induction on linear growth and pubertal development, their impact on quality of life, cardiovascular risk factors, bone health and uterine development needs to be the subject of systematic and collaborative study.

Reference List

(1) Coste J, Ecosse E, Lesage C, Chaussain JL, Carel JC. Evaluation of adolescent statural growth in health and disease: reliability of assessment from height measurement series and development of an automated algorithm. Horm Res 2002; 58(3):105-114.

(2) Berkey CS, Dockery DW, Wang X, Wypij D, Ferris B, Jr. Longitudinal height velocity standards for U.S. adolescents. Stat Med 1993; 12(3-4):403-414.

(3) Biro FM, McMahon RP, Striegel-Moore R, Crawford PB, Obarzanek E, Morrison JA et al. Impact of timing of pubertal maturation on growth in black and white female adolescents: The National Heart, Lung, and Blood Institute Growth and Health Study. J Pediatr 2001; 138(5):636-643.

(4) Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child 1969; 44(235):291-303.

(5) Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in boys. Arch Dis Child 1970; 45(239):13-23.

(6) Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B et al. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 1994; 331(16):1056-1061.

(7) Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 1995; 80(12):3689-3698.

(8) Bilezikian JP, Morishima A, Bell J, Grumbach MM. Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N Engl J Med 1998; 339(9):599-603.

(9) Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J et al. Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 1997; 337(2):91-95.

(10) Bulun SE. Aromatase deficiency and estrogen resistance: from molecular genetics to clinic. Semin Reprod Med 2000; 18(1):31-39.

(11) Zachmann M, Prader A, Sobel EH, Crigler JF, Jr., Ritzen EM, Atares M et al. Pubertal growth in patients with androgen insensitivity: indirect evidence for the importance of estrogens in pubertal growth of girls. J Pediatr 1986; 108(5 Pt 1):694-697.

(12) He Q, Karlberg J. Bmi in childhood and its association with height gain, timing of puberty, and final height. Pediatr Res 2001; 49(2):244-251.

(13) Weise M, De Levi S, Barnes KM, Gafni RI, Abad V, Baron J. Effects of estrogen on growth plate senescence and epiphyseal fusion. Proc Natl Acad Sci U S A 2001; 98(12):6871-6876.

(14) Moss ML, Noback CR. A longitudinal study of digital epiphyseal fusion in adolescence. Anat Rec 1958; 131(1):19-32.

(15) Parfitt AM. Misconceptions (1): epiphyseal fusion causes cessation of growth. Bone 2002; 30(2):337-339.

(16) Stevens DG, Boyer MI, Bowen CV. Transplantation of epiphyseal plate allografts between animals of different ages. J Pediatr Orthop 1999; 19(3):398-403.

(17) Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD et al. Catch-up growth after glucocorticoid excess: a mechanism intrinsic to the growth plate. Endocrinology 1994; 135(4):1367-1371.

(18) Walker KV, Kember NF. Cell kinetics of growth cartilage in the rat tibia. II. Measurements during ageing. Cell Tissue Kinet 1972; 5(5):409-419.

(19) Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De Levi S et al. Catch-up growth is associated with delayed senescence of the growth plate in rabbits. Pediatr Res 2001; 50(5):618-623.

(20) Schrier L, Ferns SP, Barnes KM, Emons JA, Newman EI, Nilsson O et al. Depletion of resting zone chondrocytes during growth plate senescence. J Endocrinol 2006; 189(1):27-36.

(21) Herman-Giddens ME, Slora EJ, Wasserman RC, Bourdony CJ, Bhapkar MV, Koch GG et al. Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. Pediatrics 1997; 99(4):505-512.

(22) Kaplowitz PB, Oberfield SE. Re-examination of the age limit for defining when puberty is precocious in girls in the United States: implications for evaluation and treatment. Drug and Therapeutics and Executive Committees of the Lawson Wilkins Pediatric Endocrine Society. Pediatrics 1999; 104(4 Pt 1):936-941.

(23) Parent AS, Teilmann G, Juul A, Skakkebaek NE, Toppari J, Bourguignon JP. The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. Endocr Rev 2003; 24(5):668-693.

(24) Ojeda SR, Heger S. New thoughts on female precocious puberty. J Pediatr Endocrinol Metab 2001; 14(3):245-256.

(25) Pescovitz OH, Hench KD, Barnes KM, Loriaux DL, Cutler GB, Jr. Premature thelarche and central precocious puberty: the relationship between clinical

presentation and the gonadotropin response to luteinizing hormone-releasing hormone. J Clin Endocrinol Metab 1988; 67(3):474-479.

(26) Klein KO. Precocious puberty: who has it? Who should be treated? J Clin Endocrinol Metab 1999; 84(2):411-414.

(27) Kletter GB, Kelch RP. Clinical review 60: Effects of gonadotropin-releasing hormone analog therapy on adult stature in precocious puberty. J Clin Endocrinol Metab 1994; 79(2):331-334.

(28) Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM. Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. N Engl J Med 1991; 325(24):1688-1695.

(29) Rosenthal SM, Grumbach MM, Kaplan SL. Gonadotropin-independent familial sexual precocity with premature Leydig and germinal cell maturation (familial testotoxicosis): effects of a potent luteinizing hormone-releasing factor agonist and medroxyprogesterone acetate therapy in four cases. J Clin Endocrinol Metab 1983; 57(3):571-579.

(30) Weise M, Flor A, Barnes KM, Cutler GB, Jr., Baron J. Determinants of growth during gonadotropin-releasing hormone analog therapy for precocious puberty. J Clin Endocrinol Metab 2004; 89(1):103-107.

(31) Anasti JN, Flack MR, Froehlich J, Nelson LM, Nisula BC. A potential novel mechanism for precocious puberty in juvenile hypothyroidism. J Clin Endocrinol Metab 1995; 80(1):276-279.

(32) Barnes ND, Hayles AB, Ryan RJ. Sexual maturation in juvenile hypothyroidism. Mayo Clin Proc 1973; 48(12):849-856.

(33) Pringle PJ, Stanhope R, Hindmarsh P, Brook CG. Abnormal pubertal development in primary hypothyroidism. Clin Endocrinol (Oxf) 1988; 28(5):479-486.

(34) Albanese A, Stanhope R. Predictive factors in the determination of final height in boys with constitutional delay of growth and puberty. J Pediatr 1995; 126(4):545-550.

(35) Crowne EC, Shalet SM, Wallace WH, Eminson DM, Price DA. Final height in boys with untreated constitutional delay in growth and puberty. Arch Dis Child 1990; 65(10):1109-1112.

(36) Crowne EC, Shalet SM, Wallace WH, Eminson DM, Price DA. Final height in girls with untreated constitutional delay in growth and puberty. Eur J Pediatr 1991; 150(10):708-712.

(37) Sperlich M, Butenandt O, Schwarz HP. Final height and predicted height in boys with untreated constitutional growth delay. Eur J Pediatr 1995; 154(8):627-632.

(38) Sedlmeyer IL, Hirschhorn JN, Palmert MR. Pedigree analysis of constitutional delay of growth and maturation: determination of familial aggregation and inheritance patterns. J Clin Endocrinol Metab 2002; 87(12):5581-5586.

(39) Baum WF, Schneyer U, Lantzsch AM, Kloditz E. Delay of growth and development in children with bronchial asthma, atopic dermatitis and allergic rhinitis. Exp Clin Endocrinol Diabetes 2002; 110(2):53-59.

(40) Poyrazoglu S, Gunoz H, Darendeliler F, Saka N, Bundak R, Bas F. Constitutional delay of growth and puberty: from presentation to final height. J Pediatr Endocrinol Metab 2005; 18(2):171-179.

(41) Joss EE, Schmidt HA, Zuppinger KA. Oxandrolone in constitutionally delayed growth, a longitudinal study up to final height. J Clin Endocrinol Metab 1989; 69(6):1109-1115.

(42) Kelly BP, Paterson WF, Donaldson MD. Final height outcome and value of height prediction in boys with constitutional delay in growth and adolescence treated with intramuscular testosterone 125 mg per month for 3 months. Clin Endocrinol (Oxf) 2003; 58(3):267-272.

(43) Wickman S, Sipila I, Ankarberg-Lindgren C, Norjavaara E, Dunkel L. A specific aromatase inhibitor and potential increase in adult height in boys with delayed puberty: a randomised controlled trial. Lancet 2001; 357(9270):1743-1748.

(44) Pozo J, Argente J. Delayed puberty in chronic illness. Best Pract Res Clin Endocrinol Metab 2002; 16(1):73-90.

(45) van der Werff ten Bosch JJ, Bot A. Some skeletal dimensions of males with isolated gonadotrophin deficiency. Neth J Med 1992; 41(5-6):259-263.

(46) Ratcliffe SG, Bancroft J, Axworthy D, McLaren W. Klinefelter's syndrome in adolescence. Arch Dis Child 1982; 57(1):6-12.

(47) Ranke MB, Saenger P. Turner's syndrome. Lancet 2001; 358(9278):309-314.

(48) Hook EB. Spontaneous deaths of fetuses with chromosomal abnormalities diagnosed prenatally. N Engl J Med 1978; 299(19):1036-1038.

(49) Nielsen J, Wohlert M. Chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Arhus, Denmark. Hum Genet 1991; 87(1):81-83.

(50) Hook EB, Warburton D. The distribution of chromosomal genotypes associated with Turner's syndrome: livebirth prevalence rates and evidence for diminished fetal mortality and severity in genotypes associated with structural X abnormalities or mosaicism. Hum Genet 1983; 64(1):24-27.

(51) Hall JG, Sybert VP, Williamson Nl, Reed SD. Turner's syndrome. West J Med 1982; 137:32-44.

(52) Ross JL, Long LM, Loriaux DL, Cutler GB, Jr. Growth hormone secretory dynamics in Turner syndrome. J Pediatr 1985; 106(2):202-206.

(53) Ranke MB, Blum WF, Haug F, Rosendahl W, Attanasio A, Enders H et al. Growth hormone, somatomedin levels and growth regulation in Turner's syndrome. Acta Endocrinol (Copenh) 1987; 116(3):305-313.

(54) Gravholt CH, Frystyk J, Flyvbjerg A, Orskov H, Christiansen JS. Reduced free IGF-I and increased IGFBP-3 proteolysis in Turner syndrome: modulation by female sex steroids. Am J Physiol Endocrinol Metab 2001; 280(2):E308-E314.

(55) Park E, Bailey JD, Cowell CA. Growth and maturation of patients with Turner's syndrome. Pediatr Res 1983; 17(1):1-7.

(56) Pasquino AM, Passeri F, Pucarelli I, Segni M, Municchi G. Spontaneous pubertal development in Turner's syndrome. Italian Study Group for Turner's Syndrome. J Clin Endocrinol Metab 1997; 82(6):1810-1813.

(57) Lippe B. Turner syndrome. Endocrinol Metab Clin North Am 1991; 20:121-152.

(58) Ranke MB. Growth disorder in the Ullrich-Turner syndrome. Baillieres Clin Endocrinol Metab 1992; 6(3):603-619.

(59) Laron Z. Development and biological function of the female gonads and genitalia in IGF-I deficiency -- Laron syndrome as a model. Pediatr Endocrinol Rev 2006; 3 Suppl 1:188-191.

(60) Tanaka T, Cohen P, Clayton PE, Laron Z, Hintz RL, Sizonenko PC. Diagnosis and management of growth hormone deficiency in childhood and adolescence--part 2: growth hormone treatment in growth hormone deficient children. Growth Horm IGF Res 2002; 12(5):323-341.

(61) Chiarelli F, Giannini C, Mohn A. Growth, growth factors and diabetes. Eur J Endocrinol 2004; 151 Suppl 3:U109-U117.

(62) Meikle AW. The interrelationships between thyroid dysfunction and hypogonadism in men and boys. Thyroid 2004; 14 Suppl 1:S17-S25.

(63) Pantsiouou S, Stanhope R, Uruena M, Preece MA, Grant DB. Growth prognosis and growth after menarche in primary hypothyroidism. Arch Dis Child 1991; 66(7):838-840.

(64) Zadik Z, Cooper M, Chen M, Stern N. Cushing's disease presenting as pubertal arrest. J Pediatr Endocrinol 1993; 6(2):201-204.

(65) Styne DM, Grumbach MM, Kaplan SL, Wilson CB, Conte FA. Treatment of Cushing's disease in childhood and adolescence by transsphenoidal microadenomectomy. N Engl J Med 1984; 310(14):889-893.

(66) Carroll PV, Monson JP, Grossman AB, Besser GM, Plowman PN, Afshar F et al. Successful treatment of childhood-onset Cushing's disease is associated with persistent reduction in growth hormone secretion. Clin Endocrinol (Oxf) 2004; 60(2):169-174.

(67) Davies JH, Storr HL, Davies K, Monson JP, Besser GM, Afshar F et al. Final adult height and body mass index after cure of paediatric Cushing's disease. Clin Endocrinol (Oxf) 2005; 62(4):466-472.

(68) Gustafsson JA. Estrogen receptor beta--a new dimension in estrogen mechanism of action. J Endocrinol 1999; 163(3):379-383.

(69) Cowley SM, Parker MG. A comparison of transcriptional activation by ER alpha and ER beta. J Steroid Biochem Mol Biol 1999; 69(1-6):165-175.

(70) Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 1988; 55(1):145-156.

(71) Krishnan V, Wang X, Safe S. Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. J Biol Chem 1994; 269(22):15912-15917.

(72) Yang NN, Venugopalan M, Hardikar S, Glasebrook A. Identification of an estrogen response element activated by metabolites of 17beta-estradiol and raloxifene. Science 1996; 273(5279):1222-1225.

(73) Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G et al. Mechanisms of estrogen action. Physiol Rev 2001; 81(4):1535-1565.

(74) McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW. Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Mol Endocrinol 1995; 9(6):659-669.

(75) Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC et al. Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 1994; 93(1):63-69.

(76) Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997; 337(23):1641-1647.

(77) Wijayaratne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM et al. Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 1999; 140(12):5828-5840.

(78) Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138(3):863-870.

(79) Weatherman RV, Clegg NJ, Scanlan TS. Differential SERM activation of the estrogen receptors (ERalpha and ERbeta) at AP-1 sites. Chem Biol 2001; 8(5):427-436.

(80) Nettles KW, Greene GL. Ligand control of coregulator recruitment to nuclear receptors. Annu Rev Physiol 2005; 67:309-333.

(81) Juul A, Bang P, Hertel NT, Main K, Dalgaard P, Jorgensen K et al. Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. J Clin Endocrinol Metab 1994; 78(3):744-752.

(82) Martha PM, Jr., Gorman KM, Blizzard RM, Rogol AD, Veldhuis JD. Endogenous growth hormone secretion and clearance rates in normal boys, as determined by deconvolution analysis: relationship to age, pubertal status, and body mass. J Clin Endocrinol Metab 1992; 74(2):336-344.

(83) Rose SR, Municchi G, Barnes KM, Kamp GA, Uriarte MM, Ross JL et al. Spontaneous growth hormone secretion increases during puberty in normal girls and boys. J Clin Endocrinol Metab 1991; 73(2):428-435.

(84) Albertsson-Wikland K, Rosberg S, Karlberg J, Groth T. Analysis of 24-hour growth hormone profiles in healthy boys and girls of normal stature: relation to puberty. J Clin Endocrinol Metab 1994; 78(5):1195-1201.

(85) Frohman L.A. Disorders of the anterior pituitary. In: Felig P., Baxter J.D., Frohman L.A., editors. Endocrinology and Metabolism. Ohio: McGraw-Hill, 1995.

(86) Chowen JA, Frago LM, Argente J. The regulation of GH secretion by sex steroids. Eur J Endocrinol 2004; 151 Suppl 3:U95-100.

(87) Ulloa-Aguirre A, Blizzard RM, Garcia-Rubi E, Rogol AD, Link K, Christie CM et al. Testosterone and oxandrolone, a nonaromatizable androgen, specifically amplify the mass and rate of growth hormone (GH) secreted per burst without altering GH secretory burst duration or frequency or the GH half-life. J Clin Endocrinol Metab 1990; 71(4):846-854.

(88) Eakman GD, Dallas JS, Ponder SW, Keenan BS. The effects of testosterone and dihydrotestosterone on hypothalamic regulation of growth hormone secretion. J Clin Endocrinol Metab 1996; 81(3):1217-1223.

(89) Malhotra A, Poon E, Tse WY, Pringle PJ, Hindmarsh PC, Brook CG. The effects of oxandrolone on the growth hormone and gonadal axes in boys with constitutional delay of growth and puberty. Clin Endocrinol (Oxf) 1993; 38(4):393-398.

(90) Crowne EC, Wallace WH, Moore C, Mitchell R, Robertson WH, Holly JM et al. Effect of low dose oxandrolone and testosterone treatment on the pituitary-testicular and GH axes in boys with constitutional delay of growth and puberty. Clin Endocrinol (Oxf) 1997; 46(2):209-216.

(91) Keenan BS, Richards GE, Ponder SW, Dallas JS, Nagamani M, Smith ER. Androgen-stimulated pubertal growth: the effects of testosterone and dihydrotestosterone on growth hormone and insulin-like growth factor-I in the treatment of short stature and delayed puberty. J Clin Endocrinol Metab 1993; 76(4):996-1001.

(92) Metzger DL, Kerrigan JR. Estrogen receptor blockade with tamoxifen diminishes growth hormone secretion in boys: evidence for a stimulatory role of endogenous estrogens during male adolescence. J Clin Endocrinol Metab 1994; 79(2):513-518.

(93) Vanderschueren D, Vandenput L, Boonen S, Lindberg MK, Bouillon R, Ohlsson C. Androgens and bone. Endocr Rev 2004; 25(3):389-425.

(94) Kerrigan JR, Rogol AD. The impact of gonadal steroid hormone action on growth hormone secretion during childhood and adolescence. Endocr Rev 1992; 13(2):281-298.

(95) Veldhuis JD, Roemmich JN, Rogol AD. Gender and sexual maturation-dependent contrasts in the neuroregulation of growth hormone secretion in prepubertal and late adolescent males and females--a general clinical research center-based study. J Clin Endocrinol Metab 2000; 85(7):2385-2394.

(96) Coutant R, de Casson FB, Rouleau S, Douay O, Mathieu E, Gatelais F et al. Divergent effect of endogenous and exogenous sex steroids on the insulin-like growth factor I response to growth hormone in short normal adolescents. J Clin Endocrinol Metab 2004; 89(12):6185-6192.

(97) Marin G, Domene HM, Barnes KM, Blackwell BJ, Cassorla FG, Cutler GB, Jr. The effects of estrogen priming and puberty on the growth hormone response to standardized treadmill exercise and arginine-insulin in normal girls and boys. J Clin Endocrinol Metab 1994; 79(2):537-541.

(98) Hero M, Norjavaara E, Dunkel L. Inhibition of estrogen biosynthesis with a potent aromatase inhibitor increases predicted adult height in boys with idiopathic short stature: a randomized controlled trial. J Clin Endocrinol Metab 2005; 90(12):6396-6402.

(99) Wennink JM, Delemarre-van de Waal HA, Schoemaker R, Blaauw G, van den BC, Schoemaker J. Growth hormone secretion patterns in relation to LH and estradiol secretion throughout normal female puberty. Acta Endocrinol (Copenh) 1991; 124(2):129-135.

(100) Ho KY, Evans WS, Blizzard RM, Veldhuis JD, Merriam GR, Samojlik E et al. Effects of sex and age on the 24-hour profile of growth hormone secretion in man: importance of endogenous estradiol concentrations. J Clin Endocrinol Metab 1987; 64(1):51-58.

(101) Winer LM, Shaw MA, Baumann G. Basal plasma growth hormone levels in man: new evidence for rhythmicity of growth hormone secretion. J Clin Endocrinol Metab 1990; 70(6):1678-1686.

(102) Laron Z, Lilos P, Klinger B. Growth curves for Laron syndrome. Arch Dis Child 1993; 68(6):768-770.

(103) D'Ercole AJ, Stiles AD, Underwood LE. Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc Natl Acad Sci U S A 1984; 81(3):935-939.

(104) Leung KC, Johannsson G, Leong GM, Ho KK. Estrogen regulation of growth hormone action. Endocr Rev 2004; 25(5):693-721.

(105) Green H, Morikawa M, Nixon T. A dual effector theory of growth-hormone action. Differentiation 1985; 29:195-198.

(106) Zezulak KM, Green H. The generation of insulin-like growth factor-1--sensitive cells by growth hormone action. Science 1986; 233(4763):551-553.

(107) Isaksson OG, Lindahl A, Nilsson A, Isgaard J. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocr Rev 1987; 8(4):426-438.

(108) Ohlsson C, Nilsson A, Isaksson O, Lindahl A. Growth hormone induces multiplication of the slowly cycling germinal cells of the rat tibial growth plate. Proc Natl Acad Sci U S A 1992; 89(20):9826-9830.

(109) Yakar S, Kim H, Zhao H, Toyoshima Y, Pennisi P, Gavrilova O et al. The growth hormone-insulin like growth factor axis revisited: lessons from IGF-1 and IGF-1 receptor gene targeting. Pediatr Nephrol 2005; 20(3):251-254.

(110) Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M. Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. Endocrinology 1993; 132(3):1158-1167.

(111) De Los RP, Hill DJ. Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of

the ovine fetus: possible functional implications. Can J Physiol Pharmacol 1999; 77(4):235-249.

(112) Abu EO, Horner A, Kusec V, Triffitt JT, Compston JE. The localization of androgen receptors in human bone. J Clin Endocrinol Metab 1997; 82(10):3493-3497.

(113) Ben Hur H, Thole HH, Mashiah A, Insler V, Berman V, Shezen E et al. Estrogen, progesterone and testosterone receptors in human fetal cartilaginous tissue: immunohistochemical studies. Calcif Tissue Int 1997; 60(6):520-526.

(114) Noble B, Routledge J, Stevens H, Hughes I, Jacobson W. Androgen receptors in bone-forming tissue. Horm Res 1999; 51(1):31-36.

(115) Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J et al. Localization of estrogen receptors-alpha and -beta and androgen receptor in the human growth plate at different pubertal stages. J Endocrinol 2003; 177(2):319-326.

(116) Krohn K, Haffner D, Hugel U, Himmele R, Klaus G, Mehls O et al. 1,25(OH)2D3 and dihydrotestosterone interact to regulate proliferation and differentiation of epiphyseal chondrocytes. Calcif Tissue Int 2003; 73(4):400-410.

(117) Raz P, Nasatzky E, Boyan BD, Ornoy A, Schwartz Z. Sexual dimorphism of growth plate prehypertrophic and hypertrophic chondrocytes in response to testosterone requires metabolism to dihydrotestosterone (DHT) by steroid 5-alpha reductase type 1. J Cell Biochem 2005; 95(1):108-119.

(118) Gunther DF, Underwood LE, Calikoglu AS. Androgen-accelerated bone maturation in mice is not attenuated by Faslodex, an estrogen receptor blocker. Bone 2001; 28(4):410-413.

(119) Ren SG, Malozowski S, Sanchez P, Sweet DE, Loriaux DL, Cassorla F. Direct administration of testosterone increases rat tibial epiphyseal growth plate width. Acta Endocrinol (Copenh) 1989; 121(3):401-405.

(120) Tivesten A, Moverare-Skrtic S, Chagin A, Venken K, Salmon P, Vanderschueren D et al. Additive protective effects of estrogen and androgen treatment on trabecular bone in ovariectomized rats. J Bone Miner Res 2004; 19(11):1833-1839.

(121) Phillip M, Maor G, Assa S, Silbergeld A, Segev Y. Testosterone stimulates growth of tibial epiphyseal growth plate and insulin-like growth factor-1 receptor abundance in hypophysectomized and castrated rats. Endocrine 2001; 16(1):1-6.

(122) Somjen D, Weisman Y, Harell A, Berger E, Kaye AM. Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone. Proc Natl Acad Sci U S A 1989; 86(9):3361-3365.

(123) Somjen D, Weisman Y, Mor Z, Harell A, Kaye AM. Regulation of proliferation of rat cartilage and bone by sex steroid hormones. J Steroid Biochem Mol Biol 1991; 40(4-6):717-723.

(124) Maor G, Segev Y, Phillip M. Testosterone stimulates insulin-like growth factor-I and insulin-like growth factor-I-receptor gene expression in the mandibular condyle--a model of endochondral ossification. Endocrinology 1999; 140(4):1901-1910.

(125) Turner RT, Hannon KS, Demers LM, Buchanan J, Bell NH. Differential effects of gonadal function on bone histomorphometry in male and female rats. J Bone Miner Res 1989; 4(4):557-563.

(126) Nasatzky E, Schwartz Z, Boyan BD, Soskolne WA, Ornoy A. Sex-dependent effects of 17-beta-estradiol on chondrocyte differentiation in culture. J Cell Physiol 1993; 154(2):359-367.

(127) Schwartz Z, Finer Y, Nasatzky E, Soskolne WA, Dean DD, Boyan BD et al. The effects of 17 beta-estradiol on chondrocyte differentiation are modulated by vitamin D3 metabolites. Endocrine 1997; 7(2):209-218.

(128) Rodd C, Jourdain N, Alini M. Action of estradiol on epiphyseal growth plate chondrocytes. Calcif Tissue Int 2004; 75(3):214-224.

(129) Chagin AS, Chrysis D, Takigawa M, Ritzen EM, Savendahl L. Locally produced estrogen promotes fetal rat metatarsal bone growth; an effect mediated through increased chondrocyte proliferation and decreased apoptosis. J Endocrinol 2006; 188(2):193-203.

(130) Sylvia VL, Gay I, Hardin R, Dean DD, Boyan BD, Schwartz Z. Rat costochondral chondrocytes produce 17beta-estradiol and regulate its production by 1alpha,25(OH)(2)D(3). Bone 2002; 30(1):57-63.

(131) van der Eerden BC, Emons J, Ahmed S, van Essen HW, Lowik CW, Wit JM et al. Evidence for genomic and nongenomic actions of estrogen in growth plate regulation in female and male rats at the onset of sexual maturation. J Endocrinol 2002; 175(2):277-288.

(132) Chagin AS, Savendahl L. GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. J Clin Endocrinol Metab 2007; 92:4873-4877.

(133) Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signalling. Science 2005; 307:1625-1630.

(134) Batra GS, Hainey L, Freemont AJ, Andrew G, Saunders PT, Hoyland JA et al. Evidence for cell-specific changes with age in expression of oestrogen receptor (ER) alpha and beta in bone fractures from men and women. J Pathol 2003; 200(1):65-73.

(135) Doolan CM, Condliffe SB, Harvey BJ. Rapid non-genomic activation of cytosolic cyclic AMP-dependent protein kinase activity and [Ca(2+)](i) by 17beta-oestradiol in female rat distal colon. Br J Pharmacol 2000; 129(7):1375-1386.

(136) Whitson SW, Dawson LR, Jee WS. A tetracycline study of cyclic longitudinal bone growth in the female rat. Endocrinology 1978; 103(6):2006-2010.

(137) Strickland AL, Sprinz H. Studies of the influence of estradiol and growth hormone on the hypophysectomized immature rat epiphyseal cartilage growth plate. Am J Obstet Gynecol 1973; 115(4):471-477.

(138) Gustafsson PO, Kasstrom H, Lindberg L, Olsson SE. Growth and mitotic rate of the proximal tibial epiphyseal plate in hypophysectomized rats given estradiol and human growth hormone. Acta Radiol Suppl 1975; 344:69-74.

(139) Breur GJ, Vanenkevort BA, Farnum CE, Wilsman NJ. Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. J Orthop Res 1991; 9(3):348-359.

(140) Thorngren KG, Hansson LI. Cell kinetics and morphology of the growth plate in the normal and hypophysectomized rat. Calcif Tissue Res 1973; 13(2):113-129.

(141) Turner RT, Evans GL, Wakley GK. Reduced chondroclast differentiation results in increased cancellous bone volume in estrogen-treated growing rats. Endocrinology 1994; 134(1):461-466.

(142) Svan H, Ritzen EM, Hall K, Johansson L. Estrogen treatment of tall girls: dose dependency of effects on subsequent growth and IGF-I levels in blood. Acta Paediatr Scand 1991; 80(3):328-332.

(143) Attie KM, Ramirez NR, Conte FA, Kaplan SL, Grumbach MM. The pubertal growth spurt in eight patients with true precocious puberty and growth hormone deficiency: evidence for a direct role of sex steroids. J Clin Endocrinol Metab 1990; 71(4):975-983.

(144) Deroo BJ, Korach KS. Estrogen receptors and human disease. J Clin Invest 2006; 116(3):561-570.

(145) Vidal O, Lindberg M, Savendahl L, Lubahn DB, Ritzen EM, Gustafsson JA et al. Disproportional body growth in female estrogen receptor-alpha-inactivated mice. Biochem Biophys Res Commun 1999; 265(2):569-571.

(146) Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA, Ohlsson C. Estrogen receptor specificity in the regulation of the skeleton in female mice. J Endocrinol 2001; 171(2):229-236.

(147) Chagin AS, Lindberg MK, Andersson N, Moverare S, Gustafsson JA, Savendahl L et al. Estrogen receptor-beta inhibits skeletal growth and has the capacity to mediate growth plate fusion in female mice. J Bone Miner Res 2004; 19(1):72-77.

(148) Parikka V, Peng Z, Hentunen T, Risteli J, Elo T, Vaananen HK et al. Estrogen responsiveness of bone formation in vitro and altered bone phenotype in aged estrogen receptor-alpha-deficient male and female mice. Eur J Endocrinol 2005; 152(2):301-314.

(149) Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. Bone 2002; 30(1):18-25.

(150) Tozum TF, Oppenlander ME, Koh-Paige AJ, Robins DM, McCauley LK. Effects of sex steroid receptor specificity in the regulation of skeletal metabolism. Calcif Tissue Int 2004; 75(1):60-70.

(151) Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C. Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta(-/-) mice. J Clin Invest 1999; 104(7):895-901.

(152) Windahl SH, Hollberg K, Vidal O, Gustafsson JA, Ohlsson C, Andersson G. Female estrogen receptor beta-/- mice are partially protected against age-related trabecular bone loss. J Bone Miner Res 2001; 16(8):1388-1398.

(153) Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci U S A 1998; 95(12):6965-6970.

(154) Oz OK, Zerwekh JE, Fisher C, Graves K, Nanu L, Millsaps R et al. Bone has a sexually dimorphic response to aromatase deficiency. J Bone Miner Res 2000; 15(3):507-514.

(155) Oz OK, Hirasawa G, Lawson J, Nanu L, Constantinescu A, Antich PP et al. Bone phenotype of the aromatase deficient mouse. J Steroid Biochem Mol Biol 2001; 79(1-5):49-59.

(156) Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB et al. Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. Proc Natl Acad Sci U S A 2000; 97(10):5474-5479.

(157) Vandenput L, Ederveen AG, Erben RG, Stahr K, Swinnen JV, Van Herck E et al. Testosterone prevents orchidectomy-induced bone loss in estrogen receptor-alpha knockout mice. Biochem Biophys Res Commun 2001; 285(1):70-76.

(158) Vandenput L, Swinnen JV, Boonen S, Van Herck E, Erben RG, Bouillon R et al. Role of the androgen receptor in skeletal homeostasis: the androgen-resistant testicular feminized male mouse model. J Bone Miner Res 2004; 19(9):1462-1470.

(159) Pendaries C, Darblade B, Rochaix P, Krust A, Chambon P, Korach KS et al. The AF-1 activation-function of ERalpha may be dispensable to mediate the effect of estradiol on endothelial NO production in mice. Proc Natl Acad Sci U S A 2002; 99(4):2205-2210.

(160) Blanchard O, Tsagris L, Rappaport R, Duval-Beaupere G, Corvol M. Age-dependent responsiveness of rabbit and human cartilage cells to sex steroids in vitro. J Steroid Biochem Mol Biol 1991; 40(4-6):711-716.

(161) Corvol MT, Carrascosa A, Tsagris L, Blanchard O, Rappaport R. Evidence for a direct in vitro action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex. Endocrinology 1987; 120(4):1422-1429.

(162) Macrae VE, Farquharson C, Ahmed SF. The restricted potential for recovery of growth plate chondrogenesis and longitudinal bone growth following exposure to pro-inflammatory cytokines. J Endocrinol 2006; 189(2):319-328.

(163) Polanczyk M, Zamora A, Subramanian S, Matejuk A, Hess DL, Blankenhorn EP et al. The protective effect of 17beta-estradiol on experimental autoimmune encephalomyelitis is mediated through estrogen receptor-alpha. Am J Pathol 2003; 163(4):1599-1605.

(164) Ling S, Zhou L, Li H, Dai A, Liu JP, Komesaroff PA et al. Effects of 17beta-estradiol on growth and apoptosis in human vascular endothelial cells: influence of mechanical strain and tumor necrosis factor-alpha. Steroids 2006; 71(9):799-808.

(165) Zhou X, Li F, Ge J, Sarkisian SR, Jr., Tomita H, Zaharia A et al. Retinal ganglion cell protection by 17-beta-estradiol in a mouse model of inherited glaucoma. Dev Neurobiol 2007; 67(5):603-616.

(166) Atsumi T, Miwa Y, Kimata K, Ikawa Y. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev 1990; 30(2):109-116.

(167) Shukunami C, Ishizeki K, Atsumi T, Ohta Y, Suzuki F, Hiraki Y. Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Miner Res 1997; 12(8):1174-1188.

(168) Horiguchi M, Akiyama H, Ito H, Shigeno C, Nakamura T. Tumour necrosis factor-alpha up-regulates the expression of BMP-4 mRNA but inhibits chondrogenesis in mouse clonal chondrogenic EC cells, ATDC5. Cytokine 2000; 12(5):526-530.

(169) McMillan J, Fatehi-Sedeh S, Sylvia VL, Bingham V, Zhong M, Boyan BD et al. Sex-specific regulation of growth plate chondrocytes by estrogen is via multiple MAP kinase signaling pathways. Biochim Biophys Acta 2006; 1763(4):381-392.

(170) Kinney RC, Schwartz Z, Week K, Lotz MK, Boyan BD. Human articular chondrocytes exhibit sexual dimorphism in their responses to 17beta-estradiol. Osteoarthritis Cartilage 2005; 13(4):330-337.

(171) Gruber R, Graninger W, Bobacz K, Watzek G, Erlacher L. BMP-6-induced osteogenic differentiation of mesenchymal cell lines is not modulated by sex steroids and resveratrol. Cytokine 2003; 23(4-5):133-137.

(172) Saggese G, Federico G, Cinquanta L. In vitro effects of growth hormone and other hormones on chondrocytes and osteoblast-like cells. Acta Paediatr Suppl 1993; 82 Suppl 391:54-59.

(173) Welshons WV, Wolf MF, Murphy CS, Jordan VC. Estrogenic activity of phenol red. Mol Cell Endocrinol 1988; 57(3):169-178.

(174) Houston B, Seawright E, Jefferies D, Hoogland E, Lester D, Whitehead C et al. Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes. Biochim Biophys Acta 1999; 1448(3):500-506.

(175) Jefferies D, Houston B, Lester D, Whitehead CC, Thorp BH, Botman M et al. Expression patterns of chondrocyte genes cloned by differential display in tibial dyschondroplasia. Biochim Biophys Acta 2000; 1501(2-3):180-188.

(176) Farquharson C, Lester D, Seawright E, Jefferies D, Houston B. Microtubules are potential regulators of growth-plate chondrocyte differentiation and hypertrophy. Bone 1999; 25(4):405-412.

(177) Jefferies D, Botman M, Farquharson C, Lester D, Whitehead CC, Thorp BH et al. Cloning differentially regulated genes from chondrocytes using agarose gel differential display. Biochim Biophys Acta 1998; 1396(3):237-241.

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

(179) Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 1986; 83(8):2496-2500.

(180) Berger M, Chazaud J, Jean-Faucher C, de Turckheim M, Veyssiere G, Jean C. Developmental patterns of plasma and testicular testosterone in rabbits from birth to 90 days of age. Biol Reprod 1976; 15(5):561-564.

(181) Fohr B, Schulz A, Battmann A. Sex steroids and bone metabolism: comparison of in vitro effects of 17beta-estradiol and testosterone on human osteosarcoma cell lines of various gender and differentiation. Exp Clin Endocrinol Diabetes 2000; 108(6):414-423.

(182) Ekstein J, Nasatzky E, Boyan BD, Ornoy A, Schwartz Z. Growth-plate chondrocytes respond to 17beta-estradiol with sex-specific increases in IP3 and intracellular calcium ion signalling via a capacitative entry mechanism. Steroids 2005; 70(11):775-786.

(183) Schwartz Z, Shaked D, Hardin RR, Gruwell S, Dean DD, Sylvia VL et al. 1alpha,25(OH)2D3 causes a rapid increase in phosphatidylinositol-specific PLC-beta activity via phospholipase A2-dependent production of lysophospholipid. Steroids 2003; 68(5):423-437.

(184) Sylvia VL, Hughes T, Dean DD, Boyan BD, Schwartz Z. 17beta-estradiol regulation of protein kinase C activity in chondrocytes is sex-dependent and involves nongenomic mechanisms. J Cell Physiol 1998; 176(2):435-444.

(185) Mushtaq T, Farquharson C, Seawright E, Ahmed SF. Glucocorticoid effects on chondrogenesis, differentiation and apoptosis in the murine ATDC5 chondrocyte cell line. J Endocrinol 2002; 175(3):705-713.

(186) Yu J, Eto M, Akishita M, Okabe T, Ouchi Y. A selective estrogen receptor modulator inhibits TNF-alpha-induced apoptosis by activating ERK1/2 signaling pathway in vascular endothelial cells. Vascul Pharmacol 2009.

(187) Li XL, Cheng WD, Li J, Guo XL, Guo CJ, Meng XH et al. Protective effect of estrogen on apoptosis in a cell culture model of Parkinson's disease. Clin Invest Med 2008; 31(5):E258-E264.

(188) Huppmann S, Romer S, Altmann R, Obladen M, Berns M. 17beta-estradiol attenuates hyperoxia-induced apoptosis in mouse C8-D1A cell line. J Neurosci Res 2008; 86(15):3420-3426.

(189) Zhang Y, Bhavnani BR. Glutamate-induced apoptosis in primary cortical neurons is inhibited by equine estrogens via down-regulation of caspase-3 and prevention of mitochondrial cytochrome c release. BMC Neurosci 2005; 6:13.

(190) Zhang Y, Bhavnani BR. Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC Neurosci 2006; 7:49.

(191) Leung KC, Doyle N, Ballesteros M, Sjogren K, Watts CK, Low TH et al. Estrogen inhibits GH signaling by suppressing GH-induced JAK2 phosphorylation, an effect mediated by SOCS-2. Proc Natl Acad Sci U S A 2003; 100(3):1016-1021.

(192) Yan L, Tang Q, Shen D, Peng S, Zheng Q, Guo H et al. SOCS-1 inhibits TNF-alpha-induced cardiomyocyte apoptosis via ERK1/2 pathway activation. Inflammation 2008; 31(3):180-188.

(193) Mebratu Y, Tesfaigzi Y. How ERK1/2 activation controls cell proliferation and cell death is subcellular localization the answer? Cell Cycle 2009; 8(8):1168-1175.

(194) Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J et al. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994; 94(6):2307-2316.

(195) Loeser RF, Sadiev S, Tan L, Goldring MB. Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 2000; 8(2):96-105.

(196) Finger F, Schorle C, Zien A, Gebhard P, Goldring MB, Aigner T. Molecular phenotyping of human chondrocyte cell lines T/C-28a2, T/C-28a4, and C-28/I2. Arthritis Rheum 2003; 48(12):3395-3403.

(197) Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA, Katzenellenbogen BS. Antagonists selective for estrogen receptor alpha. Endocrinology 2002; 143(3):941-947.

(198) Goldring MB. Culture of immortalized chondrocytes and their use as models of chondrocyte function. Methods Mol Med 2004; 100:37-52.

(199) Chrysis D, Zaman F, Chagin AS, Takigawa M, Savendahl L. Dexamethasone induces apoptosis in proliferative chondrocytes through activation of caspases and suppression of the Akt-phosphatidylinositol 3'-kinase signaling pathway. Endocrinology 2005; 146(3):1391-1397.

(200) Campbell-Thompson M, Lynch IJ, Bhardwaj B. Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. Cancer Res 2001; 61(2):632-640.

(201) Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O, Rice LW. Selective loss of estrogen receptor beta in malignant human colon. Cancer Res 2000; 60(2):245-248.

(202) Martineti V, Picariello L, Tognarini I, Carbonell SS, Gozzini A, Azzari C et al. ERbeta is a potent inhibitor of cell proliferation in the HCT8 human colon

cancer cell line through regulation of cell cycle components. Endocr Relat Cancer 2005; 12(2):455-469.

(203) Mushtaq T, Bijman P, Ahmed SF, Farquharson C. Insulin-like growth factor-I augments chondrocyte hypertrophy and reverses glucocorticoid-mediated growth retardation in fetal mice metatarsal cultures. Endocrinology 2004; 145(5):2478-2486.

(204) Lefebvre V, Garofalo S, Zhou G, Metsaranta M, Vuorio E, De Crombrugghe B. Characterization of primary cultures of chondrocytes from type II collagen/beta-galactosidase transgenic mice. Matrix Biol 1994; 14(4):329-335.

(205) Talwar RM, Wong BS, Svoboda K, Harper RP. Effects of estrogen on chondrocyte proliferation and collagen synthesis in skeletally mature articular cartilage. J Oral Maxillofac Surg 2006; 64(4):600-609.

(206) Ng MC, Harper RP, Le CT, Wong BS. Effects of estrogen on the condylar cartilage of the rat mandible in organ culture. J Oral Maxillofac Surg 1999; 57(7):818-823.

(207) Silbermann M, Frommer J. The nature of endochondral ossification in the mandibular condyle of the mouse. Anat Rec 1972; 172(4):659-667.

(208) Nilsson O, Falk J, Ritzen EM, Baron J, Savendahl L. Raloxifene acts as an estrogen agonist on the rabbit growth plate. Endocrinology 2003; 144(4):1481-1485.

(209) Evans G, Bryant HU, Magee D, Sato M, Turner RT. The effects of raloxifene on tibia histomorphometry in ovariectomized rats. Endocrinology 1994; 134(5):2283-2288.

(210) Wilsman NJ, Farnum CE, Leiferman EM, Fry M, Barreto C. Differential growth by growth plates as a function of multiple parameters of chondrocytic kinetics. J Orthop Res 1996; 14(6):927-936.

(211) Wilsman NJ, Farnum CE, Green EM, Lieferman EM, Clayton MK. Cell cycle analysis of proliferative zone chondrocytes in growth plates elongating at different rates. J Orthop Res 1996; 14(4):562-572.

(212) Beier F. Cell-cycle control and the cartilage growth plate. J Cell Physiol 2005; 202(1):1-8.

(213) Long F, Zhang XM, Karp S, Yang Y, McMahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 2001; 128(24):5099-5108.

(214) Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 2003; 130(5):1003-1015.

(215) Sahni M, Ambrosetti DC, Mansukhani A, Gertner R, Levy D, Basilico C. FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. Genes Dev 1999; 13(11):1361-1366.

(216) Weksler NB, Lunstrum GP, Reid ES, Horton WA. Differential effects of fibroblast growth factor (FGF) 9 and FGF2 on proliferation, differentiation and terminal differentiation of chondrocytic cells in vitro. Biochem J 1999; 342 Pt 3:677-682.

(217) Aikawa T, Segre GV, Lee K. Fibroblast growth factor inhibits chondrocytic growth through induction of p21 and subsequent inactivation of cyclin E-Cdk2. J Biol Chem 2001; 276(31):29347-29352.

(218) Ballock RT, Zhou X, Mink LM, Chen DH, Mita BC, Stewart MC. Expression of cyclin-dependent kinase inhibitors in epiphyseal chondrocytes induced to terminally differentiate with thyroid hormone. Endocrinology 2000; 141(12):4552-4557.

(219) Carlberg AL, Pucci B, Rallapalli R, Tuan RS, Hall DJ. Efficient chondrogenic differentiation of mesenchymal cells in micromass culture by retroviral gene transfer of BMP-2. Differentiation 2001; 67(4-5):128-138.

(220) Panda DK, Miao D, Lefebvre V, Hendy GN, Goltzman D. The transcription factor SOX9 regulates cell cycle and differentiation genes in chondrocytic CFK2 cells. J Biol Chem 2001; 276(44):41229-41236.

(221) Beier F, Taylor AC, LuValle P. The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. J Biol Chem 1999; 274(42):30273-30279.

(222) Stanton LA, Underhill TM, Beier F. MAP kinases in chondrocyte differentiation. Dev Biol 2003; 263(2):165-175.

(223) Prall OW, Rogan EM, Sutherland RL. Estrogen regulation of cell cycle progression in breast cancer cells. J Steroid Biochem Mol Biol 1998; 65(1-6):169-174.

(224) Prall OW, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem 1997; 272(16):10882-10894.

(225) Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN et al. Cyclin E and survival in patients with breast cancer. N Engl J Med 2002; 347(20):1566-1575.

(226) Garimella R, Sipe JB, Anderson HC. A simple and non-radioactive technique to study the effect of monophosphoesters on matrix vesicle-mediated calcification. Biol Proced Online 2004; 6:263-267.

(227) Wang W, Kirsch T. Retinoic acid stimulates annexin-mediated growth plate chondrocyte mineralization. J Cell Biol 2002; 157(6):1061-1069.

(228) Anderson HC. Matrix vesicles and calcification. Curr Rheumatol Rep 2003; 5(3):222-226.

(229) Schinke T, McKee MD, Karsenty G. Extracellular matrix calcification: where is the action? Nat Genet 1999; 21(2):150-151.

(230) Anderson HC. Molecular biology of matrix vesicles. Clin Orthop Relat Res 1995;(314):266-280.

(231) Anderson HC, Reynolds JJ. Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification. Dev Biol 1973; 34(2):211-227.

(232) Hsu HH. Further studies on ATP-mediated Ca deposition by isolated matrix vesicles. Bone Miner 1992; 17(2):279-283.

(233) Roberts S, Narisawa S, Harmey D, Millan JL, Farquharson C. Functional involvement of PHOSPHO1 in matrix vesicle-mediated skeletal mineralization. J Bone Miner Res 2007; 22(4):617-627.

(234) Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev 2001; 15(21):2865-2876.

(235) Baker TG. A quantitative and cytological study of germ cells in human ovaries. Proc R Soc Lond B Biol Sci 1963; 158:417-433.

(236) Faddy MJ. Follicle dynamics during ovarian ageing. Mol Cell Endocrinol 2000; 163(1-2):43-48.

(237) van Noord PA, Dubas JS, Dorland M, Boersma H, te VE. Age at natural menopause in a population-based screening cohort: the role of menarche, fecundity, and lifestyle factors. Fertil Steril 1997; 68(1):95-102.

(238) Welt CK. Primary ovarian insufficiency: a more accurate term for premature ovarian failure. Clin Endocrinol (Oxf) 2008; 68(4):499-509.

(239) Nelson LM. Clinical practice. Primary ovarian insufficiency. N Engl J Med 2009; 360(6):606-614.

(240) Rebar RW, Connolly HV. Clinical features of young women with hypergonadotropic amenorrhea. Fertil Steril 1990; 53(5):804-810.

(241) Nelson LM, Anasti JN, Flack MR. Premature ovarian failure. In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, surgery, and technology. Philadelphia: Lippincott-Raven, 1996: 1393-1410.

(242) Rebar RW, Erickson GF, Yen SS. Idiopathic premature ovarian failure: clinical and endocrine characteristics. Fertil Steril 1982; 37(1):35-41.

(243) Nelson LM, Anasti JN, Kimzey LM, Defensor RA, Lipetz KJ, White BJ et al. Development of luteinized graafian follicles in patients with karyotypically normal spontaneous premature ovarian failure. J Clin Endocrinol Metab 1994; 79(5):1470-1475.

(244) van Kasteren YM, Schoemaker J. Premature ovarian failure: a systematic review on therapeutic interventions to restore ovarian function and achieve pregnancy. Hum Reprod Update 1999; 5(5):483-492.

(245) Bakalov VK, Anasti JN, Calis KA, Vanderhoof VH, Premkumar A, Chen S et al. Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2005; 84(4):958-965.

(246) Singh RP, Carr DH. The anatomy and histology of XO human embryos and fetuses. Anat Rec 1966; 155(3):369-383.

(247) Cunniff C, Jones KL, Benirschke K. Ovarian dysgenesis in individuals with chromosomal abnormalities. Hum Genet 1991; 86(6):552-556.

(248) Modi DN, Sane S, Bhartiya D. Accelerated germ cell apoptosis in sex chromosome aneuploid fetal human gonads. Mol Hum Reprod 2003; 9(4):219-225.

(249) Devi AS, Metzger DA, Luciano AA, Benn PA. 45,X/46,XX mosaicism in patients with idiopathic premature ovarian failure. Fertil Steril 1998; 70(1):89-93.

(250) Schlessinger D, Herrera L, Crisponi L, Mumm S, Percesepe A, Pellegrini M et al. Genes and translocations involved in POF. Am J Med Genet 2002; 111(3):328-333.

(251) Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986; 67(4):604-606.

(252) Bachelot A, Rouxel A, Massin N, Dulon J, Courtillot C, Matuchansky C et al. Phenotyping and genetic studies of 357 consecutive patients presenting with premature ovarian failure. Eur J Endocrinol 2009; 161(1):179-187.

(253) Schoenberg B.S. Calculating Confidence Intervals for Rates and Ratios: Simplified Method Utilizing Tabular Values Based on the Poisson Distribution. Neuroepidemiology 1983; 2:257-265.

(254) Tanner J, Whitehouse R, Cameron N, Marshall W, Healy M, Goldstein H. Assessment of skeletal maturity and prediction of adult height (TW2-method). 2nd Ed ed. London: Academic Press, 1983.

(255) Massa G, Verlinde F, De Schepper J, Thomas M, Bourguignon JP, Craen M et al. Trends in age at diagnosis of Turner syndrome. Arch Dis Child 2005; 90(3):267-268.

(256) Griffin IJ, Cole TJ, Duncan KA, Hollman AS, Donaldson MD. Pelvic ultrasound measurements in normal girls. Acta Paediatr 1995; 84(5):536-543.

(257) Paterson WF, Hollman AS, Donaldson MD. Poor uterine development in Turner syndrome with oral oestrogen therapy. Clin Endocrinol (Oxf) 2002; 56(3):359-365.

(258) Doerr HG, Bettendorf M, Hauffa BP, Mehls O, Partsch CJ, Said E et al. Uterine size in women with Turner syndrome after induction of puberty with estrogens and long-term growth hormone therapy: results of the German IGLU Follow-up Study 2001. Hum Reprod 2005; 20(5):1418-1421.

(259) Snajderova M, Mardesic T, Lebl J, Gerzova H, Teslik L, Zapletalova J. The uterine length in women with Turner syndrome reflects the postmenarcheal daily estrogen dose. Horm Res 2003; 60(4):198-204.

(260) Bannink EM, van Sassen C, van Buuren S, de Jong FH, Lequin M, Mulder PG et al. Puberty induction in Turner syndrome: results of oestrogen treatment on development of secondary sexual characteristics, uterine dimensions and serum hormone levels. Clin Endocrinol (Oxf) 2009; 70(2):265-273.

(261) McDonnell CM, Coleman L, Zacharin MR. A 3-year prospective study to assess uterine growth in girls with Turner's syndrome by pelvic ultrasound. Clin Endocrinol (Oxf) 2003; 58(4):446-450.

(262) Piippo S, Lenko H, Kainulainen P, Sipila I. Use of percutaneous estrogen gel for induction of puberty in girls with Turner syndrome. J Clin Endocrinol Metab 2004; 89(7):3241-3247.

(263) Press F, Shapiro HM, Cowell CA, Oliver GD. Outcome of ovum donation in Turner's syndrome patients. Fertil Steril 1995; 64(5):995-998.

(264) Li TC, Dockery P, Ramsewak SS, Klentzeris L, Lenton EA, Cooke ID. The variation of endometrial response to a standard hormone replacement therapy in women with premature ovarian failure. An ultrasonographic and histological study. Br J Obstet Gynaecol 1991; 98(7):656-661.

(265) Khastgir G, Abdalla H, Thomas A, Korea L, Latarche L, Studd J. Oocyte donation in Turner's syndrome: an analysis of the factors affecting the outcome. Hum Reprod 1997; 12(2):279-285.

(266) Bath LE, Critchley HO, Chambers SE, Anderson RA, Kelnar CJ, Wallace WH. Ovarian and uterine characteristics after total body irradiation in childhood and adolescence: response to sex steroid replacement. Br J Obstet Gynaecol 1999; 106(12):1265-1272.

(267) Sas TC, de Muinck Keizer-Schrama SM, Stijnen T, Jansen M, Otten BJ, Hoorweg-Nijman JJ et al. Normalization of height in girls with Turner syndrome after long-term growth hormone treatment: results of a randomized dose-response trial. J Clin Endocrinol Metab 1999; 84(12):4607-4612.

(268) Rosenfeld RG, Attie KM, Frane J, Brasel JA, Burstein S, Cara JF et al. Growth hormone therapy of Turner's syndrome: beneficial effect on adult height. J Pediatr 1998; 132(2):319-324.

(269) Guyda HJ. Four decades of growth hormone therapy for short children: what have we achieved? J Clin Endocrinol Metab 1999; 84(12):4307-4316.

(270) Stephure DK. Impact of growth hormone supplementation on adult height in turner syndrome: results of the Canadian randomized controlled trial. J Clin Endocrinol Metab 2005; 90(6):3360-3366.

(271) Nilsson KO, Albertsson-Wikland K, Alm J, Aronson S, Gustafsson J, Hagenas L et al. Improved final height in girls with Turner's syndrome treated with growth hormone and oxandrolone. J Clin Endocrinol Metab 1996; 81(2):635-640.

(272) Hamelin CE, Anglin G, Quigley CA, Deal CL. Genomic imprinting in Turner syndrome: effects on response to growth hormone and on risk of sensorineural hearing loss. J Clin Endocrinol Metab 2006; 91(8):3002-3010.

(273) Reiter EO, Blethen SL, Baptista J, Price L. Early initiation of growth hormone treatment allows age-appropriate estrogen use in Turner's syndrome. J Clin Endocrinol Metab 2001; 86(5):1936-1941.

(274) Quigley CA, Crowe BJ, Anglin DG, Chipman JJ. Growth hormone and low dose estrogen in Turner syndrome: results of a United States multi-center trial to near-final height. J Clin Endocrinol Metab 2002; 87(5):2033-2041.

(275) Ranke MB, Partsch CJ, Lindberg A, Dorr HG, Bettendorf M, Hauffa BP et al. Adult height after GH therapy in 188 Ullrich-Turner syndrome patients: results of the German IGLU Follow-up Study 2001. Eur J Endocrinol 2002; 147(5):625-633.

(276) Rochiccioli P, Battin J, Bertrand AM, Bost M, Cabrol S, le Bouc Y et al. Final height in Turner syndrome patients treated with growth hormone. Horm Res 1995; 44(4):172-176.

(277) Massa G, Heinrichs C, Verlinde S, Thomas M, Bourguignon JP, Craen M et al. Late or delayed induced or spontaneous puberty in girls with Turner syndrome

treated with growth hormone does not affect final height. J Clin Endocrinol Metab 2003; 88(9):4168-4174.

(278) Chernausek SD, Attie KM, Cara JF, Rosenfeld RG, Frane J. Growth hormone therapy of Turner syndrome: the impact of age of estrogen replacement on final height. Genentech, Inc., Collaborative Study Group. J Clin Endocrinol Metab 2000; 85(7):2439-2445.

(279) Price DA, Ranke MB. Growth hormone in Turner syndrome. Arch Dis Child 2001; 84(6):525.

(280) Cacciari E, Mazzanti L. Final height of patients with Turner's syndrome treated with growth hormone (GH): indications for GH therapy alone at high doses and late estrogen therapy. Italian Study Group for Turner Syndrome. J Clin Endocrinol Metab 1999; 84(12):4510-4515.

(281) van Pareren YK, de Muinck Keizer-Schrama SM, Stijnen T, Sas TC, Jansen M, Otten BJ et al. Final height in girls with turner syndrome after long-term growth hormone treatment in three dosages and low dose estrogens. J Clin Endocrinol Metab 2003; 88(3):1119-1125.

(282) Apter D, Lenko HL, Perheentupa J, Soderholm A, Vihko R. Subnormal pubertal increases of serum androgens in Turner's syndrome. Horm Res 1982; 16(3):164-173.

(283) Gravholt CH, Svenstrup B, Bennett P, Sandahl CJ. Reduced androgen levels in adult turner syndrome: influence of female sex steroids and growth hormone status. Clin Endocrinol (Oxf) 1999; 50(6):791-800.

(284) Haeusler G, Schmitt K, Blumel P, Plochl E, Waldhor T, Frisch H. Growth hormone in combination with anabolic steroids in patients with Turner syndrome: effect on bone maturation and final height. Acta Paediatr 1996; 85(12):1408-1414.

(285) Bareille P, Massarano AA, Stanhope R. Final height outcome in girls with Turner syndrome treated with a combination of low dose oestrogen and oxandrolone. Eur J Pediatr 1997; 156(5):358-362.

(286) Stahnke N, Keller E, Landy H. Favorable final height outcome in girls with Ullrich-Turner syndrome treated with low-dose growth hormone together with oxandrolone despite starting treatment after 10 years of age. J Pediatr Endocrinol Metab 2002; 15(2):129-138.

(287) Freeman JV, Cole TJ, Chinn S, Jones PR, White EM, Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. Arch Dis Child 1995; 73(1):17-24.

(288) Tanner JM, Whitehouse RH. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. Arch Dis Child 1976; 51(3):170-179.

(289) Whincup PH, Gilg JA, Odoki K, Taylor SJ, Cook DG. Age of menarche in contemporary British teenagers: survey of girls born between 1982 and 1986. BMJ 2001; 322(7294):1095-1096.

(290) Tanner JM. Trend towards earlier menarche in London, Olso, Copenhagen, the Netherlands and Hungary. Nature 1973; 243(5402):95-96.

(291) Ranke MB, Pfluger H, Rosendahl W, Stubbe P, Enders H, Bierich JR et al. Turner syndrome: spontaneous growth in 150 cases and review of the literature. Eur J Pediatr 1983; 141(2):81-88.

(292) Massarano AA, Brook CG, Hindmarsh PC, Pringle PJ, Teale JD, Stanhope R et al. Growth hormone secretion in Turner's syndrome and influence of oxandrolone and ethinyl oestradiol. Arch Dis Child 1989; 64(4):587-592.

(293) Link K, Blizzard RM, Evans WS, Kaiser DL, Parker MW, Rogol AD. The effect of androgens on the pulsatile release and the twenty-four-hour mean concentration of growth hormone in peripubertal males. J Clin Endocrinol Metab 1986; 62(1):159-164.

(294) Stanhope R, Hindmarsh P, Pringle PJ, Holownia P, Honour J, Brook CG. Oxandrolone induces a sustained rise in physiological growth hormone secretion in boys with constitutional delay of growth and puberty. Pediatrician 1987; 14(3):183-188.

(295) Loche S, Corda R, Lampis A, Puggioni R, Cella SG, Muller EE et al. The effect of oxandrolone on the growth hormone response to growth hormone releasing hormone in children with constitutional growth delay. Clin Endocrinol (Oxf) 1986; 25(2):195-200.

(296) Loche S, Colao A, Cappa M, Bellone J, Aimaretti G, Farello G et al. The growth hormone response to hexarelin in children: reproducibility and effect of sex steroids. J Clin Endocrinol Metab 1997; 82(3):861-864.

(297) Chagin AS, Vannesjo J, Savendahl L. Androgen receptor modulation does not affect longitudinal growth of cultured fetal rat metatarsal bones. Horm Res 2009; 71(4):219-227.

(298) Mashchak CA, Lobo RA, Dozono-Takano R, Eggena P, Nakamura RM, Brenner PF et al. Comparison of pharmacodynamic properties of various estrogen formulations. Am J Obstet Gynecol 1982; 144(5):511-518.

(299) Janfaza M, Sherman TI, Larmore KA, Brown-Dawson J, Klein KO. Estradiol levels and secretory dynamics in normal girls and boys as determined by an

ultrasensitive bioassay: a 10 year experience. J Pediatr Endocrinol Metab 2006; 19(7):901-909.

(300) Norjavaara E, Ankarberg C, Albertsson-Wikland K. Diurnal rhythm of 17 beta-estradiol secretion throughout pubertal development in healthy girls: evaluation by a sensitive radioimmunoassay. J Clin Endocrinol Metab 1996; 81(11):4095-4102.

(301) Nilsson S, Kuiper G, Gustafsson JA. ERbeta a Novel Estrogen Receptor Offers the Potential for New Drug Development. Trends Endocrinol Metab 1998; 9(10):387-395.

(302) Rosenfield RL, Perovic N, Devine N, Mauras N, Moshang T, Root AW et al. Optimizing estrogen replacement treatment in Turner syndrome. Pediatrics 1998; 102(2 Pt 3):486-488.

(303) Rosenfield RL, Devine N, Hunold JJ, Mauras N, Moshang T, Jr., Root AW. Salutary effects of combining early very low-dose systemic estradiol with growth hormone therapy in girls with Turner syndrome. J Clin Endocrinol Metab 2005; 90(12):6424-6430.

(304) Bellantoni MF, Vittone J, Campfield AT, Bass KM, Harman SM, Blackman MR. Effects of oral versus transdermal estrogen on the growth hormone/insulin-like growth factor I axis in younger and older postmenopausal women: a clinical research center study. J Clin Endocrinol Metab 1996; 81(8):2848-2853.

(305) Raudaskoski T, Knip M, Laatikainen T. Plasma insulin-like growth factor-I and its binding proteins 1 and 3 during continuous nonoral and oral combined hormone replacement therapy. Menopause 1998; 5(4):217-222.

(306) Vestergaard P, Hermann AP, Orskov H, Mosekilde L. Effect of sex hormone replacement on the insulin-like growth factor system and bone mineral: a cross-sectional and longitudinal study in 595 perimenopausal women participating in the Danish Osteoporosis Prevention Study. J Clin Endocrinol Metab 1999; 84(7):2286-2290.

(307) Orme ML, Back DJ, Breckenridge AM. Clinical pharmacokinetics of oral contraceptive steroids. Clin Pharmacokinet 1983; 8(2):95-136.

(308) Piippo S, Lenko H, Kainulainen P, Sipila I. Use of percutaneous estrogen gel for induction of puberty in girls with Turner syndrome. J Clin Endocrinol Metab 2004; 89(7):3241-3247.

(309) Ankarberg-Lindgren C, Elfving M, Wikland KA, Norjavaara E. Nocturnal application of transdermal estradiol patches produces levels of estradiol that mimic those seen at the onset of spontaneous puberty in girls. J Clin Endocrinol Metab 2001; 86(7):3039-3044.

Scotland A Research Ethics Committee

Secretariat
Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9026
Fax 0131 536 9346
www.corec.org.uk

NHS SCOTLAND

Dr M Donaldson
Senior Lecturer in Child Health
Department of Child Health
Yorkhill NHS Trust
Royal Hospital for Sick Children
Yorkhill
Glasgow
G3 8SJ

Date: 22 September 2008
Your Ref.:
Our Ref.: 98/0/092

Enquiries to: Walter Hunter
Extension: 89026
Direct Line: 0131 536 9026
Email: walter.hunter@lhb.scot.nhs.uk

Dear Dr Donaldson

Study title: **Prospective UK collaborative study of growth promoting treatment in Turner's Syndrome; impact of using consistent dose of growth hormone and benefit of combination treatment with oxandrolone, and early or late oestrogen induction.**

REC reference: **98/0/092**

Thank you for your letter of 14 July 2008. I apologise for the delay in replying.

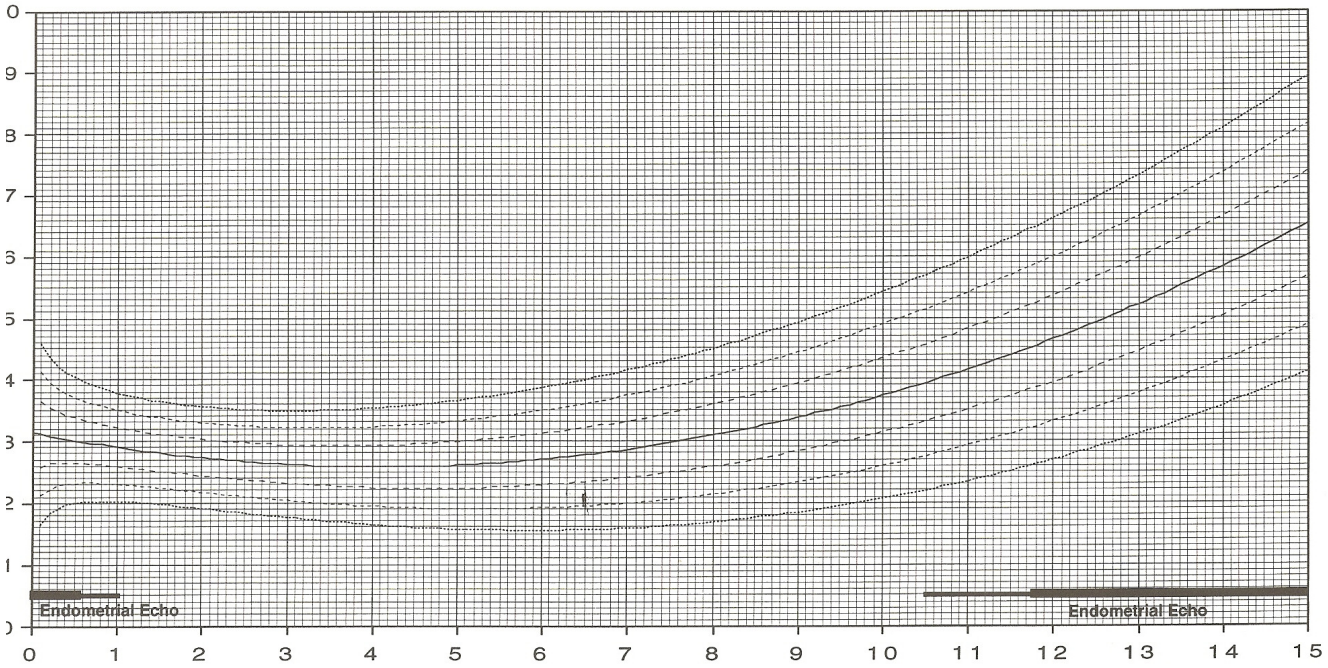
The Committee is content that the consent already obtained for the above study will cover Dr Perry's intended work to use the raw anonymised data that has been collected for the purposes of examining height velocity in the cohort group in relation to oestrogen treatment.

REC reference number: 98/0/092-Please quote this number on all correspondence

Yours sincerely

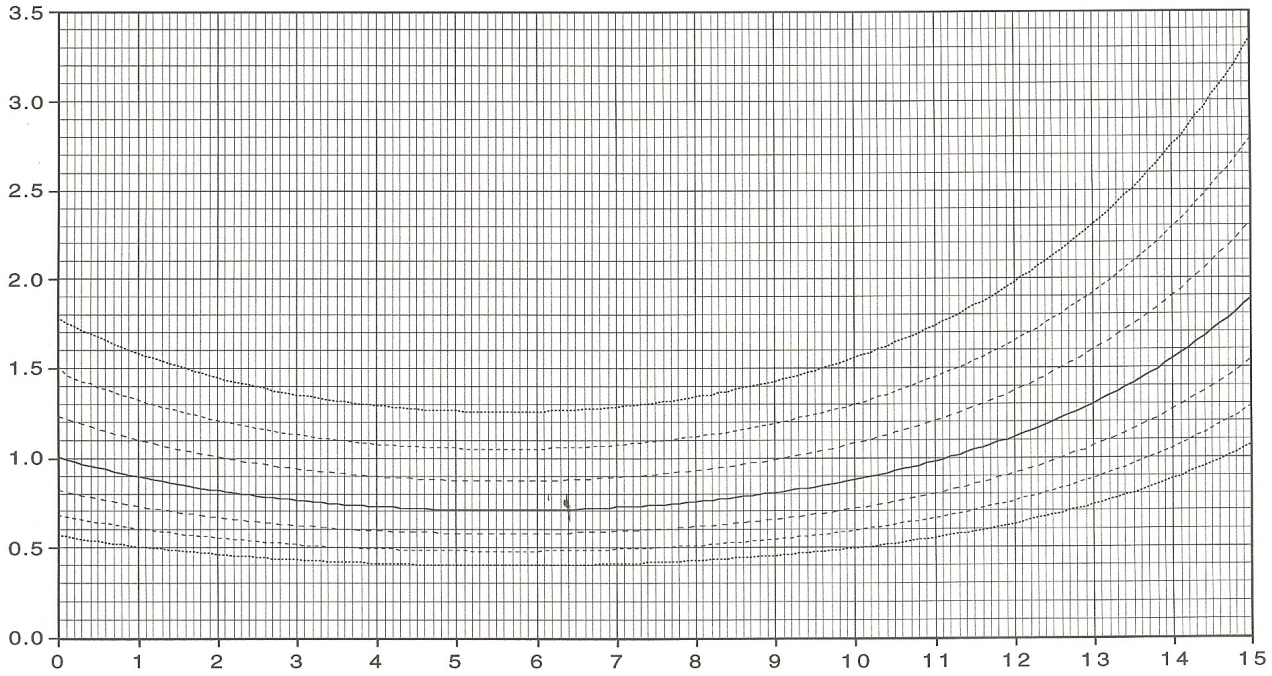
WALTER HUNTER
Committee Co-ordinator

Chairman Professor Kennedy Lees
Vice-Chairman Dr Malcolm Booth



Smoothed reference centile curves for uterine length versus age (3rd, 10th, 25th, 50th, 75th, 90th and 97th centile lines) at different stages of puberty (From Griffin *et al*, 1995 with permission)

FUNDAL CERVICAL RATIO	Comments
Name.	
Unit Number.	
Date of Birth. / / .	



Smoothed reference centile curves for fundal cervical ratio versus age (3rd, 10th, 25th, 50th, 75th, 90th and 97th centile lines) at different stages of puberty (From Griffin *et al*, 1995 with permission)