Declaration

The research described in this thesis is the sole work of the author, except where acknowledgement is made and is not being submitted in support of another degree or qualification at the University of Edinburgh or any other educational institute.

Catriona Paul
August 2007
Acknowledgements

I would firstly like to thank my supervisors Professor Philippa Saunders and Professor David Melton for giving me the opportunity to work on such an interesting project. Philippa for always keeping her door open and for providing me with all the support I could ask for and David for his help and input throughout.

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Abstract
Infertility affects ~20% of couples in Europe and in 50% of cases the problem lies with the male. The development of assisted reproductive technologies (ART) such as \textit{in vitro} fertilisation (IVF) and intra-cytoplasmic spermatozoa injection (ICSI) has allowed some couples to overcome male-factor infertility. However concerns remain over the increasing use of ART as elevated levels of DNA damage in sperm from infertile men have been reported and a link between DNA damage in sperm and early embryonic failure has been demonstrated. DNA damage in sperm, caused by oxidative stress may also be passed on from father to child resulting in an increased incidence of childhood cancer. This has led to fears that the use of damaged sperm in ART could contribute to early embryonic failure and/or birth defects. The studies described in this thesis used mouse models to investigate the relationship between DNA integrity in male germ cells and male fertility. This was achieved by studying both the effects of targeted ablation of genes involved in DNA repair and the impact of scrotal heat stress on testicular function and sperm DNA integrity.

Three lines of transgenic mice with deletions in genes involved in genomic integrity (\textit{Ercc1}, \textit{Msh2} and \textit{p53}) were studied. All three genes are expressed in the testis. These studies confirmed and extended studies on \textit{Ercc1} knockout (-/-) mice showing reduced germ cell complement, increased apoptosis, an increased percentage of damaged sperm and demonstrated for the first time that depletion of \textit{Ercc1} results in an increased incidence of unrepaired double strand DNA breaks (DSB) in pachytene spermatocytes. The persistence of DSBs in spermatocytes and abnormal sperm chromatin structure confirmed that the repair functions of \textit{Ercc1} are essential for normal germ cell maturation. In the \textit{p53}^{-/-} mice these studies showed for the first time that there was an increase in DSBs in spermatocytes and an increase in numbers of sperm with damaged DNA. The level of apoptosis was also increased in the testes suggesting that caspase-3 mediated apoptosis is not entirely p53 dependent as been previously suggested. These studies demonstrated for the first time that targeted ablation of \textit{Msh2} compromises germ cell complement and as in the \textit{Ercc1}^{-/-} this resulted in gaps in the seminiferous epithelium consistent with clonal loss of germ
cells. Consistent with a role for MSH2 in mismatch repair no DSBs were detected in spermatocytes from $Msh2^{-/-}$.

Testicular function is temperature dependent and due to their location in the scrotum testes are normally kept between 2°C and 8°C below core body temperature. In mice transient scrotal heat stress (30 minutes at 38°C, 40°C and 42°C) disrupted testicular function. Analysis of sperm and testis parameters revealed that stress at 38°C was sufficient to have subtle effects on epididymal function but the higher temperatures had additional consequences for testicular function which resulted in DNA damage in spermatocytes, germ cells loss and increased apoptosis. Further studies into the pathways of apoptosis demonstrated that the mitochondrial/intrinsic pathway plays a role in heat stress response. The fertility of males was altered in those heated to 42°C resulting in reduced pregnancy rate and litter size. Given that the paternal genome is reported to be required for the development of extraembryonic tissues and this will influence growth of the embryo, it was interesting to note an increase in resorption sites in pregnancies using 40°C males. IVF was used to demonstrate that embryos formed using sperm from males stressed at 42°C were compromised between the 4-cell and blastocyst stage suggesting that though sperm with DNA damage are still capable of fertilisation, the paternal DNA was introducing genomic instability to the embryo and having fatal effects on development. These studies have also shown that one possible underlying cause of the disturbance in testicular function is hypoxia, as a marked increase in Hif1 alpha (a marker of hypoxia) mRNA and relocalisation of the protein was observed in the testis.

In conclusion, DNA damage in the male germ line caused either by induced stress, or by targeted ablation of DNA repair genes, can disrupt testicular architecture, function and therefore the fertility of mice. These data have demonstrated that deletion of $Ercc1$, $Msh2$ and $p53$ can have differential but overlapping affects on germ cell function and sperm production and that increased scrotal temperature can cause subfertility in male mice. This study has provided further confirmation of possible male-mediated effects on embryo survival and these findings should be taken into
consideration when using sperm from infertile men in IVF/ICSI treatments where the normal quality control processes involved in fertilisation are bypassed.
Presentations relating to this thesis

Evidence of an increase in unrepaired DNA breaks in germ cells and spermatozoa from ERCC1 knockout mice.
Oral presentation at the 14th European Testis Workshop, Bad Aibling, Germany, April 2006.

Mild scrotal heat stress causes DNA damage and subfertility in mice.

Publications relating to this thesis

Paul, C., Povey, JE., Lawrence, NJ., Selfridge, J., Melton, DW., Saunders, PTK. Targeted deletion of genes implicated in protecting the integrity of male germ cells have differential effects on the incidence of DNA breaks and germ cell loss. *Plos One*, submitted.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>Axial elements</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junctions</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>AO</td>
<td>Acridine orange</td>
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<td>AOI</td>
<td>Area of interest</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BER</td>
<td>base excision repair</td>
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<td>CdK</td>
<td>cyclin dependent kinase</td>
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<td>CE</td>
<td>central element</td>
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<td>CS</td>
<td>cockayne syndrome</td>
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<td>dHJ</td>
<td>double Holliday junctions</td>
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<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DSB</td>
<td>double strand breaks</td>
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<td>e</td>
<td>embryonic day</td>
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<td>EGA</td>
<td>embryonic genome activation</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>FACS</td>
<td>fluorescent assisted cell sorting</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<tr>
<td>GGR</td>
<td>global genome repair</td>
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<tr>
<td>GR</td>
<td>glutathione reductase</td>
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<td>h</td>
<td>hours</td>
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<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
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<tr>
<td>Hsp</td>
<td>heat shock protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<td>ICSI</td>
<td>intracytoplasmic spermatozoa injection</td>
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<td>INSL</td>
<td>insulin-like peptide</td>
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<td>IVF</td>
<td>in vitro fertilisation</td>
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<td>KO</td>
<td>knockout</td>
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<td>luteinising hormone</td>
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<td>MPF</td>
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<td>NER</td>
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<td>normal goat serum</td>
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<td>periodic acid Schiff</td>
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<td>PVP</td>
<td>polyvinylpirrolidone</td>
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<td>reactive oxygen species</td>
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<td>seconds</td>
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<td>SC</td>
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</tr>
<tr>
<td>SCO</td>
<td>Sertoli cell only</td>
</tr>
<tr>
<td>SCP</td>
<td>synaptonemal complex protein</td>
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<td>SCSA</td>
<td>sperm chromatin structure assay</td>
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<tr>
<td>SDF</td>
<td>sperm diluting factor</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSBR</td>
<td>single strand break repair</td>
</tr>
<tr>
<td>TCR</td>
<td>transcription-coupled repair</td>
</tr>
<tr>
<td>TF</td>
<td>transverse filament</td>
</tr>
<tr>
<td>TP</td>
<td>transition protein</td>
</tr>
<tr>
<td>TTD</td>
<td>trichothiodystrophy</td>
</tr>
<tr>
<td>TTP</td>
<td>time to pregnancy</td>
</tr>
<tr>
<td>XP (a,b,c,d,f and g)</td>
<td>xeroderma pigmentosum (complementation group)</td>
</tr>
<tr>
<td>ZP</td>
<td>zona pellucida</td>
</tr>
</tbody>
</table>
# Table of contents

Declaration.................................................................................................i
Acknowledgements .................................................................................. ii
Abstract ...................................................................................................... iii
Presentations relating to this thesis........................................................... vi
Publications relating to this thesis ............................................................ vi
Abbreviations ............................................................................................. vii
Table of contents ....................................................................................... ix
List of Figures ........................................................................................... xvii
List of Tables .............................................................................................. xxi

1 Literature Review .................................................................................... 1

1.1 General introduction ............................................................................ 1

1.1.1 DNA damage transmission to offspring ......................................... 1

1.2 The adult testis: structure and function ............................................. 2

1.2.1 Organisation of the adult testis ....................................................... 2

1.2.2 Somatic cells ..................................................................................... 5

1.2.2.1 Sertoli cells .................................................................................. 6

1.2.2.2 Leydig cells ................................................................................... 8

1.2.3 Germ cell maturation ......................................................................... 9

1.2.3.1 Spermatogenesis .......................................................................... 9

1.2.3.1.1 First wave of spermatogenesis .............................................. 10

1.2.3.1.2 Spermatogenesis in the adult mouse .................................. 10

1.2.3.2 Spermatogonial phase ................................................................. 12

1.2.3.3 Spermatocyte phase ................................................................... 13

1.2.3.3.1 Meiotic crossing over (recombination) ................................ 18

1.2.3.4 Mouse models of infertility: lessons from mice deficient in meiosis-specific proteins ......................................................... 21

1.2.3.5 Spermiogenesis .......................................................................... 22

1.2.4 The epididymis ................................................................................... 24

1.2.4.1 Structure and function ............................................................... 24
1.2.4.2 Sperm maturation in the epididymis ........................................... 25
  1.2.4.2.1 Protective function of epididymis ........................................... 26
1.2.4.3 Sperm – structure ................................................................. 27
1.2.4.4 Chromatin structure ............................................................... 28
1.3 Maintenance of normal testis function ........................................... 29
  1.3.1 DNA damage - origins and consequences ........................................ 30
  1.3.2 Susceptibility to damage .......................................................... 31
  1.3.3 DNA repair pathways ............................................................... 32
    1.3.3.1 Nucleotide excision repair pathway (NER) ............................... 32
    1.3.3.2 Mismatch repair pathway (MMR) .......................................... 35
  1.3.4 Base excision repair pathway (BER) ........................................... 37
  1.3.5 Lessons from mice deficient in DNA repair genes ........................... 38
1.4 Stress response in the testis ......................................................... 40
  1.4.1.1 Heat, the testis and its response to stress .................................... 40
  1.4.1.2 Heat-induced changes in gene expression .................................... 42
  1.4.1.3 Animal models of heat stress .................................................. 43
  1.4.2 Hypoxic stress ........................................................................... 44
  1.4.3 Oxidative stress .......................................................................... 45
  1.4.4 Apoptosis ................................................................................... 45
1.5 Male fertility ................................................................................... 49
  1.5.1 Fertilisation in the mouse ............................................................. 49
  1.5.2 Normal embryo development ....................................................... 52
    1.5.2.1 Embryonic gene expression and paternal genome reactivation .... 54
      1.5.2.1.1 DNA repair in the preimplantation embryo .......................... 56
1.6 General aims of this thesis ............................................................. 58
2 General Materials and Methods ....................................................... 59
  2.1 Animals ......................................................................................... 59
    2.1.1 Ercc1 mice ................................................................................ 59
    2.1.2 Msh2 mice ................................................................................. 59
    2.1.3 p53 mice .................................................................................. 60
    2.1.4 Animal accommodation ............................................................ 60
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.5</td>
<td>Sacrifice of animals</td>
<td>60</td>
</tr>
<tr>
<td>2.1.6</td>
<td>Tissue collection</td>
<td>61</td>
</tr>
<tr>
<td>2.2</td>
<td>Treatments</td>
<td>61</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Anaesthetic</td>
<td>61</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Testicular heating method</td>
<td>62</td>
</tr>
<tr>
<td>2.2.2.1</td>
<td>Time points</td>
<td>63</td>
</tr>
<tr>
<td>2.3</td>
<td>Tissue fixation and processing</td>
<td>63</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Tissue fixation</td>
<td>63</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Tissue processing and paraffin embedding</td>
<td>63</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Sectioning of paraffin blocks</td>
<td>64</td>
</tr>
<tr>
<td>2.4</td>
<td>Tissue staining and immunohistochemistry</td>
<td>64</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Haematoxylin and eosin staining</td>
<td>64</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Immunohistochemistry for paraffin embedded tissue</td>
<td>64</td>
</tr>
<tr>
<td>2.4.2.1</td>
<td>Antigen retrieval</td>
<td>65</td>
</tr>
<tr>
<td>2.4.2.2</td>
<td>Blocking non specific sites</td>
<td>65</td>
</tr>
<tr>
<td>2.4.2.3</td>
<td>Primary antibodies</td>
<td>66</td>
</tr>
<tr>
<td>2.4.2.4</td>
<td>Secondary antibodies</td>
<td>66</td>
</tr>
<tr>
<td>2.4.2.5</td>
<td>Antigen detection and counterstaining</td>
<td>67</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Automated immunostaining</td>
<td>68</td>
</tr>
<tr>
<td>2.4.4</td>
<td>TUNEL</td>
<td>68</td>
</tr>
<tr>
<td>2.4.5</td>
<td>Counting TUNEL positive cells</td>
<td>69</td>
</tr>
<tr>
<td>2.5</td>
<td>Spermatozoa preparation</td>
<td>69</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Biggers, Whitten and Whittingham solution</td>
<td>69</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Preparation of Murine spermatozoa</td>
<td>69</td>
</tr>
<tr>
<td>2.5.3</td>
<td>Sperm Counts</td>
<td>70</td>
</tr>
<tr>
<td>2.5.4</td>
<td>Morphology of sperm</td>
<td>70</td>
</tr>
<tr>
<td>2.5.5</td>
<td>Spermatozoa Chromatin Structure Assay (SCSA)</td>
<td>71</td>
</tr>
<tr>
<td>2.5.5.1</td>
<td>FACS analysis</td>
<td>71</td>
</tr>
<tr>
<td>2.6</td>
<td>Meiotic spreads</td>
<td>72</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Germ cell extraction from murine testes</td>
<td>72</td>
</tr>
<tr>
<td>2.6.1.1</td>
<td>Immunostaining of meiotic spreads</td>
<td>72</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>2.6.2 Counting $\gamma$H2AX foci as an indication of DNA damage</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.6.3 Fluorescent microscopy</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7 Protein expression analysis</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.1 RIPA extraction from murine tissue</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.2 Protein quantification</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.3 Acrylamide gel electrophoresis</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.7.4 Transfer of proteins</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.7.5 Probing the membrane</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.7.6 Protein expression analysis – LiCor technology</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.8 RNA analysis</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.8.1 RNA extraction from murine tissue</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.8.2 RNA quantification</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>2.8.3 Reverse transcription of RNA (RT-PCR)</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>2.9 Taqman-quantitative PCR</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2.9.1 Taqman Q-RT-PCR reaction</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.9.2 Roche Universal Probe Library™ – Q-RT-PCR</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.9.2.1 PCR reaction for Roche Universal Probe Library™</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.9.3 Analysis of results</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.9.3.1 Statistical analysis</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2.10 Mating studies</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>2.10.1 Mice</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>2.10.2 Treatments</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>2.10.3 Timed matings</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>2.10.4 Collection of blastocysts from mated females</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>2.11 In vitro fertilisation (IVF)</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>2.11.1 Mice</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>2.11.2 Dissection media</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>2.11.3 T6 preparation</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>2.11.4 KSOM preparation</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>2.11.5 Sperm preparation</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>
2.11.6 Superovulation of females ............................................................... 87
2.11.7 Oocyte collection ......................................................................... 87
2.11.8 Assessment of sperm ................................................................. 88
2.11.9 Fertilisation .................................................................................. 88
2.11.10 Embryo development ................................................................. 88
  2.11.10.1 Development to 2-cell stage.................................................. 88
  2.11.10.2 Development to blastocyst stage .......................................... 89
2.11.11 Assessment of developing embryos ......................................... 89
2.11.12 Fixation of embryos ................................................................. 89
2.11.13 Immunostaining of embryos ..................................................... 89
2.12 Commonly used solutions .............................................................. 90
  2.12.1 RIPA lysis buffer ....................................................................... 90
  2.12.2 T6 media stock solutions .......................................................... 90
  Stock B (100ml) ................................................................................ 90
  Stock C (10ml) .................................................................................. 91
  Stock D (10ml) ................................................................................ 91
  2.12.3 KSOM media stock solutions ..................................................... 91
  Stock A (100ml) ................................................................................ 91
  2.12.4 Phosphate buffered saline (PBS) ................................................. 92
3 Differential testicular function in mice with impaired DNA integrity ...... 93
  3.1 Introduction...................................................................................... 93
  3.1.1 Mouse models for DNA damage ................................................ 94
    3.1.1.1 The NER pathway and Ercc1 ................................................. 94
    3.1.1.2 The MMR pathway and Msh2 .............................................. 95
    3.1.1.3 Tumor suppressor gene p53 ............................................... 95
    3.1.2 Aims of this chapter.................................................................... 96
  3.2 Materials and Methods .................................................................. 96
    3.2.1 Animals .................................................................................... 96
    3.2.2 Preparation of Murine spermatozoa ......................................... 96
    3.2.3 Sperm counts ............................................................................ 97
    3.2.4 Study of morphology of sperm ................................................ 97
3.2.5 Spermatozoa chromatin structure assay (SCSA) ............................ 97
3.2.6 Histology and Immunohistochemistry ...................................... 98
  3.2.6.1 Measurement of tubule diameters ..................................... 98
  3.2.6.2 Detection of apoptotic cells using immunolocalisation of Caspase 3 98
3.2.7 Meiotic Spreads ........................................................................ 99
  3.2.7.1 Immunostaining of meiotic spreads ..................................... 100
  3.2.7.2 γH2AX foci counts ............................................................. 100
3.2.8 Statistical analysis ...................................................................... 100
3.3 Results .......................................................................................... 101
  3.3.1 Testicular phenotypes of mice lacking Ercc1, Msh2 or p53 .......... 101
  3.3.2 Tubule diameter distribution .................................................... 102
  3.3.3 Immunodetection of MVH and p63: characterisation of GC loss ... 105
  3.3.4 Apoptosis ............................................................................... 107
  3.3.5 DNA damage in spermatocytes ................................................. 110
  3.3.6 Sperm counts .......................................................................... 113
  3.3.7 Sperm morphologies ................................................................. 114
  3.3.8 Sperm chromatin abnormalities ................................................. 116
3.4 Discussion ...................................................................................... 118
4 Mild scrotal heat stress causes DNA damage and subfertility .......... 124
  4.1 Introduction ............................................................................... 124
  4.1.1 Temperature, the testis and spermatogenesis ............................ 124
  4.1.2 Aims of this chapter ................................................................. 125
  4.2 Materials and Methods ............................................................... 126
  4.2.1 Animals and treatments .......................................................... 126
  4.2.2 Tissue recovery ....................................................................... 126
  4.2.3 Terminal deoxynucleotide transferase mediated deoxy-UTP nick end labelling (TUNEL) ................................................................. 127
  4.2.4 Meiotic spreads and immunostaining of spermatocytes ............ 127
  4.2.5 Sperm preparation for counts, morphology and SCSA ............... 127
  4.2.6 Fertility analysis ..................................................................... 128
4.2.7 IVF .................................................................................. 128
4.2.8 Blastocyst retrieval from mated females ...................... 128
4.2.9 Immunostaining of blastocysts .................................... 129
4.2.10 Statistical analysis ......................................................... 129

4.3 Results ............................................................................... 130
4.3.1 Heat induced altered testicular architecture .................. 130
4.3.2 TUNEL ........................................................................ 133
4.3.3 Impaired DNA repair in spermatocytes ...................... 135
4.3.4 Sperm counts ................................................................. 138
4.3.5 Sperm morphologies ....................................................... 139
4.3.6 Heat stress induced chromatin abnormalities in sperm .... 141
4.3.7 Reduced fertility in heat stressed males ...................... 142
4.3.8 IVF ........................................................................... 145
4.3.9 Embryo development .................................................. 147

Discussion ............................................................................. 151

4.4 Conclusions ...................................................................... 160

5 Mild Scrotal heat stress and the stress response .................... 161
5.1 Introduction ..................................................................... 161
5.1.1 Aims of this chapter .................................................. 164
5.2 Materials and Methods .................................................. 165
5.2.1 Animals and tissue recovery ...................................... 165
5.2.2 Immunohistochemistry .............................................. 165
5.2.3 Western blotting ........................................................ 166
5.2.4 Taqman QRT-PCR ...................................................... 167
5.2.5 Statistical analysis ....................................................... 167

5.3 Results ............................................................................... 168
5.3.1 Heat induced hypoxia ................................................ 168
5.3.1.1 Mild transient heat stress induces hypoxia .......... 168
5.3.1.2 Heme oxygenase 1 (HO1) .................................... 170
5.3.2 Stress response in the testis ........................................ 174
5.3.2.1 Stip1 .................................................................... 174
5.3.2.2 Hsp105 .................................................................................. 177

5.3.3 Heat induced apoptosis .................................................................. 179
  5.3.3.1 Bax ..................................................................................... 179
  5.3.3.2 Caspase-9 .......................................................................... 181
  5.3.3.3 Cleaved caspase-3 immunostaining ........................................ 183

5.4 Discussion ...................................................................................... 186

5.5 Conclusions .................................................................................... 192

6 Final Discussion .................................................................................. 193
  6.1 DNA repair pathways and testicular function .................................. 194
  6.2 Scrotal heat stress, DNA damage and male fertility ......................... 196
  6.3 Stress response in the testis ............................................................ 201
  6.4 General conclusions ....................................................................... 203

References ........................................................................................... 206
List of Figures

Figure 1-1 Overall appearance of a seminiferous tubule ................................................................. 3
Figure 1-2 Spermatogenic cycle of the mouse ......................................................................................... 4
Figure 1-3 A schematic diagram of a Sertoli cell with its associated germ cells in the seminiferous epithelium ................................................................. 5
Figure 1-4 Schematic diagram of junctions present in the testis .............................................................. 7
Figure 1-5 Schematic diagram showing the division of spermatogenesis ........................................... 9
Figure 1-6 Summary diagram of the mammalian mitotic cell cycle ...................................................... 11
Figure 1-7 Stages of mammalian mitosis ............................................................................................... 12
Figure 1-8 Schematic diagram of stages in Meiosis I ............................................................................ 15
Figure 1-9 Model of synaptonemal complex structure ......................................................................... 16
Figure 1-10 Schematic diagram of stages in Meiosis II ......................................................................... 18
Figure 1-11 Schematic diagram of the homologous recombination (HR) pathway ... 20
Figure 1-12 Schematic diagram of the formation of the acrosomal vesicle and nuclear remodelling ............................................................................................................................................... 24
Figure 1-13 Structure of a human sperm ................................................................................................. 28
Figure 1-14 Comparison of the packaging of DNA in somatic versus sperm nuclei 29
Figure 1-15 Schematic diagram of the NER pathway ........................................................................... 34
Figure 1-16 A simplified schematic diagram of the mismatch repair pathway (MMR) ............................................................................................................................................... 36
Figure 1-17 Schematic diagram of the base excision repair (BER) pathway ....................................... 38
Figure 1-18 Simplified summary diagram of apoptosis ........................................................................... 47
Figure 1-19 Embryo development in the mouse .................................................................................... 54
Figure 1-20 Gene expression in embryo development diagram showing maternal to zygotic gene transition in the mouse ............................................................................................................................................... 56
Figure 2.1 Testicular heating experiment .............................................................................................. 62
Figure 2.2 Schematic diagram of the immunohistochemistry protocol ................................................. 67
Figure 2.3 Western blot apparatus: arrangement of gel-membrane sandwich .................................... 76
Figure 2.4 A typical amplification plot after a successful Taqman run ............................................ 83
Figure 3.1 Histological evaluation of testes from adult mice ................................................................ 102
Figure 3.2 High power histological analysis of knock out testis tubular architecture.............................................................103
Figure 3.3 Distribution of seminiferous tubule diameters .................................................................104
Figure 3.4 MVH immunoexpression in the testis ..................................................................................106
Figure 3.5 p63 immunoexpression in the testis ....................................................................................107
Figure 3.6 Detection of apoptotic cells .................................................................................................109
Figure 3.7 Quantification of caspase-3 positive germ cells .................................................................110
Figure 3.8 Double strand breaks in pachytene spermatocytes detected using γH2AX immunohistochemistry on germ cell spreads .................................................................112
Figure 3.9 Quantification of double strand breaks in pachytene spermatocytes detected using γH2AX Immunohistochemistry on germ cell spreads .................................................................113
Figure 3.10 Epididymal sperm counts in Ercc1, Msh2 and p53 lines ..................................................114
Figure 3.11 Morphology of epididymal sperm from TG-Ercc1 ..........................................................115
Figure 3.12 Morphology of epididymal sperm from Msh2 .................................................................115
Figure 3.13 Morphology of epididymal sperm from p53 .................................................................116
Figure 3.14 Susceptibility of epididymal sperm to acid denaturation detected by the SCSA in TG-Ercc1, Msh2 and p53 lines ...........................................................................................................117
Figure 4.1 Histological evaluation of testes from adult mice subjected to scrotal heating and their controls .........................................................................................................................131
Figure 4.2 Histological evaluation of higher powered magnification (x100) of testes from adult mice subjected to scrotal heating ........................................................................................................132
Figure 4.3 TUNEL stained testicular cross sections from adult mice subjected to transient scrotal heat stress ..............................................................................................................................134
Figure 4.4 Quantification of cells with fragmented DNA using TUNEL staining ..................................................135
Figure 4.5 Double strand breaks in pachytene spermatocytes detected using γH2AX immunohistochemistry on germ cell spreads .................................................................137
Figure 4.6 Quantification of double strand breaks in pachytene spermatocytes detected using γH2AX Immunohistochemistry on meiotic germ cell spreads .................................................................138
Figure 4.7 Epididymal sperm counts in 38°C, 40°C and 42°C groups ..................................................139
Figure 4.8 Morphology of epididymal sperm from 38°C heated males .............................................140
Figure 4.9 Morphology of epididymal sperm from 40°C heated males .............................................140
Figure 4.10 Morphology of epididymal sperm from 42°C heated males

Figure 4.11 Susceptibility of epididymal sperm to acid denaturation detected by the SCSA in 38°C, 40°C and 42°C groups.

Figure 4.12 Pregnant females (e14.5) previously mated to control males or those that have been subjected to a transient scrotal heat stress (40°C or 42°C).

Figure 4.13 Pregnancy rate of females mated to control males or males that have been subjected to a transient scrotal heat stress.

Figure 4.14 Effect of transient scrotal heat stress (40°C or 42°C) on number of viable fetuses per female.

Figure 4.15 Occurrence of fetal resorptions in female mice mated to control males or males that have been subjected to a transient scrotal heat stress.

Figure 4.16 Embryo development following in vitro fertilisation (IVF).

Figure 4.17 Embryo development following in vitro fertilisation (IVF).

Figure 4.18 Blastocyst stage embryos.

Figure 4.19 Z-stack images of blastocyst stage embryos immunostained with Oct3/4 and ZO-1.

Figure 5.1 Hif1 alpha mRNA expression in testes from adult mice subjected to transient scrotal heat stress.

Figure 5.2 Immunodetection of Hif1 alpha on testicular cross sections from adult mice subjected to transient scrotal heat stress.

Figure 5.3 HO1 mRNA expression in testes from adult mice subjected to transient scrotal heat stress.

Figure 5.4 HO1 protein expression in adult mice subjected to transient scrotal heat stress.

Figure 5.5 Immunodetection of Stip1 on testicular cross sections from adult mice subjected to transient scrotal heat stress.

Figure 5.6 Stip1 expression in testes from control and heated mice.

Figure 5.7 Expression of Hsp105. Protein expression in testes from adult mice subjected to transient scrotal heat stress.

Figure 5.8 Bax protein expression in adult mice subjected to transient scrotal heat stress.
Figure 5.9 Caspase-9 protein expression after heat stress in adult mice subjected to transient scrotal heat stress ................................................................. 183
Figure 5.10 Detection of apoptotic cells ................................................................. 184
Figure 5.11 Quantification of caspase-3 positive germ cells ................................. 185
List of Tables

Table 2-1 Summary of anaesthetic and reversal agent dose administered to mice....61
Table 2-2 Summary of primary antibodies used for immunohistochemistry ..........66
Table 2-3 Summary of secondary antibodies used in Immunohistochemistry ........66
Table 2-4 Summary of primary antibodies used in meiotic spreads .....................73
Table 2-5 Summary of secondary antibodies used in meiotic spreads .................73
Table 2-6 Summary of primary antibodies used in Western blots ......................76
Table 2-7 Summary of secondary antibodies used on Western blots for LiCor detection ........................................................................................................77
Table 2-8 Primer and probe sequences for transcripts studied using the Roche Universal Probe Library™ .............................................................................81
Table 5-1 Summary of primary antibodies used for immunohistochemistry .......166
Table 5-2 Summary of primary antibodies used for Western blotting ...............166
Table 5-3 Primer/probe details for HO-1 expression analysis using the Roche Universal Probe Library™ .................................................................167
1 Literature Review

1.1 General introduction

The primary functions of the adult testis are the synthesis and release of steroids and the production of mature sperm, without which paternal genetic information could not be passed on (Sharpe, 1994). Germ cell maturation (spermatogenesis) is a complex, multi-step process during which the DNA of the cells is replicated and exchanged during homologous recombination. Errors can be introduced into the DNA during this process and can not only compromise the survival of the germ cells but if not repaired can be passed to the offspring.

The genetic integrity of the male germ line impacts on fertility, the progress of pregnancy and, ultimately, the health and well-being of the offspring. Human infertility represents a major clinical problem and affects between 17 and 25% of couples in Europe (Dunson et al., 2004); in 50% of cases the problem has been attributed to the male partner. The development of the techniques of in vitro fertilisation (IVF) and in particular intra-cytoplasmic spermatozoa injection (ICSI) has offered opportunities to overcome some cases of male-factor infertility (Campbell and Irvine, 2000). There are, however, some concerns over the safety of assisted reproductive technology (ART) (Hansen et al., 2002; Kurinczuk, 2003; Powell, 2003) as previous studies have demonstrated elevated levels of DNA damage in sperm from infertile men (Irvine et al., 2000). This has lead to fears that the use of damaged sperm in ICSI could contribute to early embryonic failure and/or birth defects (Gosden et al., 2003; Lopes et al., 1998a). The primary aim of this thesis is to use mouse models to provide a better understanding of the relationship between DNA integrity in male germ cells and male fertility.

1.1.1 DNA damage transmission to offspring

It is well known that sperm quality influences fertilisation and embryo development although there is some debate over whether DNA damage contained within the
sperm chromatin can be passed on to the embryo and cause adverse effects to the offspring. There are however numerous studies showing malformations of fetuses and increased incidences in disease in offspring generated from damaged sperm. For example gamma ray treated sperm resulted in fetal malformations (Muller et al., 1999) also treatment of rats with cyclophosphamide resulted in an elevated level of abnormalities in their offspring (Hales et al., 1992). Smoking and paternal age are also risk factors for the transmission of damage. A number of genetic diseases have been shown to increase with paternal age (Crow, 1995; Crow, 1997) and retrospective studies have demonstrated an association between increasing paternal age and the incidence of breast cancer and cancers of the nervous system, however no correlation was shown with other cancers (Hemminiki and Kyyronen, 1999; Hemminiki et al., 1999). In addition to this, studies on mice have shown that mutation frequency increases during spermiogenesis in postreplicative cell types in older mice (Walter et al., 1998). A number of studies have shown that DNA damage in sperm, for example that found in smoking fathers, can be passed to the offspring following ICSI/IVF treatment (Zenzes, 2000; Zenzes et al., 1999). In addition this has been reported to lead to an increase in incidence of childhood cancers (Ji et al., 1997; Sorahan et al., 1997).

1.2 The adult testis: structure and function

1.2.1 Organisation of the adult testis

The adult testis has two main functions: the production of mature sperm (spermatogenesis) and the synthesis and secretion of hormones. In adult mammals the testis is enclosed in a scrotal sac that lies outside the abdominal cavity and comprises of two distinct compartments: the seminiferous tubules (Figure 1-1), which contain the Sertoli cells and germ cells, and the interstitium, which is made up of the Leydig cells, fibroblasts, peritubular myoid cells (PTM), immune cells such as macrophages and blood vessels. These compartments are separated by cellular barriers, which limit the exchange of water-soluble materials (Sharpe, 1994). The testicular tissue is enclosed in a capsule or tunica.
The seminiferous tubules are long, highly convoluted tubes containing maturing germ cells, which are highly organised and are traditionally divided into a number of ‘stages’ based on their germ cell complement as determined by histological evaluation of periodic acid Schiff (PAS) stained plastic sections of the testis (see Figure 1-1). The cycle of the epithelium was first defined in the rat by Leblond and Clermont (1952) and was described as having 14 stages. Different species have seminiferous epithelia, which have different numbers of fixed groups of germ cell stages with the mouse testis having 12 (Clermont, 1972) (Figure 1-2) and the human 6 (Clermont, 1966). For example, a cross-section of a stage VII tubule in the mouse contains preleptotene spermatocytes, mid pachytene spermatocytes and step 7 and 16 spermatids (Russell et al., 1990a) (Figure 1-2).
The organisation of spermatogenesis along the seminiferous tubule is known as the spermatogenic wave. In rodents, spermatogenesis follows a longitudinal pattern and in transverse sections through the testis whole segments are at the same stage of the cycle and adjacent segments are one stage ahead or behind (Leblond and Clermont, 1952). The spermatogenic wave in the human tubule, however, is said to be organised in a helical pattern (Schulze and Rehder, 1984) but this view has been challenged and there are suggestions that the stages are organised at random (Johnson, 1994) and also a recent study characterised in detail the organisation of spermatogenesis into clones in non-human primates (Ehmcke et al., 2005).

Within the seminiferous tubules there is a close association between Sertoli cells and the germ cells, which are at different stages of development (spermatogonia, spermatocytes, spermatids, and elongated spermatids) (Figure 1-3). This results in
extensive communications taking place between these cells at the biochemical and molecular level (Cheng and Mruk, 2002). In contrast to somatic cells at the end of cytokinesis germ cells remain linked by intercellular cytoplasmic bridges, which connect daughter cells in a syncytium. These are produced in both the mitotic (Huckins and Oakberg, 1978) and meiotic (Burgos and Fawcett, 1955) stages thus linking the cytoplasm of generations of daughter cells. This can result in the sharing of gene products between cells and may allow compensation for deficiencies in some haploid cells caused by heterozygosity (Braun et al., 1989). In the seminiferous tubule the least mature diploid germ cells are positioned at the basement membrane and as cells mature and differentiate they move closer to the luminal surface where the elongated sperm are released (Figure 1-1, Figure 1-3).

![Diagram](image_url)

**Figure 1-3** A schematic diagram of a Sertoli cell with its associated germ cells in the seminiferous epithelium. Arrowhead denotes tight junctions. Adapted from Cooke and Saunders (2002).

### 1.2.2 Somatic cells

There are three main types of somatic cell within the testis: Sertoli cells, Leydig cells and peritubular myoid cells (PTM).
1.2.2.1 Sertoli cells

The Sertoli cells are irregular shaped columnar cells and are located within the seminiferous epithelium and extend from the basement membrane to the lumenal surface. These cells play a central role in the development of the testis and in the regulation of spermatogenesis (Sharpe et al., 2003). The Sertoli cells are the first ‘male’ cell type to differentiate in the bipotential fetal gonad where they enclose the gonocytes within the testicular cords (Mackay, 2000). Sertoli cells continue to proliferate until the start of the first wave of spermatogenesis when they undergo a final differentiation (Griswold, 1998). In adulthood each Sertoli cell is found in association with a fixed number of germ cells, which are dependent on it for their survival and development (de Franca et al., 1993). Therefore there is a direct correlation between the number of Sertoli cells, the number of germ cells, the testicular volume and amount of sperm produced (Russell and Peterson, 1984; Sharpe, 1994). The number of germ cells that each Sertoli cell supports varies between species (Griswold et al., 2001). Following proliferation the Sertoli cells differentiate and their nucleolus becomes more prominent. Concomitant with this the formation of specialised tight junctions between Sertoli cells establishes the blood-testis barrier (reviewed in Gondos and Berndston, 1993).

Extensive interactions take place between the Sertoli cells and the germ cells throughout spermatogenesis. The entire process of germ cell development, except the earlier stages of spermatogonia to leptotene spermatocytes, is segregated from the systemic circulation by the blood-testis barrier, which is created by tight junctions between Sertoli cells near the basal lamina (Setchell et al., 1969) (Figure 1-4). The tight junctions connecting adjacent Sertoli cells enable strict regulation of the transport of substances between these two compartments. There is thus an elaborate network of cell-cell communications so that Sertoli cells can provide germ cells with the required nutrients: in vitro studies have demonstrated a bidirectional trafficking system between the Sertoli cells and germ cells and that each cell type regulates the function of the other (Aravindan et al., 1997; Aravindan et al., 1996; Jégou, 1993). During spermatogenesis, the preleptotene and leptotene spermatocytes must migrate
from the basal to the adluminal compartment therefore crossing the blood-testis barrier. This event is accompanied by the restructuring of cell-cell actin based adherens junctions (AJ) while maintaining the blood testis barrier (Yan and Cheng, 2005). There are three main types of junctions in the testis: occluding junctions (e.g. tight junctions), anchoring junctions (e.g. adherens junctions) and also communicating or gap junctions (Cheng and Mruk, 2002). The localisation of these within the seminiferous epithelium is summarised in Figure 1-4.

![Figure 1-4 Schematic diagram of junctions present in the testis. Adapted from Goossens and van Roy (2005).](image)

The Sertoli cells also have a phagocytic function including phagocytosis and elimination of residual cytoplasmic bodies discarded during sperm maturation (Russell and Griswold, 1993) (Mizuno et al., 1996). Apoptotic germ cells are also rapidly removed by phagocytosis. For further details on apoptosis see section 1.4.4.
1.2.2.2 Leydig cells

The Leydig cells are situated in the interstitium together with the PTM cells, macrophages, blood vessels and their endothelial cells (Saez, 1994). The primary function of the Leydig cells is steroid synthesis and they are the site of synthesis of the androgens, such as testosterone and also insulin-like growth factor 3 (INSL3). In the mouse there are two populations of Leydig cells. The first is present during fetal life and seen soon after testis differentiation (around embryonic day (e) 12.5) and is responsible for masculinisation of the fetus. The second population develops after birth and are responsible for biosynthesis of the steroids (androgens and oestrogens) required for normal adult reproductive function and male behaviour (Baker and O'Shaughnessy, 2001; O'Shaughnessy et al., 2002). Biosynthesis of testosterone by the Leydig cells is stimulated by luteinising hormone (LH), which is released by the pituitary. Binding of LH to its receptor on the cell surface stimulates the production of cyclic AMP (cAMP) (Cooke et al., 1981). cAMP release stimulates testosterone synthesis by stimulating the transport of cholesterol into the Leydig cell mitochondria and by inducing expression of steroidogenic enzymes (Stocco and Clark, 1996).

The PTM cells are stratified around the seminiferous tubules external to the basement membrane in concentric layers. These cells are modified myofibroblast cells, express smooth muscle actin and are capable of spontaneous contractions that propel fluid secreted by the Sertoli cells along with the spermatids into the lumen and through the tubule to the rete testis (Clermont, 1958; Schlatt et al., 1997). PTM cells also deposit extracellular matrix elements, which maintain mesenchymal-epithelial interactions with Sertoli cells (Verhoeven et al., 2000) and separate the testicular cords from the interstitial compartment during development (Tilmann and Capel, 1999). In addition to Leydig cells and PTM cells the interstitium also contains blood vessels which are involved in thermoregulation of the testis and in the delivery of hormones such as LH to the Leydig cells and macrophages which are involved in mediating the immune response of the testis.
1.2.3 Germ cell maturation

1.2.3.1 Spermatogenesis

One of the essential functions of the testis is the production of mature, highly differentiated haploid sperm from primitive, diploid, spermatogonial stem cells. Spermatogenesis is generally divided into the following phases: mitotic proliferation, meiotic division and spermiogenesis (Figure 1-5) followed by the release of mature spermatozoa (spermiation) (Sharpe, 1994) and takes 35 days in mice or 75 days in humans (Clermont, 1963).

Figure 1-5 Schematic diagram showing the division of spermatogenesis into spermatogonial (mitotic), spermatocyte (meiosis) and spermiogenesis stages in the rat. Provided by Professor Philippa Saunders.
1.2.3.1.1 **First wave of spermatogenesis**

Early in postnatal life the first wave of spermatogenesis is initiated when gonocytes differentiate into spermatogonia between birth and day 5 post partum (pp) in the mouse (Bellve et al., 1977). These spermatogonia correspond to adult In and B spermatogonia and this is followed by the sequential appearance of the differentiating cells (Bellve et al., 1977; Kluin et al., 1982) (de Rooij, 1998). It is well accepted that the first wave of spermatogenesis during puberty is less efficient than that of adults, and that it exhibits massive apoptosis (Kluin et al., 1982; Mori et al., 1997). This apoptosis is thought to reflect the adjustment in the number of germ cells to that which the Sertoli cells can maintain (Orth et al., 1988). It is also suspected that the first round of spermatogenesis may not produce fertile spermatozoa.

1.2.3.1.2 **Spermatogenesis in the adult mouse**

Mitosis is the process whereby cells divide to produce two daughter cells with the same chromosome complement as the original cell and forms part of the mammalian mitotic cell cycle (Figure 1-6). This involves a series of transitions that can be divided into four phases including S-phase (when DNA synthesis occurs) and M-phase (when cells undergo mitosis). There are also two gap (G) phases between these stages known as G2 (between S and M) and G1 (between M and S). The successful progression of mitosis through these stages is dependent on a series of highly ordered and tightly controlled enzymatic reactions. These include cyclins, cyclin dependent kinases (Cdks), which are both involved in cell cycle transitions and also Aurora kinases. These ensure the formation of a bipolar mitotic spindle and correct segregation of chromosomes (Adams et al., 2001; Kimura et al., 1999). There are also a number of checkpoints in place at the G1, S and G2 phases to ensure that any cell containing DNA damage does not continue to proliferate until this damage is repaired. If the damage cannot be repaired the cell dies (MacLachlan et al., 1995; Waldman et al., 1996).
The process of mitosis (M) is generally split into five distinct subphases: prophase, metaphase, anaphase, telophase and cytokinesis (Figure 1-7). These phases are similar to those found in meiosis II (described below). Briefly, the chromosomes line up along the centre of the cell as sister chromatids prepare to separate from one another. The centromere connecting the chromatids then divides and they separate with each one moving to opposite ends of the cell. The original cell then divides into two new daughter cells each containing 46 chromosomes and is genetically identical to the parent cell.

Figure 1-6 Summary diagram of the mammalian mitotic cell cycle. Reproduced and adapted from Clinical Tools Inc. (www.geneticsolutions.co.uk).
1.2.3.2 Spermatogonial phase

The adult testis contains a population of spermatogonial stem cells, which are located on the basal membrane of the seminiferous tubules outside the blood-testis barrier. Upon division, spermatogonial stem cells can give rise to two types of daughter cells. The first type is capable of self-renewal and maintains the stem cell population to ensure that the demand for a continuous supply of spermatogonia is met. The second type become type A spermatogonia (de Rooij and de Boer, 2003; de Rooij and Russell, 2000), which in the mouse undergo six further mitotic divisions into type B spermatogonia before they differentiate (de Rooij, 2001). The B spermatogonia
differentiate further and give rise to the preleptotene spermatocytes. These cells duplicate their DNA and are then recruited by the Sertoli cells into the adluminal compartment where they enter meiosis.

1.2.3.3 Spermatocyte phase

The spermatocytes resulting from the spermatogonial phase (see above) have now made their way into the adluminal compartment of the seminiferous epithelium where they enter a lengthy prophase. Prior to this the pre-leptotene spermatocytes undergo a bulk DNA synthesis. The spermatocyte phase of spermatogenesis is characterised by the meiotic maturation of germ cells. This involves two cell divisions: meiosis I and meiosis II. Meiosis I is the reductional division as the chromosome number is halved resulting in a haploid chromosome complement. Meiosis II is equational as the chromosome number remains the same in each cell before and after the second division. Meiosis I is divided into four stages: prophase I, metaphase I, anaphase I and telophase I (Figure 1-8). Prophase I is the longest stage and is split into five phases: leptotene, zygotene, pachytene, diplotene and diakinesis (reviewed in Cohen and Pollard, 2001). Each stage is defined by the appearance of the chromosomes in the cell. During prophase I chromosomes must find their homolog, synapse, undergo recombination events and desynapse to give rise to viable gametes that display genetic diversity. The meiotic-specific structure that facilitates these events is the synaptonemal complex (SC), which tethers homologous chromosomes together.

The SC is a proteinaceous, zip-like structure (Figure 1-9) composed of three parts including two parallel lateral elements (LE) and a central element (CE). The LEs and the CE are tethered together by fine fibres called transverse filaments (TF) (Schmekel and Daneholt, 1995). Early in prophase I, precursors of the SC begin to appear as the axial elements (AE) first become visible (the lateral elements are called axial elements prior to pairing of the homologous chromosomes). An AE forms a proteinaceous core that associates with the two sister chromatids of a homologous
chromosome. The SC is composed of three main proteins, synaptonemal complex proteins 1, 2, and 3 (SCP1, SCP2, and SCP3) (Dobson et al., 1994; Lammers et al., 1994; Meuwissen et al., 1992; Offenberg et al., 1998; Yuan et al., 2000). SCP1 is a major component of the TFs (Liu et al., 1996b). SCP2 and SCP3 are both components of the axial/lateral element of the SC and are also associated with the centromeres in meiotic metaphase I cells (Lammers et al., 1994; Offenberg et al., 1998; Schalk et al., 1998). The CE of the SC has recently been reported to be composed of two proteins known as SYCE1 and SYCE2 (Costa et al., 2005).
Figure 1-8 Schematic diagram of stages in Meiosis I. Reproduced and adapted from Clinical Tools, Inc (www.geneticsolutions.co.uk).
The initiation of synapsis is dependent in DNA breaks (DSBs) during the leptotene and zygotene stages (Mahadevaiah et al., 2001). The leptotene spermatocytes are characterised by visible condensed chromatin of sister chromatids and during this time the axial elements of the SC are formed. In the zygotene spermatocytes, maternal and paternal homologues begin to align, sites of recombination become apparent and the central element of the SC tethers the axial elements and brings the homologues together. In the zygotene and pachytene spermatocytes, the LEs of homologous chromosomes become connected along their entire length by the TFs, and a CE is formed in the middle which facilitates synapsis. Synapsis is complete in pachytene spermatocytes and homologues are fully joined by the SC. During this stage recombination, transcription and chromatin remodelling also occur (Ashley, 2004).

Near the end of prophase I, the SC disintegrates in the diplotene spermatocytes, chromosomes desynapse and the sites of recombination become apparent as individual chiasmata. These hold the homologues together until they align along the mid plate of the cell. At diakinesis homologous chromosomes shorten and condense.
and begin to move onto the metaphase plate during metaphase I. The chiasmata are then released which allows the homologues to move to opposite poles of the spermatocyte during anaphase I, the spindle breaks down (telophase I) and division of the cytoplasm (cytokinesis) occurs producing two secondary spermatocytes, each containing a single set of chromosomes that then enter meiosis II (Johnson and Everitt, 1995). The second meiotic division more closely resembles mitosis (without the intervening S phase of DNA replication). Meiosis II is split into four stages as in mitosis and meiosis I: prophase II, metaphase II, anaphase II and telophase II. The chromosomes separate and move to the opposite ends of the second meiotic spindle before dividing to yield four round spermatids (Johnson and Everitt, 1995; Sharpe, 1994) (Figure 1-10). Following this the spermatids must undergo a differentiation process to yield mature sperm before being released into the epididymis, this is detailed in section 1.2.3.5.
1.2.3.3.1 Meiotic crossing over (recombination)

In addition to the segregation of homologous chromosomes (detailed above), one important process during meiosis is homologous recombination (HR) or crossing over. During meiotic prophase parts of the DNA from the paternal and maternal homologous chromosomes are recombined. The recombination events result in the
exchange of genetic information between non-sister chromatids resulting in crossovers that are essential for the correct segregation of the chromosomes and play an important role in creating genetic diversity among individuals within a population by creating new and potentially beneficial combinations of paternal and maternal alleles (Baarends et al., 2001). HR also removes deleterious mutations that could result in meiotic failure.

The recombination process is initiated by the formation of double-strand breaks (DSBs) early in prophase I, (Sun et al., 1989) and this is mediated by the topoisomerase Spo11 (Celerin et al., 2000; Keeney et al., 1997). The broken DNA ends are then resected by degradation of their 5’-ended strands to generate single stranded tails with 3’ overhangs. One of these tails then locates its equivalent sequence on its homologous chromosome and invades it to form a D-loop and is extended by DNA synthesis (Figure 1-11). This enables it to anneal to the single stranded tail on the opposite side of the DSB forming two four-way DNA junctions - a structure called the double Holliday junction (dHJ)(Schwacha and Kleckner, 1995) (Figure 1-11). The final step in the recombination process is the resolution of dHJs by the cleavage of a pair of symmetrical strands at each junction. Cleavage of a different pair of strands at each junction splices together maternal DNA flanking one side of the dHJ with paternal DNA flanking the other side. This type of recombinant product is called a crossover (reviewed in Morelli and Cohen, 2005; Svetlanov and Cohen, 2004; Szostak et al., 1983; Whitby, 2005). It constitutes a new combination of alleles and establishes chiasmata, which are thought to be stabilised by the Mut L homolog, Mlh1 together with Mlh3 (Kolas and Cohen, 2004). A crossover in one region of a chromosome reduces the likelihood of another crossover nearby. However if the dHJ is resolved by cleavage of the same pair of strands at each junction, then crossing over does not occur. These do not contribute to chiasma formation.

Crossing over is essential both for disjunction of the maternal from the paternal chromosome as well as for production of the exchanges that will hold them together
until the onset of anaphase I. Many of the enzymes that catalyse these processes have been identified including Spo11 (as mentioned above), the Mre-Rad50-Nbs1 (MRN) complex (involved in strand resection) (Dolganov et al., 1996; Dong et al., 1999; Petrini, 1999), Rad51 which together with Dmc1 and others drives homologous pairing and strand invasion that leads to the formation of the dHJs. (Bishop et al., 1992; Shinohara et al., 1992).

![Schematic diagram of the homologous recombination (HR) pathway.](image)

*Figure 1-11 Schematic diagram of the homologous recombination (HR) pathway.*

Diagram shows double Holliday junction formation (dHJ) and involvement of key proteins in the pathway. Adapted from Aguilera and Boulton (2007).
Failure to introduce DSBs into homologs results in recombination and synopsis failure and ultimately failure of meiosis and infertility as illustrated by the testicular phenotype of Spo11 knockout mice (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000) (see below). If the DSB’s created during HR are not repaired they can result in permanent cell cycle arrest, apoptosis, or tumorigenesis (Pierce et al., 2001). If recombination fails, chromosome disjunction also frequently fails, with disastrous consequences for gamete formation.

1.2.3.4 Mouse models of infertility: lessons from mice deficient in meiosis-specific proteins

There are several mouse models where inactivation of key proteins required during meiosis results in meiotic failure, apoptosis and infertility. These mutations can also lead to female fertility phenotypes such as embryo loss and infertility. Some examples of these models are discussed below.

In mice, deletion of Spo11, results in infertility in both males and females as meiosis fails at the zygotene stage in both sexes (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000). Likewise a mutation in Dmc1 causes infertility due to failure of germ cells to progress past the zygotene stage (Pittman et al., 1998). A null mutation in the SCP3 gene has been shown to cause homozygous mutant males to be sterile due to massive apoptotic cell death during meiotic prophase. The SCP3+/- male mice failed to form axial/lateral elements and SCs, and the chromosomes in the mutant spermatocytes did not synapse (Kolas et al., 2004; Yuan et al., 2000). Female SCP3+/- mice, however, are fertile (Yuan et al., 2000). Inactivation of Mlh1, the protein product of which is involved in recombination and in marking sites of crossing over, results in both male and female infertility (Baker et al., 1996; Edelmann et al., 1996). Mlh1+- spermatocytes are able to progress through the pachytene stage, however, at diplotene the central element of the SC breaks down and the chromosomes are no longer held together at their chiasmata so that most
appear as univalents resulting in apoptotic elimination and a lack of mature sperm (Eaker et al., 2002).

### 1.2.3.5 Spermiogenesis

The small round haploid spermatids generated at the end of meiosis II (see section 1.2.3.3) are then remodelled into mature spermatozoa in a process called spermiogenesis. This process is divided into a number of phases: Golgi phase, cap phase, acrosome phase, and maturation phase and begins with the production of small vesicles in the Golgi body. These fuse together to form a single larger proacrosomal vesicle, which forms the acrosomal vesicle over the nucleus (Dooher and Bennett, 1973). During the Golgi phase the acrosomal vesicle attaches to the nuclear envelope and grows as a result of ongoing fusion of newly arriving Golgi-derived vesicles (Russell et al., 1990b; Tang et al., 1982). The head cap then appears, expanding around the acrosomic granule and gradually grows over the surface of the nucleus. The Golgi apparatus, which contributes glycoprotein material to the acrosomic system, eventually separates to float freely in the cytoplasm (Clermont and Leblond, 1955). During the later stages (acrosome and cap phase) the acrosomal vesicle flattens and spreads over the nucleus, covering up to two thirds of its surface (Figure 1-12). During this process acrosomal proteins condense and are packed in a paracrystalline structure termed the acrosomal granule, which eventually forms the acrosomal matrix in mature sperm (Anakwe and Gerton, 1990; Escalier et al., 1991). The nucleus then undergoes elongation and condensation to form the head of the sperm. The cytoplasm of the cell is redistributed to the luminal pole of the cell, away from the acrosome, thus forming an elongated cell with the nucleus at one end and cytoplasmic tail at the other. The manchette forms within the cytoplasmic tail as a cylindrical sheath of microtubules attached to the nucleus and surrounding the initial segment of flagellum. The flagellum, which forms the neck and tail regions, is surrounded by nine electron dense fibres (reviewed in de Kretser et al., 1998). Mitochondria migrate to form a sheath around these fibres from the neck region to
the annulus, a ring-like structure part of the way down the tail. This region is termed the mid-piece.

During spermiogenesis there are also a number of modifications to the nucleus to permit nuclear condensation. During the early stages of spermiogenesis the nucleus is round, contains histones as the major basic nuclear proteins, and is transcriptionally active. However as the sperm matures through the last stages of spermiogenesis and during epididymal maturation there are dramatic changes in chromatin structure, nuclear shaping, and condensation. After nuclear elongation starts, transcription ceases and the histones are removed and replaced by other proteins. In mammals, the histones are first replaced by the transition proteins (TPs). During human (Gusse et al., 1986) and mouse (Bellve et al., 1988) spermiogenesis, the TPs are then replaced by two protamines, protamine 1 (P1) and protamine 2 (P2) which are arginine and cysteine rich (Balhorn et al., 1999; Oliva and Dixon, 1991). The replacement of histones with these protamines involves major remodeling of the chromatin, which is presumably why RNA transcription and translation during this stage ceases (Kierszenbaum and Tres, 1975).

During the final maturation phase the nucleus completes its condensation and takes on its definitive shape. The sperm loses its Golgi apparatus and much of its cytoplasm by Sertoli cell phagocytosis (de Kretser and Kerr, 1994; Johnson and Everitt, 1995). After shedding its residual cytoplasm the elongated spermatid is released from its association with the Sertoli cell and leaves the testis with a small cytoplasmic droplet which is shed into the lumen of the epididymis and endocytosed by the clear cells in the cauda epididymis (Hermo et al., 1988; Robaire and Hermo, 1988).
1.2.4 The epididymis

1.2.4.1 Structure and function

The efferent ducts (a series of tubules) arising from the rete testis come together to form a highly convoluted duct system called the epididymis. The epididymis comprises a highly specialized structure subdivided essentially into three major regions: caput, corpus and cauda. The vas deferens extends from the cauda as a straight tube connecting with the urethra, which empties to the outside of the body: the route via which sperm are released (Robaire and Hermo, 1988). The main function of the epididymis is the transport, maturation (see below), storage and release of mature sperm and this function is thought to be highly dependent on circulating testosterone as in the absence of androgens the sperm become immotile, lose the ability to fertilise and die (Dyson and Orgebin-Crist, 1973; White, 1932).
1.2.4.2 Sperm maturation in the epididymis

Following release from the testis into the epididymis sperm are neither fully motile nor able to recognize or fertilize an egg. In order to achieve this they first need to undergo a maturation process, which occurs in the epididymis. Sperm first enter the epididymis at the initial segment of the caput, then pass through the caput proper, and subsequently the corpus and cauda epididymis. The cauda epididymis is located in the most ventral and coolest region of the scrotum. This is the region where storage of sperm occurs and this has given rise to the notion that one of the reasons for the evolution of the scrotum was to provide optimal conditions for sperm storage (Bedford, 1978).

During epididymal transit the sperm undergo distinct morphological modifications such as shedding of the cytoplasmic droplet, physiological changes to the membrane and biochemical maturation including structural stabilisation of the chromatin and other organelles with disulphide bonds. These changes are fundamental to their ability to swim progressively, bind to the zona pellucida, penetrate and fertilise the egg and produce viable offspring. The fluid microenvironment within the epididymis has been suggested to promote the maturation of the spermatozoa. It has been shown that the pH is quite acidic with values from 6.5 to 6.8 and that the fluid contains intracellular second messengers such as cAMP and calcium as well as protein kinases and inhibitors of protein phosphatases that promote motility (Bedford and Hoskins, 1990; Garbers and Kopf, 1980; Hoskins et al., 1983; Hoskins et al., 1978; Tash and Bracho, 1994; Vijayaraghavan et al., 1996). These mediators presumably act through protein phosphorylation. The ability of sperm to bind and fertilise the egg develops in parallel with motility (Eddy and O'Brian, 1993; Yanagimachi, 1994). The same protein kinases and protein phosphatases responsible for regulating motility are also likely to play a role in signalling events during fertilization. Supporting this idea is the observation that many factors and treatments shown to be essential for maintaining and promoting the fertilizing ability of spermatozoa are also those that stimulate sperm motility (Cooper et al., 1986). These factors include bicarbonate, calcium, phosphodiesterase inhibitors, and protein phosphatase inhibitors. For
example, the protein phosphatase-inhibitors calyculin A and okadaic acid not only stimulate motility but also promote sperm hyperactivation (Si and Okuno, 1999) and the zona-induced sperm acrosome reaction (Furuya et al., 1993). After maturation the epididymal sperm may be stored for a period of time in the distal epididymis and vas deferens before ejaculation. There are some studies in mice, however, that report that transit through the epididymis may not be essential for maturation. For example, sperm removed from the caput region of the mouse epididymis were found to be capable of binding the zona pellucida of eggs provided the sperm are capacitated in vitro first (Biegler et al., 1994; Kawai et al., 1991). However these sperm would have been exposed to some parts of the epididymis and other studies analysing sperm removed from different parts along the human epididymis show marked maturational changes associated with how far they have travelled (Bedford, 1994) including the development of motility (Yeung et al., 1993).

1.2.4.2.1 Protective function of epididymis

As sperm can be stored in the epididymis for long periods (1-2 weeks in some mammals), the epididymis must play a protective role to allow sperm survival. As in the testis, there is a blood-epididymis barrier composed of tight junctions, which only allows selective entry of substances into the epididymal lumen (Robaire and Hermo, 1988). A number of epididymal proteins have been identified that probably play a role in the protection of sperm from reactive oxygen species (ROS) and proteases (Hinton et al., 1995; Hinton et al., 1996). These proteins are mainly secreted in the proximal regions of the epididymis although it is not known whether these are released only to protect sperm during storage or if they also play a role in sperm maturation. For example, they may regulate the ROS generating activity in order to mediate capacitation (Aitken and Vernet, 1998). It has also been demonstrated that a number of antioxidants are expressed in the epididymis in a region-specific manner (Potts et al., 1999; Zini and Schlegel, 1997). As with the testis (section 1.4.1.1) the function of the epididymis is also adversely affected by elevated temperatures (Bedford, 1991) and elevated temperature has been reported to result in a change in
protein composition of the epididymal fluid (Esponda and Bedford, 1986). For example, studies on mammals such as the dog and on epididymal cell cultures have reported that some epididymal gene products, such as CD52, are particularly sensitive to small temperature changes (Gebhardt et al., 1999; Kirchhoff et al., 2000; Pera et al., 1996).

1.2.4.3 Sperm – structure

A mature sperm is composed of the head, the flagellum (tail) and the neck as shown in Figure 1-13. The head contains very little cytoplasm and the highly compacted and inactive DNA, which will be passed on to the oocytes during fertilisation. The packaging of the DNA in sperm is extremely specialised to allow optimum functioning of the sperm. The DNA in the sperm nucleus is bound to protamines, which allows the large volume of DNA to be packaged into a small space (Ward and Coffey, 1991). The organisation of chromatin in sperm is discussed in section 1.2.4.4. The tail of the mature sperm consists of a central axoneme, surrounded by dense fibres, which form the cytoskeleton of the tail. The head also contains the acrosome, which contains the enzymes required for the penetration of the egg. The tail contains little cytoplasm but is abundant in mitochondria, which provide the essential energy required by the sperm for motility (Eddy and O'Brian, 1993). Subsequent to sperm release from the epididymis, the sperm must undergo further maturation to be capable of fertilisation, see section 1.5.1.
Figure 1-13 Structure of a human sperm. N.B. Mature mouse sperm have a hooked head rather than a rounded head.

1.2.4.4 Chromatin structure

Sperm DNA is organised in a specific manner that keeps the nuclear chromatin compact and stable. It is packaged with a type of small basic protein into a tight almost crystalline structure that is six times more highly condensed than in mitotic chromosomes (Fuentes-Mascorro et al., 2000). It is not packaged into nucleosomes as in other cells, as this volume would be too large for the sperm nucleus. The nucleosomal-type chromatin is transformed into a smooth fibre and condensed. Epididymal maturation involves the final stages of chromatin reorganisation in which protamines become cross-linked by disulphide bonds, which help to make this the most condensed eukaryotic DNA (Ward and Coffey, 1990). The packaging of DNA in a sperm nucleus is summarised in Figure 1-14.
Figure 1-14 Comparison of the packaging of DNA in somatic versus sperm nuclei. Adapted from www.compbio.med.wayne.edu/image/chro.jpg

1.3 Maintenance of normal testis function

As mentioned before one of the main functions of the testis is to produce mature sperm, which can subsequently fertilise oocytes to result in viable offspring. Spermatogenesis is a highly ordered process that is dependent on the appropriate expression and action of specific genes at multiple stages of germ cell development and any disruption to these processes can lead to impaired fertility. In addition to the
steroid hormones that are required for normal spermatogenesis, such as testosterone and 5 α-dihydrotestosterone (DHT), the testis expresses a number of factors involved in protection of the DNA that is to be passed on to future generations. These include proteins involved in checkpoints, DNA damage recognition, DNA repair and also antioxidants.

1.3.1 DNA damage - origins and consequences

The stability and integrity of genetic information in the form of DNA is of vital importance for the normal function and reproduction of all living organisms. The DNA double helix is constantly under attack from endogenous and exogenous factors that can induce a wide range of DNA abnormalities/lesions. DNA lesions, such as DSBs, occur during processes such as transcription, recombination, and replication. However they can also be induced by irradiation, exposure to chemicals and high levels of ROS. During spermatogenesis germ cells are capable of producing ROS (Fisher and Aitken, 1997). Maturing epididymal sperm generate low levels of ROS, a process thought to be related to capacitation (de Lamirande and Gagnon, 1993; Visconti et al., 1995). This involves a redox-regulated tyrosine phosphorylation cascade (Aitken et al., 1998b; de Lamirande and Gagnon, 1993). However, if sperm are exposed to or generate excessive levels of ROS then fertilisation capacity and DNA integrity are compromised (Aitken and Clarkson, 1987; Aitken et al., 1998a). ROS generation has also been associated with failure to remove the cytoplasmic droplet during maturation, which can result in premature release and loss of sperm function (Gomez et al., 1996). ROS can induce a variety of DNA lesions. One of the most abundant is the oxidative lesion, 8-oxoG (reviewed in Lindahl, 1993), which is strongly mutagenic. A complex anti-oxidant defence system exists in the testis (Bauche et al., 1994), complemented by DNA repair systems as well as both p53-dependent and -independent apoptotic pathways (Embree-Ku et al., 2002; Yin et al., 2002).
There are reports that oxidative damage to DNA plays a role in mutagenesis and increased risk of developing cancerous tumors (Loft and Poulsen, 1996). Oxidative damage linked to cigarette smoke has been reported to cause DNA damage in sperm which can be passed from the father to the offspring (Zenzes, 2000; Zenzes et al., 1999) and that this is associated with an increased incidence of childhood cancer in their offspring (Ji et al., 1997; Sorahan et al., 1997). The testis has a number of different repair pathways in place to deal with many different types of DNA damage/alterations.

1.3.2 Susceptibility to damage

DNA is constantly under attack from endogenous and exogenous factors that can induce a wide range of DNA abnormalities/lesions. In sperm, the chromatin is normally a highly organised, condensed and compact structure consisting of DNA and nuclear proteins (see section 1.2.4.4). These properties should protect genetic integrity and enable the transport of the paternal genome through the male and female reproductive tracts (Manicardi et al., 1998). There are several factors that can lead to DNA damage in spermatozoa, including environmental stress, genetic mutations and chromosomal abnormalities. However, the exact mechanisms by which chromatin abnormalities/DNA damage arise in spermatozoa in the human are not well understood. There are however a number of possibilities, including oxidative stress (from cigarette smoking) and defective chromatin packaging (during spermiogenesis). A high level of DNA strand breaks are formed during histone removal and protamine replacement (McPherson and Longo, 1993) and although most are repaired by ligation (Kovtun and McMurray, 2001) (Boissonneault, 2002), sperm appear to contain higher levels of DNA strand breaks than most somatic cells (Haines et al., 1998; van der Schans et al., 2000). Mature sperm are also exposed to ROS in the male and female reproductive tract. ROS at high levels can be toxic to sperm quality and function (see section 1.3.1, (Saleh and Agarwal, 2002)).
1.3.3 DNA repair pathways

DNA repair is required for meiotic recombination and correction of DNA damage in developing germ cells (Baarends et al., 2001). The expression of proteins able to undertake DNA repair is essential to repair any mistakes/mismatches generated, for example, during meiotic crossing over or via exogenously induced lesions. More than 130 genes have been identified in humans that are involved in protecting genome integrity (Wood et al., 2001). There are numerous pathways involved in DNA repair in mammals (and other eukaryotes) in addition to HR (section 1.2.3.3.1). These include mismatch repair (MMR), which is involved in repairing small mismatches or loops, nucleotide excision repair (NER), which is associated with repair of UV-induced DNA lesions and base excision repair (BER), which is involved in replacement of aberrant (including oxidised) bases in DNA. Proteins involved in MMR, NER, and BER are all expressed in the testis (reviewed in Jaroudi and SenGupta, 2007). For example, histone variant 2AX (H2AX) is involved in the DNA damage response to DSBs (Downs et al., 2000) and acts by recruiting DNA repair factors to DNA damage sites where it is rapidly phosphorylated resulting in formation of γ-H2AX foci (Rogakou et al., 1999). In response to DNA damage, cell cycle checkpoints, which slow down or arrest cell cycle progression can be activated. This allows the cell to repair or prevent the transmission of damaged DNA. Checkpoint machineries can also initiate pathways leading to apoptosis and the removal of damaged cells.

1.3.3.1 Nucleotide excision repair pathway (NER)

The NER pathway is one of the most versatile repair pathways protecting cells from DNA damage and involves more than 25 different proteins (Petit and Sancar, 1999). NER is involved primarily in the repair of UV induced lesions and bulky chemical DNA adducts. NER is present at all stages of germ cell maturation (Xu et al., 2005) and expression of NER proteins is elevated in the testis (Hsia et al., 2003; Li et al., 1996; Weeda et al., 1991). NER consists of two distinct sub-pathways: global genome repair (GGR) and transcription coupled repair (TCR). GGR is responsible
for the repair of DNA damage throughout the genome and TCR is targeted to transcription-arresting lesions on the transcribed strand of transcriptionally active genes. The NER pathway consists of an initial damage recognition step, followed by dual incisions either side of the lesion. The damaged segment is then removed on a short oligonucleotidetide before repair synthesis takes place (summarised in Figure 1-15).

Xeroderma pigmentosum complementation group C (Xpc) initiates the NER process (in GGR), complexed to hHR23B and acts as a DNA damage sensor and repair-recruitment factor (Sugasawa et al., 1998). The general transcription factor complex TFIIH, containing the Xpb and Xpd helicases, catalyses strand separation at the site of the lesion (Evans et al., 1997; Mu et al., 1997). Xpa verifies and binds the damage in an open DNA conformation and facilitates the assembly of the remainder of the repair machinery (Miyamoto et al., 1992; Tanaka et al., 1990). Replication protein A (RPA) stabilizes the opened DNA complex and is involved in positioning the Xpg and Ercc1-Xpf endonucleases responsible for the DNA incisions around the lesion (de Laat et al., 1998). The two proteins Ercc1 and Xpf (Ercc4) act together in a complex, which is a structure specific endonuclease that catalyses the incision on the 5’ side of the lesion (Bessho et al., 1997a; Bessho et al., 1997b) and Xpg makes the 3’ incision during NER (O’Donovan et al., 1994). The final steps of NER involve DNA synthesis to fill the gap and ligation of the newly synthesised strand to the original sequence. This involves the activities of PCNA, DNA polymerases and finally, DNA ligase (Hunting et al., 1991; Shivji et al., 1992). NER is active throughout spermatogenesis but declines in post-meiotic cells, for example Ercc1 and XPF are expressed until the round spermatid stage (reviewed in Jaroudi and SenGupta, 2007).

NER deficiency is found in three rare heritable disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) and all three diseases have the common characteristic of sun-sensitivity (reviewed in de Boer and Hoeijmakers, 2000; Friedberg, 2001; Lehmann, 2003). XP patients exhibit increased
skin cancer risk, however the others do not (de Boer and Hoeijmakers, 2000; Kraemer et al., 1994). The consequences of NER protein deficiency are discussed below (section 1.3.5).

Figure 1-15 Schematic diagram of the NER pathway. Adapted from de Laat (1999).
1.3.3.2 Mismatch repair pathway (MMR)

The MMR system acts to remove base-base mismatches and small loops that occur as a result of strand slippage during mitotic replication and that escape the proof reading of polymerases (reviewed in Stojic et al., 2004). In addition to this mismatches can also arise during S phase in meiosis as well as during meiotic recombination. MMR provides several genetic stabilisation functions; it corrects DNA synthesis errors, ensures the fidelity of genetic recombination, and participates in the earliest steps of checkpoint and apoptotic responses to several classes of DNA damage (reviewed in Iyer et al., 2006; Jiricny, 2006; Kunkel and Erie, 2005). The MMR pathway in prokaryotes involves three proteins MutS, MutL, and MutH (Cohen and Pollard, 2001). In humans and mice there are many MutS and MutL homologs and their names are based on their homology to the prokaryotic proteins. For example in the mouse MMR pathway (summarised in Figure 1-16), Msh2, Msh3, Msh4, Msh5 and Msh6 are MutS homologs and Mlh1, Mlh3, Pms1 and Pms2 are MutL homologs (Cohen and Pollard, 2001; Svetlanov and Cohen, 2004). In the mouse, Msh2 to Msh6 function as heterodimers to initiate repair activity; Msh2/Msh6 and Msh2/Msh3 recognise replicative mismatches, whereas Msh4/Msh5 is a meiosis-specific complex essential for processing recombination intermediates (reviewed in Kunkel and Erie, 2005). It is thought that Msh4 and 5 are involved in processing recombination intermediates in meiosis as disruptions of either of the genes that encode these proteins results in meiotic failure (during prophase I) and infertility (Edelmann et al., 1999; Kneitz et al., 2000). The MMR pathway is active throughout spermatogenesis though it declines after meiosis and becomes undetectable in elongated spermatids, for example Msh2, Msh3 and Pms2 are expressed in spermatocytes though are not readily detectable in round spermatids (reviewed in Jaroudi and SenGupta, 2007).
The process of MMR commences with mismatch recognition, which, in human cells, is mediated predominantly by the hMSH2-MSH6 heterodimer (Jiricny, 1998) followed by binding to mismatched DNA and distinguishing between the template and the new strand though it is not understood how discrimination occurs but may simply involve recognition of nicks or gaps during replication. This dimer then undergoes an ATP-dependent conformational change, which converts into a sliding clamp that has been shown to move along the DNA backbone in vitro (Gradia et al., 1999; Iaccarino et al., 2000). This complex is then bound by a second heterodimer, composed of hMLH1 and hPMS2 (Li and Modrich, 1995). This complex has the ability to search for mismatches in any direction. The sliding clamp has also been

Figure 1-16 A simplified schematic diagram of the mismatch repair pathway (MMR). Adapted from http://www.biochemsoctrans.org/bst/033/0689/bst0330689f01.htm
shown to stimulate the exonuclease EXO1 (Genschel and Modrich, 2003), which is involved in the degradation of nucleotides starting from a nick 5’ to the mismatch and travelling towards the unpaired strands. The region of single stranded DNA is then stabilised by the replication protein A (RPA). When the region of DNA degraded by EXO1 contains the mismatch the degradation ceases (Genschel and Modrich, 2003). This is followed by replicative polymerases filling in the gap, which leaves a final nick that is sealed by DNA ligase as in NER.

Cells lacking MMR show an increased mutation frequency and microsatellite instability (MSI) (Modrich and Lahue, 1996). Defects in this pathway are linked to nonpolyposis colon cancer (Peltomaki, 2005) but may also play a role in the development of sporadic tumors that occur in a number of tissues (Peltomaki, 2003). Sporadic gastrointestinal cancer may also be associated with deficiencies of DNA mismatch repair (Planck et al., 2003; Planck et al., 2002).

### 1.3.4 Base excision repair pathway (BER)

BER is another major pathway of DNA repair though as this is not a major theme in this study it will not be described in depth. BER is involved in replacement of aberrant (including oxidised) bases in DNA induced by hydrolysis, ROS or other metabolites. The pathway is initiated by one of a number of DNA glycosylases specific for individual modified bases to form a basic apurinic/apyrimidinic (AP) site, which is followed by an AP lyase reaction and ligation of the nick (summarised in Figure 1-17 and reviewed in Sancar et al., 2004). For example, the enzyme 8-oxoguanine DNA glycosylase 1 (Ogg1) initiates BER of 8-oxoG, the most frequent mutagenic lesion caused by oxygen free radicals, by cleavage of a chemical bond to release the damaged base from the DNA (Klungland et al., 1999). In the mouse, the highest levels of Ogg1 occur in testis in the spermatocytes and lowest in spermatids (Olsen et al., 2003).
1.3.5 Lessons from mice deficient in DNA repair genes

A number of mice deleted in genes encoding proteins involved in DNA repair have been generated and provide invaluable information about the impact of DNA repair on spermatogenesis (and other processes). Xpa knockout mice have been shown to have an NER pathway defect and become very sensitive to UVB (Boonstra et al., 2001). Xpa null males appear to be subfertile compared with wild-type controls (Tsai et al., 2005), with smaller than average surviving litters, however the authors did not investigate at what stage embryonic loss occurred. Both male and female Ercc1 knockout mice (with a corrected liver phenotype to prevent death before the first
wave of spermatogenesis (Selfridge et al., 2001)) had gonads with a depleted germ cell complement (Hsia et al., 2003). The male mice were infertile and had a high frequency of DNA strand breaks in their sperm as detected by the single cell COMET assay. There was also an increase in oxidative damage as there was a threefold higher level of the oxidised base 7,8-dihydro-8-oxoguanine (8-oxoG) in the testis thus suggesting an additional role for the NER protein, Ercc1, in the repair of 8-oxoG (Selfridge et al., 2001). In contrast to the Ercc1 knockout mice, the Xpa deficient mice appeared to have normal spermatogenesis (de Vries et al., 1995; de Vries and van Steeg, 1996). Mutations in RPA result in defects in DNA double-strand break repair and homozygous deletion in mice results in embryonic lethality before implantation and impaired cell proliferation. Heterozygous mice for RPA had decreased survival, chromosomal instability, impaired double strand break repair, and developed lymphomas (Wang et al., 2005).

Ogg1 null mice are viable and fertile but have elevated levels of 8-oxoG and an increased mutation rate (Klungland et al., 1999; Minowa et al., 2000). Whilst results from studies in mice suggest that defects in NER and BER do not always block sperm production, those mice that are still fertile might allow mutations to be carried into the zygote with consequences for embryonic development although this has not yet been investigated.

Studies on mice with deletion of the MMR genes (Msh4 or Msh5) have revealed that they exhibit meiotic failure and infertility (Edelmann et al., 1999; Kneitz et al., 2000). Msh2 is highly expressed in mouse spermatogonia and leptotene/zygotene spermatocytes (Richardson et al., 2000) and Msh2 knockout mice manifest an enhanced predisposition to cancer (de Wind et al., 1995), but no abnormalities in spermatogenesis have been reported. Notably, sperm from patients with hereditary nonpolyposis colon cancer, heterozygous for an MSH2 mutation, had a significantly increased frequency of aneuploidy (Martin et al., 2000). Deficiency for Mlh1 has been described previously in section 1.2.3.4.
1.4 Stress response in the testis

In the testis a variety of mechanisms are triggered on exposure to stress, such as DNA repair (as discussed above), the heat shock response, the oxidative stress response and also apoptosis or cell death. During spermatogenesis germ cells show a high degree of variation in their susceptibility to different forms of stress. For example it has been reported that spermatogonia are the most sensitive to irradiation (Clifton and Bremner, 1983) and spermatocytes and round spermatids are the most sensitive to heat stress (reviewed in Setchell, 1998). Discussed below are the responses of the testis to heat, oxidative stress, hypoxia and also the mechanism of apoptosis in response to these stresses.

1.4.1.1 Heat, the testis and its response to stress

Spermatogenesis and normal testicular function are both temperature dependant and in most mammals the testes are kept between 2 and 8°C below core body temperature (Harrison and Weiner, 1948). Testicular temperature is regulated by a heat exchange system between the pampiniform plexus and the testicular artery. The scrotum has no subcutaneous fat, which also helps to dissipate heat to the exterior as required (Glad Sorensen et al., 1991). The fact that mice have the ability to retract their testes into the body cavity unlike adult humans may mean that mice are not as able to cope with wide fluctuations in temperature and humans may have evolved a more efficient pampiniform plexus to withstand larger fluctuations. However, this may also mean that the mouse testis is exposed to higher temperatures within the body cavity more regularly and therefore may not be as susceptible as the human testis to higher temperatures.

In man, raised scrotal temperature can occur as a result of occupational exposure, lifestyle or a clinical disorder (Mieusset et al., 1987). Men with scrotal temperatures above the normal range often exhibit fertility problems and their ejaculates have been shown to contain an increased incidence of abnormal and immature sperm (Mieusset et al., 1987). A number of epidemiological studies have investigated the effects of
occupational exposure on male fertility. Occupational exposure can occur in men who work in high temperature environments such as bakers and welders (reviewed in Thonneau et al., 1998) and also occupations that involve long periods in a sedentary position such as professional drivers. For example, at one site in Hungary it was found that 10% of infertile men were professional drivers whereas this profession accounted for less than 4% of the general population (Sas and Szollosi, 1979). Another study in Italy (Figa-Talamanca et al., 1992) reported that twice as many heat-exposed workers from the ceramics industry had difficulties in conceiving and that these men had a lower percentage of normal motility sperm. Retrospective studies have also shown that when comparing the time to pregnancy (TTP), the partners of professional drivers and men exposed to heat because of their occupation, took significantly longer to achieve a pregnancy than those male partners that were not employed in these jobs (Thonneau et al., 1997).

A recent study by Mieusset et al. (2007) has also reported that posture and clothing can cause an increase in scrotal temperature. This study reported that the lowest scrotal temperature in naked men was found in the standing position and in the supine or seated position the temperature was raised (Mieusset et al., 2007). Clothing was also shown to increase the temperature of the scrotum. There are also implications that lifestyle may contribute to raised scrotal temperatures. For example, in men who take regular hot baths and saunas (Jockenhovel et al., 1990). As with occupational exposure studies, there are reports that normal car drivers have increased scrotal temperatures compared to those experienced whilst walking (Bujan et al., 2000).

There are a number of clinical disorders that can result in increased scrotal temperature. For example cryptorchidism, where one or both testes fail to descend into the scrotum and remain in the abdominal cavity, results in the exposure of the testes to abdominal temperatures. It has been shown that following treatment to rectify this condition these men still have a raised scrotal temperature in adult life and impaired spermatogenesis (Mieusset et al., 1995). In addition to this, patients
with varicocele, caused by an enlargement of the spermatic vein and thus venous draining abnormalities, have been shown to exhibit higher testis temperatures than control patients in some studies (Lerchl et al., 1993; Salisz et al., 1991) although this has been disputed by others (Lund and Nielsen, 1996).

1.4.1.2 Heat-induced changes in gene expression

One of the responses to heat stress is the heat shock response, which is induced to enable the cell to cope with the harmful effects of protein-damaging stresses (Hartl and Hayer-Hartl, 2002; Young et al., 2003). This response also occurs as a result of exposure to other factors such as chemical agents, nutrient withdrawal and UV radiation (Mailhos et al., 1993; Simon et al., 1995). The heat shock response is characterised by a general down-regulation of gene transcription and protein synthesis, whilst the transcription of a specific subset of genes called the heat shock genes is induced resulting in the synthesis of a number of heat shock proteins (Hsps)(Lindquist and Craig, 1988). Hsps participate in repair of damaged proteins but they can also induce apoptosis (Hartl, 1996). For example, during heat shock, the Hsps function as molecular chaperones that bind to, and aid the re-folding of, damaged proteins, thereby preventing protein aggregation (Hartl and Hayer-Hartl, 2002; Young et al., 2003). The induction of Hsps is mainly regulated by a family of heat shock transcription factors (HSFs), which bind to the heat shock elements (HSEs) on heat shock genes and other target genes to regulate the response (Pirkkala et al., 2001; Wu, 1995). In contrast, decreased expression of oxidative stress-induced antioxidants has been reported to occur in the testis after heat stress (Rockett et al., 2001), this may leave the germ cells more susceptible to oxidative damage during hyperthermia. In addition, expression of a number of DNA repair genes such *Ogg1* (involved in BER), *Xpg* (involved in NER) and *Rad54* are also downregulated, which may impair the repair of any heat-induced DNA damage (Rockett et al., 2001). Other studies have shown decreased expression of poly(ADPR)polymerases (PARP) in the rat testis in response to heat stress (Tramontano et al., 2000). These proteins are
involved in detection of strand breaks and signalling in both the BER and NER pathways (Flohr et al., 2003; Schreiber et al., 2002).

1.4.1.3 Animal models of heat stress

A number of animal models designed to study the impact of heat stress on the testis have been developed. These include transient exposure of the testes to elevated temperatures (typically greater than 40°C) or placing the testes and epididymides within the body cavity (surgical inducing cryptorchidism) resulting in long-term exposure of the testes to core body temperature (37°C). Both methods have been reported to cause a variety of disturbances in testicular function, including a decrease in testis weight, increased apoptosis, germ cell loss and altered fertilisation capability of sperm (Lue et al., 1999; McLaren et al., 1994; Setchell et al., 1998; Setchell et al., 1996). For example, localised scrotal heating of mice at 40°C and 42°C for 60 min is reported to cause a decrease in testicular weight and an increase in DNA damage in sperm at 40°C at 3, 7, 11 and 14 days after heat stress as determined by the sperm chromatin structure assay (SCSA). Those heated to 42°C had no sperm at these time points for use in the SCSA (Sailer et al., 1997). In another study heat stress (42°C for 30 min) caused an increase in DNA damage in sperm as analysed by the COMET assay with maximum damage observed at 4h after heat stress suggesting that this could have been mediated by alterations in epididymal function. They also showed an increase in germ cell apoptosis using Apotag (Banks et al., 2005). In addition to this, a comprehensive study carried out by Rockett et al. (2001) found that a single transient (20 min) heat stress of 43°C resulted not only in an increase in apoptosis of spermatocytes but also the stress-inducible proteins Hsp70-1 and Hsp70-3 in spermatocytes 4h after heat and reduced litter sizes in control females mated to heated males 23 to 28 days later consistent with an effect on spermatocytes. This study also used DNA microarrays to establish the upregulation of 27 and downregulation of 151 genes in testes 4h after heat shock (Rockett et al., 2001). There are other reports of hyperthermia affecting fertility in mice causing both reduced pregnancy rate and embryo weight as well as reduced fertilisation rate in
vitro, using sperm from heated males (Jannes et al., 1998). There are also several models of induced cryptorchidism showing similar increases in apoptosis and alterations in testicular architecture (Vigodner et al., 2003; Yin et al., 1997; Yin et al., 1998b).

### 1.4.2 Hypoxic stress

Hypoxia occurs when the oxygen tension drops below that required for normal cellular function in a particular tissue (Hockel and Vaupel, 2001). This may be caused by inadequate blood flow or reduced oxygen transport capacity. Hypoxia has been shown to lead to cell cycle arrest and apoptosis in endothelial cells (Iida et al., 2002), embryonic stem cells and mouse embryo fibroblasts (Carmeliet et al., 1998; Green et al., 2001). The hypoxia-inducible factor (Hif)-1 is a transcriptional regulator that is critical in the regulation of genes involved in the response to hypoxia. Hif1 is composed of the α subunit, the expression of which is oxygen dependent and the β subunit, which is constitutively expressed (Wang et al., 1995). The high proliferative and transcriptional activity of germ cells in the adult testis suggests that as an organ it would have high oxygen consumption and it has been reported that sperm functions such as capacitation and fertilisation can occur under anaerobic conditions (Fraser and Quinn, 1981). Although the influence of hypoxia on germ cells is not widely reported there are studies on the effects on the development of the testis: simulated hypoxic stress of postnatal rats resulted in a decrease in testosterone production at post natal day 21 and enlargement of the mitochondria in the Leydig cells (Liu and Du, 2002). Exposure of mice to hypobaric (hypoxic) conditions induces expression of Hif1α in spermatocytes and Sertoli cells (Marti et al., 2002). Following upregulation of Hif1 the activated protein binds to hypoxia response elements in genes involved in vasodilation, angiogenesis and glycolysis, which has the net effect of restoring oxygen homeostasis (Shweiki et al., 1992; Wenger, 2002). This, however, has mostly been studied in cell culture and there does not appear to be any studies of Hif1 activity/regulation in testicular tissue.
1.4.3 Oxidative stress

The production of free radicals or ROS such as the superoxide anion, hydrogen peroxide as well as nitric oxide, induce important changes in sperm function, including hyperactivation, capacitation and the acrosome reaction *in vitro* (reviewed in de Lamirande et al., 1997). However, induction of high levels of ROS within the testis can cause altered tissue physiology and oxidative damage to DNA. There have been several reports linking oxidative stress to male infertility (Aitken, 1995; Aitken et al., 1998a; Ong et al., 2002).

The testis expresses a number of antioxidants and these contribute to mechanisms that protect its cells from oxidative damage. These include superoxide dismutase (SOD), glutathione reductase (GR), peroxidase and heme oxygenase 1 (HO1) (Bauche et al., 1994; Gu and Hecht, 1996) and they all have the capacity to act as ROS scavengers. However, if the balance between ROS generation and scavenging is disrupted i.e. the amount of ROS exceeds that of the ROS scavenging capacity, testicular function may be disturbed. For example, treatment with pro-oxidants such as organic hydroperoxide caused DNA damage within the testis, the production of abnormal sperm and also reduced litter size consistent with a significant effect on male fertility (Rajesh Kumar et al., 2002). Furthermore, oxidative stress in humans caused for example by smoking has been shown to cause an increase in oxidation of sperm DNA and poor antioxidant levels in semen consistent with the potential relationship between oxidative damage and male reproductive dysfunction (Fraga et al., 1996; Saleh and Agarwal, 2002). Other studies have shown that this type of oxidative DNA damage can be passed onto offspring (Zenzes et al., 1999).

1.4.4 Apoptosis

Apoptosis is characterised by chromatin condensation, DNA fragmentation, release of nuclear proteins, cytoplasmic shrinking, cytoplasmic vacuolization, and the break up of the cell into membrane-bound remnants termed apoptotic bodies (Majno and Joris, 1995). Although this is not the only mechanism of cell death it is the principal
means by which germ cells die in the testis, presumably as this mechanism does not cause an inflammatory response which would inevitably have deleterious consequences on testis function. Necrosis is considered an un-regulated form of cell death and is induced by failure of physiological pathways involved in, for example, regulation of ion transport and pH balance. It is characterised by vacuolisation of the cytoplasm and inflammation around the dying cell (Proskuryakov et al., 2002). Apoptotic cells can be discriminated from necrotic cells, in addition to the characteristics mentioned above, by DNA fragmentation. This fragmentation occurs as a result of enzymatic cleavage of DNA at the nucleosomes and results in a characteristic ‘DNA ladder’ when genomic DNA is extracted from apoptotic cells and subjected to electrophoresis (Savitz and Rosenbaum, 1998). There are two major pathways of apoptosis in mammalian cells: intrinsic and extrinsic (Hengartner, 2000; Reed, 2000) (summarised in Figure 1-18). The intrinsic pathway involves the mitochondria and the release of cytochrome c into the cytoplasm where it binds apoptotic protease activating factor 1 (Apaf 1). This activates the initiator caspase-9 which subsequently activates the executioner caspases 3, 6 and 7 (Orrenius, 2004). The extrinsic pathway involves the ligation of the death receptor Fas to its ligand FasL, which recruits the Fas-associated death domain (FADD). This complex then binds to the initiator caspase-8 or 10 which then go on to activate the executioner caspases 3, 6 and 7 (Zimmermann et al., 2001). Thus both pathways have caspase-3 activation in common.
All of the aforementioned types of stress can induce cell death via apoptosis. During normal spermatogenesis apoptosis plays an important role in maintaining a balance between the numbers of germ cells and the numbers of Sertoli cells (de Franca et al., 1993). The apoptotic response to stress is usually mediated through the mitochondrial or intrinsic pathway. This is modulated by proteins from the Bcl2 family: Bcl2 itself is an inhibitor of apoptosis whereas Bax is proapoptotic (Adams and Cory, 1998; Borner, 2003). Previous studies have shown a redistribution of Bax following heat stress (King, 2003; Yamamoto et al., 2000) and it is likely to be this redistribution that induces apoptosis (Yamamoto et al., 2000). The transcription factor p53, which is highly expressed in the testis, mediates apoptosis in response to diverse stimuli including hypoxia, oxidative stress, and DNA damage (Fridman and Lowe, 2003). It has been shown that p53 can induce expression of Bax and downregulate that of Bcl2 (Miyashita et al., 1994). Previous studies have demonstrated that germ cell apoptosis can be induced by various conditions.
including heat stress, oxidative stress and hypoxia. For example, testicular heat stress has been shown to cause apoptosis of spermatogonia, pachytene spermatocytes and early spermatids (Hikim et al., 2003; Yin et al., 1997) although spermatogonia appear relatively resistant (Blanco-Rodriguez and Martinez-Garcia, 1998). In the rat, analysis of temporal and stage specific changes in apoptosis following heat stress demonstrated that the most susceptible cell types were pachytene spermatocytes and early spermatids at stages I to IV and pachytene, diplotene and dividing spermatocytes at stages XII to IIV and that these were undergoing apoptosis by 6h after heat stress (Yamamoto et al., 2000). Some studies suggest that the intrinsic pathway is induced after heat and that the Fas-FasL pathway is redundant (Hikim et al., 2003; Vera et al., 2004). However, others have suggested that the testis induces the Fas signalling pathway as the main pathway of apoptosis following heat stress (Miura et al., 2002). It has also been shown that transient heat shock induces the increased immunoexpression of Hsp105 in the testis (King, 2003) and that induced cryptorchidism causes HSP 105 to bind to p53 providing another potential mechanism for induction of apoptosis (Kumagai et al., 2000). Oxidative stress can be caused by the production of ROS during the reoxygenation of hypoxic tissue and also by hypoxia alone. For example, experiments in cardiac cells have shown that both hyopxia and reoxygenation induce an increase in apoptosis (Kang et al., 2000; Li et al., 1999). Oxidative stress, by way of ozone exposure has also been shown to result in the loss of round spermatids and spermatocytes in the rat and treatment with vitamin E but not C can rescue this phenotype (Jedlinska-Krakowska et al., 2006). Further studies in mice demonstrated that oxidative stress, in a model of ischemia-reperfusion, resulted in germ-cell-specific apoptosis and that this apoptosis occurred via the mitochondrial/intrinsic pathway (Lysiak et al., 2007). Hypoxia has also been shown to induce apoptosis (in jurkat cell lines) via the mitochondrial/intrinsic pathway and using cells lacking components of the extrinsic/Fas pathway (caspase-8 and FADD) it was demonstrated that this pathway (extrinsic) does not play a role in hypoxia-induced apoptosis (Weinmann et al., 2004). The cell cycle (mechanism of cell replication and proliferation, discussed in section 1.2.3) is directly linked to apoptosis via a number of cell cycle checkpoints at the G1/S, S and G2/M phases.
(MacLachlan et al., 1995). These checkpoints are a series of control systems that enable continued proliferation only if the conditions are favourable: DNA damage and chromosomal misalignment can activate these checkpoints (Weinert and Hartwell, 1989). The cell cycle is then paused for DNA repair but if the damage cannot be repaired then apoptosis of the cell is initiated (Waldman et al., 1996).

1.5 Male fertility

The fertility of an individual male is dependent on both his ability to produce mature sperm and also their capacity to fertilise an egg, which then develops normally to result in the generation of viable offspring. The dynamics of fertilisation and embryo development are discussed below (1.5.1, 1.5.2, 1.5.2.1).

1.5.1 Fertilisation in the mouse

Fertilisation is defined as the fusion of two gametes: one from the male (sperm) and one from the female (egg). Approximately 60 million sperm are released into the female reproductive tract during ejaculation in the mouse (Hogan et al., 1994). The meeting of the egg and the sperm takes place in the ampulla region of the oviduct where fertilisation takes place (Suarez, 1987; Suarez and Osman, 1987). The sperm, however, are not ready for fertilisation; they must first undergo a further maturation step known as capacitation, which involves a number of biochemical changes that prepare the sperm for the acrosome reaction (see below). Sperm acquire the ability to fertilise the egg through the process of capacitation as they migrate through the female reproductive tract. Capacitation consists of a number of processes including: the functional coupling of the signal transducing pathways that regulate the initiation of the acrosome reaction by zona pellucida glycoprotein 3 (ZP3); alterations in flagellar motility that may be required to penetrate the zona pellucida (ZP); development of the capacity to fuse with the egg. This is accompanied by changes in metabolism, membrane characteristics, protein phosphorylation and in pH and calcium levels. Firstly in this process there is a cholesterol efflux from the sperm
membrane, which initiates many aspects of the capacitation process (Visconti et al., 1998). Following this many sperm proteins are tyrosine phosphorylated via a cAMP dependent mechanism (Visconti et al., 2002). Elevations in pH, bicarbonate and calcium levels along with the associated cAMP production may activate cyclic nucleotide-gated channels present in the sperm flagella/tail and these are linked to tail movement and sperm motility (Wiesner et al., 1998) and may be involved in the hyperactivation of motility: a characteristic of capacitated sperm (Suarez et al., 1987) (Aoki et al., 1999). Finally, hyperpolarisation of membrane potential occurs, which involves the opening of calcium channels allowing sperm to participate in ZP3 signal transduction (Arnoult et al., 1999). After completing the aforementioned processes the sperm is now capable of fertilisation and binding of the egg ZP. This is initiated by one of the three ZP proteins. The ZP is composed of three major glycoproteins: ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980b). It is thought to be ZP3 that mediates both the initial binding of the sperm and the induction of the acrosome reaction (Bleil and Wassarman, 1980a) as its deletion leads to complete loss of a functional ZP and infertility (Liu et al., 1996a) and several high affinity binding proteins for ZP3 have been identified on sperm (Hardy and Garbers, 1994; Hardy and Garbers, 1995). The ZP3 signal transduction, to initiate the acrosome reaction, involves the opening of calcium channels (Arnoult et al., 1996a) resulting in a transient calcium influx, the activation of phospholipase C (Tomes et al., 1996), and an increase in intracellular pH (Arnoult et al., 1996b) culminating in a sustained calcium influx that directly drives exocytosis of the acrosome (Florman, 1994). This acrosome reaction is a necessary step to allow the fusion of the sperm with the egg plasma membrane (Florman et al., 1999). The fusion with the plasma membrane is thought to involve a series of proteins: sperm fertilin-α (ADAM1), fertilin-β (ADAM2), cyritestin (ADAM3) and CRISP1 (Cho et al., 1998; Cuasnicu et al., 2001; Evans, 1999; Nishimura et al., 2001). Integrins found on the egg surface are thought to be receptors for the sperm ADAMs (Evans, 2001; Zhu and Evans, 2002). The integrin-associated protein CD9 on the egg surface is also important for egg fusion (Le Naour et al., 2000; Miyado et al., 2000). Once fertilised the egg becomes activated and can initiate embryonic development (Runft et al., 2002). An early step
Chapter 1  

Literature Review

51

in this activation process is an increase in cytosolic calcium concentration, which occurs as a ‘wave’ that travels across the egg (Runft et al., 2002; Stricker, 1999). This increase in calcium concentration induces exit from meiotic arrest of the egg, progression into mitosis and also the exocytosis of the egg’s cortical granules, which modify the ZP to prevent fertilisation by more sperm (Schultz and Kopf, 1995).

Many changes in the sperm head components occur upon exposure to the egg cytoplasm. As described earlier (section 1.2.4.4), the sperm chromatin is packaged into a highly condensed structure with protamines in place of histones. In addition to this the sperm nucleus is surrounded by a structure called the perinuclear theca, which is composed of a number of different proteins (Bellve et al., 1992). Soon after the sperm and egg fuse the sperm nuclear envelope breaks down and the perinuclear theca is removed from around the chromatin (Sutovsky et al., 1996; Sutovsky et al., 1997). The chromatin then undergoes decondensation beginning in the posterior region of the sperm head and progressing to the anterior region (Terada et al., 2000). This process requires the breakdown of disulphide bonds, which is accomplished by glutathione in the egg cytoplasm (Perreault et al., 1988) and during this the sperm protamines are replaced by egg-derived histones (Perreault, 1990). The sperm tail also enters the egg at fertilisation and the mitochondria dissociate from the midpiece of the tail (Sutovsky et al., 1996) but sperm mitochondria do not persist past the primiplication stages and only maternal mitochondria are inherited (Cummins et al., 1997).

In the ovary limited numbers of eggs enter meiosis, organise their meiotic spindle and extrude the first polar body leading to the production of mature fertilisable eggs (Voronina and Wessel, 2003). Prior to fertilisation the meiotic cell cycle of the egg is arrested at metaphase II due to the presence of active maturation promoting factor (MPF) (Verlhac et al., 1993), which is a cell cycle modulator responsible for inducing spindle assembly, chromatin condensation and nuclear envelope breakdown (Murray and Hunt, 1993). Soon after the fusion of the sperm and egg MPF is inactivated allowing the female chromatin to enter anaphase II (Murray and Hunt,
Several hours after fertilisation the calcium oscillations described above cease and the male and female pronuclei form, a process, which has been shown to involve tr-kit (a truncated form of the C-kit tyrosine kinase), which is also involved in the release from metaphase arrest (Sette et al., 1997). A characteristic decrease in MAP kinase activity is also thought to be involved in pronucleus formation (Moos et al., 1995). For example, mouse eggs arrested in metaphase II have high levels of MAP and cdc2/cyclinB1 protein kinases and following fertilisation, the resumption of meiosis and extrusion of the second polar body appear to be cdc2/cyclinB1 dependent and the formation of the male and female pronuclei appear to be MAP kinase dependent (Moos et al., 1995; Moos et al., 1996). The pronuclei move towards the centre of the egg and at this time DNA replication takes place over a period of about 24h during which the parental chromosomes remain separated but within the same cytoplasmic environment (Thibault, 1996). This replication is followed by nuclear envelope breakdown, which has also been shown to be dependent on the mobilisation of, and transient rises in, calcium (Kao et al., 1990) (Kono et al., 1996). The final phase of fertilisation, syngamy, involves the combination of the two haploid chromosomes, which until now have remained separate, into a single diploid nucleus. Soon after this the spindle forms, mitosis begins and proceeds through metaphase, anaphase, telophase, and cleavage of the 1-cell zygote to form the 2-cell conceptus (Johnson and Everitt, 1995).

1.5.2 Normal embryo development

After fertilisation the embryo rapidly divides but without an increase in overall size resulting in the formation of numerous smaller nucleated cells called blastomeres. Each stage of division is characterised by the doubling of the number of blastomeres although because the blastomeres do not always divide in synchrony embryos frequently contain odd numbers of cells. In spite of this, embryo cleavage stages are defined as the 2-cell, 4-cell and the 8-cell stage and throughout these stages the blastomeres form a loose arrangement with plenty of space between them. However, after the 8-cell stage the blastomeres undergo a characteristic change in their
association with each other known as compaction when all the cells cluster together
to form a compact ball of cells (Fleming et al., 2001). It is thought that compaction
is mediated by E-cadherin since it’s disruption results in failure of this process
(Riethmacher et al., 1995). The compact arrangement of blastomeres is stabilised by
tight junctions that form between the outside cells of the ball, sealing off the inside.
After compaction, the next round of cell divisions tends to occur along the apical-
basal axis of the blastomeres, resulting in the formation of a 16-cell morula
consisting of small inner cells enclosed within larger outer cells (Barlow et al.,
1972). Most of the outer cells are then epithelialized and become trophoderm
(TE), whereas the inner cells go on to generate the inner cell mass (ICM) in
blastocysts (Fleming, 1987). Analysis of cell fate revealed that the ICM gives rise to
all of the embryonic cells and the extraembryonic endoderm, whereas TE forms the
embryonic portion of the placenta (Pedersen et al., 1986). By the 64-cell stage the
two groups of cells (ICM and trophoderm) are two distinct separate layers
(Fleming, 1987). During trophoderm differentiation tight junctions (TJ) are
formed and are characterised by the sequential expression and membrane assembly
of the ZO-1α- isoform at the 8-cell stage (Sheth et al., 1997), cingulin at the 16-cell
stage (Fleming et al., 1993; Javed et al., 1993) and the ZO-1α+ isoform and
occluding junctions around the 32-cell stage (Sheth et al., 1997; Sheth et al., 2000).
The TJ seal between adjacent TE cells is required for epithelial integrity and the
successful generation of the blastocoelic cavity by transepithelial ion transport
processes mediated by the TE. It was originally suggested that the trophoblast
secreted fluid into the morula to create a blastocoelic cavity (Wiley, 1984) and a
more recent study demonstrated that protein kinase C (PKC) contributes to the
regulation of embryo cavitation via target proteins including Na+/K+ ATPase
(Eckert et al., 2004). This cavitation process results in the characteristic blastocyst
stage embryo consisting of a ball of trophoderm cells surrounding the ICM. These
cleavages occur as the cilia of the oviduct push the embryo, which is still enclosed in
the ZP towards the uterus. Once the embryo reaches the uterus it must hatch from the
ZP in order to adhere to the wall and begin the process of implantation. The initiation
of implantation in the mouse involves the attachment of the blastocyst to the uterus,
the initiation of contact between the blastocyst trophectoderm and the luminal epithelium and occurs between midnight of day 4 and early morning of day 5 of pregnancy (Das et al., 1994). Further studies have demonstrated that heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) may be involved in mediating the adhesion of the blastocyst to the uterus during the process of implantation (Raab et al., 1996). In mice following implantation the embryo develops further as the fetus until birth at day 21. The development and implantation of a mouse embryo is summarised in (Figure 1-19).

![Figure 1-19](image_url)

**Figure 1-19 Embryo development in the mouse.** A schematic diagram of embryo development from fertilisation to implantation taking around 4.5 days in the mouse. Reproduced from www.stemcells.nih.gov/info/scireport.

### 1.5.2.1 Embryonic gene expression and paternal genome reactivation

As detailed above, fertilisation triggers the completion of meiosis in the egg and the formation of a 1-cell embryo, which contains a set of paternal and a set of maternal chromosomes. The initiation of meiotic maturation triggers the degradation of
maternal transcripts, which is 90% complete by the two-cell stage (Nothias et al., 1995). This marks a switch to transcription from the newly formed embryonic genome, known as embryonic genome activation (EGA) although protein synthesis from some maternal mRNA transcripts continues into the 8-cell stage. The EGA occurs in two phases: a minor activation before cleavage and a major activation at the two-cell stage (Hamatani et al., 2004). The minor EGA occurs in the male pronucleus (Aoki et al., 1997) but this level of transcription is low and results in the synthesis of a small set of polypeptides that transiently increase at the 2-cell stage (Latham et al., 1991). The major EGA, however, promotes a dramatic reprogramming of gene expression pattern along with the generation of novel transcripts (reviewed in Kanka, 2003). Studies investigating gene expression in the preimplantation embryo have demonstrated a wave of reprogramming at the 4- to 8-cell stage (Hamatani et al., 2004; Wang et al., 2004). Further studies have shown specific changes in gene expression related to the stage of embryo development. These studies have identified an upregulation of genes for cytoskeletal, cell adhesion and junction proteins, for example Cadherin 1 and Claudin 7, were identified in the morula stage compared to 4-cell stage embryos. In addition they also demonstrated genes involved in ion channels, membrane traffic and lipid metabolism were higher in blastocysts compared to morulae (Cui et al., 2007). Other studies have shown that paternal gene expression starts as early as the 1-cell fertilised embryo, however paternally derived protein is not synthesised until the 2-cell stage (Matsumoto et al., 1994). While reports on the activation of paternal genes describe active transcripts as early as the 1-cell stage, others suggest that the paternal genome is not required before the 8-cell stage in mice (Renard et al., 1991). It is well known that there is a paternal effect on the development and survival of the embryo. For example studies looking at the effects of irradiation-induced DNA damage in sperm on bovine embryonic development demonstrated normal fertilisation but embryo arrest before blastocyst formation (Fatehi et al., 2006). The paternal genome is also implicated in the development of extraembryonic tissues, the trophoblast and the general growth of the embryo: one of the first studies on imprinting showed that embryos with two
female pronuclei can implant but undergo resorption and exhibit poor extraembryonic membrane and trophoblast formation (Surani et al., 1984).

Figure 1-20 Gene expression in embryo development diagram showing maternal to zygotic gene transition in the mouse. Maternal events are indicated in red, paternal in blue and zygotic in green. Open bars apply to both parents. The ICM is highlighted in yellow, and trophectoderm cells in orange. Periods of transcription are highlighted with hatched bars. Reproduced from www.depamphilislab.nichd.nih.gov/gene_fig1.jpg

1.5.2.1.1 DNA repair in the preimplantation embryo

As mentioned earlier in this chapter, DNA repair plays a crucial role in the maintenance of genome integrity and embryonic development relies upon the
genomic integrity of the gametes that form the embryo. However it is inevitable that some mutations or damaged DNA are passed on to the newly fertilised embryo and also with the large amount of DNA synthesis and cell division taking place it is important that these embryos have in place DNA repair mechanisms to overcome this. Repair in the early preimplantation embryo is thought to rely entirely on maternal transcripts and proteins translated in the oocyte prior to ovulation (Baumann et al., 2007; Gurtu et al., 2002; Vinson and Hales, 2002).

There is evidence that fertilisation with sperm exposed to a DNA damaging agents can alter the expression of repair genes in the preimplantation embryo as early as the 1-cell stage (Harrouk et al., 2000). Repair proteins involved in MMR, BER, NER and DSB repair have been shown to be expressed at specific stages throughout embryo development. However, not all proteins for each pathway are expressed and considering most repair pathways require the action of protein complexes this may limit the embryos ability of repairing damage (reviewed in Jaroudi and SenGupta, 2007). Previous studies have also shown that paternal DNA damage can be translated into chromosome aberrations at the first-cleavage metaphase stage in the zygote (Matsuda et al., 1989). Paternal transmission of DNA damage was discussed earlier in this chapter (section 1.1.1).
1.6 General aims of this thesis

The main hypotheses of this thesis are that any alterations to DNA integrity either by deletion of genes involved in DNA repair or by exacerbation of DNA damage using a mild environmental (heat) stress will result in abnormalities in germ cell development (spermatogenesis) and possibly result in the production of sperm with DNA damage which will inevitably cause problems with fertility.

The overall aim of the studies described in this thesis was to further characterise the relationship between DNA damage, repair and infertility in the male. The studies employed a number of mouse models and the main aims were:

- To characterise testicular function in three contrasting mouse models with impaired DNA integrity.
  - The three specific mouse models used have undergone targeted disruption of *Ercc1*, *Msh2* or *p53*. (*Ercc1* and *Msh2* are involved in DNA repair and *p53* is a cell cycle checkpoint gene, which has been implicated in the elimination of germ cells with DNA damage). The effects of these genetic defects on testicular function were evaluated both by investigating testicular germ cells (germ cell death and DNA integrity) and the sperm that are produced (numbers, morphology and DNA integrity).
- To characterise the effects of increased scrotal temperature on testicular function (using the same parameters as above) and fertility using natural matings and IVF.
- To determine the mechanisms of heat induced DNA damage by examining the stress response in the testis including hypoxia, oxidative stress and apoptosis related pathways.


2 General Materials and Methods

2.1 Animals

All animals used in these studies were maintained and treated in accordance with the Animals Scientific Procedures Act, 1986. All procedures were carried out according to Home Office regulations under project licence 60/3544 held by Professor Philippa Saunders.

2.1.1 Ercc1 mice

Ercc1 (excision repair cross-complementing gene) is essential for the NER pathway, where it acts in a complex with Xpf (Ercc4), and also in HR, DSB repair and repair of interstrand crosslinks (Hsia et al., 2003; Schiestl and Prakash, 1990). The key role of Ercc1 (and Xpf in complex) in both the NER pathway and the HR pathway in meiosis is the ability to cleave single-stranded 3’ tails projecting from DNA duplexes.

Ercc1 knockout mice were generated by the insertion of a neomycin cassette into exon 5 of the gene (McWhir et al., 1993). These mice have lost their NER ability and mitotic recombination is impaired. The Ercc1 (-/-) mice die before the first wave of spermatogenesis due to liver failure. To allow studies on the role of Ercc1 in other organs the liver phenotype has been corrected by introduction of an Ercc1 transgene under the control of a liver-specific promoter (transthyretin) (Selfridge et al., 2001). The mice used in this study were on a segregating BALB/c X 129Ola X C57BL/6 background and provided by Professor David Melton, Molecular Medicine Centre, University of Edinburgh.

2.1.2 Msh2 mice

Msh2 (Mut S homolog2) is involved in the mismatch repair pathway. Msh2 recognises and binds mismatched DNA and distinguishes between the template and the new strand. It recruits Mlh1 (Mut L homolog 1), which nicks the new strand and an exonuclease removes the mismatch. The production of Msh2-/- mice has been
described previously by de Wind et al. (1995), which involved the insertion of a hygromycin resistance gene between codons 588 and 589 of one allele of \(Msh2\) to disrupt the gene sufficiently that no \(Msh2\) transcript was detected by southern blotting. These mice were on a C57BL/6 background and were provided by Professor David Melton, Molecular Medicine Centre, University of Edinburgh.

### 2.1.3 \(p53\) mice

The \(p53\) tumor suppressor protein is highly expressed in the testis and is involved in mitotic checkpoints (reviewed in Hartwell, 1992; Stewart et al., 1995) and maintenance of genomic stability during mitotic proliferation (Fukasawa et al., 1997). The \(p53\) knockout mice were maintained as heterozygotes and produced by insertion of a \(pgk\) (phosphoglycerate kinase promoter)-neomycin cassette to disable the gene as described by Clarke et al. (1993) and were on a CBA/Ca background. The animals were provided by Professor David Melton, Molecular Medicine Centre, University of Edinburgh.

### 2.1.4 Animal accommodation

All mice were bred and maintained at University of Edinburgh Animal Facilities (George Square, BRF (Little France) or Western General hospital) and were kept under standard conditions of 12 hour (h) light/12h dark cycle, in an ambient temperature of 20-25°C and humidity kept at 55%. Male mice were housed in groups of up to five or singly housed following experimental procedures. Female mice were housed in groups of up to six. Animals had free access to food and water.

### 2.1.5 Sacrifice of animals

Adult animals were killed by inhalation of \(CO_2\) followed by cervical dislocation and fetuses by decapitation in accordance with Schedule one of the Home Office regulations.
2.1.6 Tissue collection

Tissue from adult male mice was collected using dissection scissors by carefully cutting the skin directly under the penis, and opening the body cavity. The testes, epididymides and vas deferens were located and pulled from the scrotal sac by the fat pad. The vas deferens was pulled taught and cut midway down to remove the gonads and reproductive tracts from the animal.

Fetuses were removed from pregnant female mice by opening the abdomen and dissecting the uterine horns including ovaries and oviducts into Phosphate Buffered Saline (PBS, see section 2.12.4) using fine dissection scissors. Each fetus was cut from the uterus in its own fetal amniotic sac. The amniotic sac was cut open and the fetus detached from the placenta before decapitation.

2.2 Treatments

2.2.1 Anaesthetic

Animals were anaesthetised with intraperitoneal injections of Vetalar® (Pharmacia and Upjohn, Corby, UK) and Domitor (Pfizer, Kent, UK). Both were diluted in hospital grade sterile water (IVEX Pharmaceuticals, County Antrim, NI) at 1:6.5 and 1:5 respectively. Following treatment the animals were administered Antisedan® (Pharmacia and Upjohn); an anaesthetic reversal agent diluted 1:10 in hospital grade sterile water. The dose of anaesthetic and reversal agent given was determined by body weight (Table 2-1).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vetalar®</td>
<td>60</td>
</tr>
<tr>
<td>Domitor®</td>
<td>1</td>
</tr>
<tr>
<td>Antisedan®</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2-1 Summary of anaesthetic and reversal agent dose administered to mice
2.2.2 Testicular heating method

A water bath (Clifton; VWR, Lutterworth, UK) was filled with fresh tap water, heated to the required temperature and checked regularly using a thermometer. Following anaesthesia, the lower part of the body (hind legs, tail and scrotum) of each animal was passed through a hole in a polystyrene raft (Figure 2.1). The animals were secured using a rubber band and 2 needles (Figure 2.1). The raft was placed gently in the water bath at 38°C, 40°C or 42°C and timed for 30 min. The animals were removed from the raft, dried with a paper towel and given the appropriate dose of Antisedan®. The animals were returned to their cages, which were placed on a warm mat to facilitate recovery. Control mice were given a dose of anaesthetic and left at room temperature for 30 min before being given the reversal agent. All mice were housed singly after this procedure.

Figure 2.1 Testicular heating experiment. Anaesthetised mice secured on polystyrene raft with lower third of body submerged in water.
2.2.2.1 Time points

For each time point a minimum of three mice per group were studied. Although at least four mice were heated or kept as controls a small number of animals died during the recovery period a number of days following the experiment and the majority of deaths occurred in the control group. These animals appeared to be very cold compared with non-experimental animals suggesting that they did not recover to normal body temperature following anaesthetic treatment despite being kept on a warm mat for 24h after. Animals were sacrificed at various time points after heating: 3h, 6h, 24h, 48h, 7 days, 14 days and 28 days (40°C and 42°C). At each time point one testis was removed and fixed in Bouins (2.3.1), one removed and halved with one half frozen on dry ice and the other placed in PBS for germ cell extraction (2.6.1). The epididymides were used for sperm retrieval (2.5).

2.3 Tissue fixation and processing

2.3.1 Tissue fixation

Testes from adult male mice were fixed in Bouin’s solution (Triangle Biomedical Sciences, Lancashire, UK) for 8h, cut in half and then transferred to 70% ethanol before being embedded in paraffin wax. Fetuses were fixed in 4% paraformaldehyde (PFA) for 24h at 4°C before being transferred to 70% ethanol. Fixed fetuses were stored in ethanol at 4°C until use. Ovaries were fixed in Bouins for 4h and transferred to ethanol as above.

2.3.2 Tissue processing and paraffin embedding

Using an automated Leica TP-1050 processor (Leica Microsystems, Milton Keynes, UK) the tissue was processed and dehydrated through a series of graded alcohols on a 17.5h cycle. Processed tissue was saturated and embedded by hand in liquid paraffin wax and cooled. Wax blocks were stored at room temperature prior to use.
2.3.3 Sectioning of paraffin blocks

5µm paraffin sections were cut from chilled wax blocks using a hand-operated microtome (RM2135; Leica). Sections were floated in a water bath (Lamb RA, model E/65) at 45°C ± 5°C. Sections were mounted on positively charged glass slides (BDH, Lutterworth, UK) and stacked in a metal rack before being dried overnight in an oven (Lamb RA, model E28.5) at 55°C.

2.4 Tissue staining and immunohistochemistry

2.4.1 Haematoxylin and eosin staining

The tissue sections were dewaxed by washing 2 x 5 min in xylene (Triangle Biomedical Sciences). Sections were rehydrated by passing through a series of graded alcohols (100%, 95%, and 75% (v/v)) before being washed in tap water. The slides were immersed in Harris’s haematoxylin (Triangle Biomedical Sciences) for 4-5 min, which stains cell nuclei blue. The slides were rinsed in tap water before being briefly (2-3 sec) immersed in 1% acid alcohol to remove non-specific cytoplasmic staining and rinsed in tap water. Slides were immersed in Scott’s tap water or Blueing solution for 30 sec to allow the blue colour to develop. Adequate colour was checked using a light microscope. Slides were then immersed in a bath of eosin Y (1% aqueous solution mixed with 1% alcohol solution at a ratio of 3:1 respectively; Triangle Biomedical Sciences) for 30 sec to stain the cytoplasm pink and rinsed in tap water. Sections were subsequently dehydrated in a series of alcohols from 75% to absolute ethanol (see above). Finally the slides were cleared in xylene (2 x 5 min) and coverslips (VWR) mounted using Pertex (Cell Path, Hemel Hempstead, UK). Slides were air dried and stored in cardboard slide trays before microscope analysis.

2.4.2 Immunohistochemistry for paraffin embedded tissue

Immunohistochemistry was carried out according to standard protocols to detect the presence and/or location of specific proteins in the tissue mentioned previously.
Sections of tissue (2.3.3) were dewaxed and rehydrated in graded alcohols (2.4.1) before being washed in water.

2.4.2.1 Antigen retrieval

Some antibodies require a temperature-induced antigen retrieval step (Norton et al. 1994) to break the cross-links formed during fixation, which would otherwise mask antigenic sites, thereby enabling the recognition of the corresponding antigen. This was achieved by pressure-cooking in 0.01M citrate buffer, pH 6.0 for 5 min at full pressure, after which sections were left to stand undisturbed for 20 min. Sections were then cooled under running tap water.

2.4.2.2 Blocking non specific sites

Non-specific binding of antibodies or detection reagents was blocked deliberately prior to the addition of the primary antibodies. Following the rehydration of sections or in some cases antigen retrieval the slides were washed in Tris-buffered saline (TBS; 0.05M Tris-HCl, pH 7.4, 0.85% NaCl) for 5 min. Endogenous peroxidase activity, which would otherwise interfere with horseradish peroxidase amplification (2.4.2.4), was blocked with 3% hydrogen peroxide in methanol (both BDH laboratory supplies) for 30 min at room temperature. Slides were rinsed in tap water followed by 5 min in TBS.

Non-specific binding of the secondary antibody was prevented by incubation with the appropriate non-immune block. This consisted of a solution containing serum from the species in which the secondary antibody was raised. For example, if the secondary antibody was raised in goat the slides were incubated in normal goat serum (NGS; Autogen Bioclear UK Ltd, Wiltshire, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Sigma). Slides were removed from TBS wash and dried carefully around the section using tissue paper. Blocking serum was added to each section for 30 min during which slides were kept in a humidified chamber at room temperature.
2.4.2.3 Primary antibodies

The blocking buffer was removed by tapping the slides on tissue paper and replaced with the primary antibody diluted in the appropriate blocking serum. The slides were incubated overnight in a humidified chamber at 4°C. Table 2-2 summarises antibodies and the conditions used for each. Negative controls were included in each run where the primary antibody was replaced with the non-immune block. The slides were subsequently washed in TBS (2 x 5 min). To further validate the immunostaining specificity, it would be of use to include pre-absorbed antibody controls, however this was not performed here due to lack of available peptides except for MVH though this was not used due to specific germ cell staining.

**Table 2-2 Summary of primary antibodies used for immunohistochemistry**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
<th>Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved caspase-3</td>
<td>Rabbit IgG</td>
<td>CellSignalling</td>
<td>1:200</td>
<td>Citrate</td>
</tr>
<tr>
<td>Hif 1 alpha</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:200</td>
<td>Citrate</td>
</tr>
<tr>
<td>Stip1</td>
<td>Mouse</td>
<td>BDH Biosciences</td>
<td>1:100</td>
<td>Citrate</td>
</tr>
<tr>
<td>MVH</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:500</td>
<td>Citrate</td>
</tr>
<tr>
<td>P63</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>1:500</td>
<td>Citrate</td>
</tr>
</tbody>
</table>

2.4.2.4 Secondary antibodies

The sections were incubated with the appropriate biotinylated secondary antibody. The secondary antibodies used were raised against the host species of the primary antibodies. Table 2-3 summarises antibodies used and their conditions.

**Table 2-3 Summary of secondary antibodies used in Immunohistochemistry**

<table>
<thead>
<tr>
<th>Host species</th>
<th>Target species</th>
<th>Source</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Rabbit</td>
<td>DAKO</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Goat</td>
<td>Vector</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat</td>
<td>Mouse</td>
<td>DAKO</td>
<td>1:500</td>
</tr>
</tbody>
</table>
Following 2 x 5 min washes in TBS all sections were incubated with avidin-biotin-horseradish peroxidase (ABC-HRP; DAKO) diluted in 0.05M Tis-HCl (pH 7.4) for 30 min followed by 2 x 5 min washes in TBS. Peroxidase-labelled avidin has a high affinity for the biotin component of the secondary antibody thereby giving highly specific antibody localisation Figure 2.2.

![Figure 2.2 Schematic diagram of the immunohistochemistry protocol. The primary antibody recognises specific antigens on the protein of interest and binds. A biotin-labelled secondary antibody binds to species-specific sites on the primary and an avidin biotin complex (ABC) associates with the conjugated biotin. The oxidation of DAB by the peroxidase component of ABC-HRP produces the characteristic brown colour.](image)

### 2.4.2.5 Antigen detection and counterstaining

Sections were incubated with liquid 3,3’-diaminobenzidine (DAB)$^+$ substrate chromogen system (DAKO) for 1-5 min. This produces an insoluble, stable dark brown end product at sites of antibody localisation. This is due to DAB being an electron donor that oxidises in the presence of peroxidase in order for a colour change to take place. The colour reaction was stopped by immersion in water.

Sections were counterstained by immersion in Harris’s haematoxylin to stain the cell nuclei blue. Slides were rinsed in tap water and subsequently dehydrated in graded alcohols and cleared in two 5 min washes of xylene (2.4.1). Slides were then mounted with glass coverslips (VWR) using pertex (Cell Path).
Immunostained sections were photographed using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a digital camera (Canon DS6031; Canon Europe, Amsterdam). Captured images were then transferred to a PC and compiled using Adobe Photoshop 7.0 (Adobe Systems Inc, CA, USA).

2.4.3 Automated immunostaining

An automated Bond-X immunostaining machine (Vision Biosystems, Newcastle, UK) was also used for immunodetection of proteins on sections. This machine allowed a high throughput of slides (up to 30 per run) in a reproducible manner. The conditions for each individual antibody were optimised using titre runs. All reagents used were provided as kits purchased from Vision Biosystems. As before, sections were dewaxed and rehydrated (2.4.1) followed by antigen retrieval (2.4.2.1) if required. The machine uses a specific polymer programme, which applies the same principles as described above (2.4.2). Slides were blocked with peroxidase (5 min) and incubated (2h) with primary antibody diluted in antibody diluent (Vision Biosystems) then incubated with the post primary reagent for 15 min followed by polymer reagent also for 15 min to increase the detection sensitivity of DAB (incubated for 10 min). Finally the machine counterstains the slides with haematoxylin followed by dehydrating and mounting by hand (2.4.1).

2.4.4 TUNEL

Sections were dewaxed in xylene and rehydrated through a series of graded alcohols before being blocked with 3% hydrogen peroxide in methanol (2.4.2). Slides were washed 2 x 5 min in PBS (0.01M/pH7.4) and dried carefully around the section using tissue paper. Slides were placed on an ice-cold metal tray and 50µl of reaction mix (30mM HCl, 140mM Na Cacodylate, 1.5mM CoCl) containing 1µl/ml terminal d-transferase (TdT; Roche, Lewes, UK) and 5µl/ml digoxigenin-11-dUTP (Roche) was added to each slide. Sections were sealed under coverslips with cow gum/hexane and incubated at 37°C for 30 min. The coverslips were removed and following two subsequent washes in PBS the sections were blocked in 20% NRS in PBS for 10 min.
at room temperature. The block was replaced with sheep anti-Dig antibody (1:100 in NRS/PBS) and slides incubated in a humidified chamber for 90 min at room temperature. Following two 5 min washes in PBS, biotinylated rabbit anti-sheep IgG (1:500 in TBS) was added to the sections and incubated at room temperature for 30 min. The ABC-HRP/DAB+ chromogen detection method was used as in section 2.4.2.4 and 2.4.2.5. The slides were counterstained with Harris’s haematoxylin and dehydrated prior to mounting coverslips in pertex.

### 2.4.5 Counting TUNEL positive cells

In order to determine the extent of apoptosis in the testis, counts of TUNEL-positive cells were made. This was achieved by counting the total number of positive cells in 100 tubule cross sections per animal.

### 2.5 Spermatozoa preparation

#### 2.5.1 Biggers, Whitten and Whittingham solution

Biggers, Whitten and Whittingham solution (BWW) was prepared fresh before each experiment and kept at 4°C. The solution was made to the following recipe; 90mM NaCl, 4.5mM KCl, 1.6mM CaCl$_2$, 1.1mM KH$_2$PO$_4$, 1.1mM MgSO$_4$.7H$_2$O, 25mM NaHCO$_3$ (all BDH), 5.6mM glucose (Sigma), 55µM sodium pyruvate (Sigma), 0.2% sodium lactate (Sigma), 20000 IU penicillin/streptomycin (Calbiochem, UK), 20mM Hepes buffer (Gibco), 0.3% human serum albumin (Baxter Healthcare, Norfolk, UK). The solution was corrected to pH 7.6.

#### 2.5.2 Preparation of Murine spermatozoa

Epididymides were carefully dissected out into 500µl BWW using iridectomy scissors after removing any adherent fat. The epididymides were minced using iridectomy scissors until a cloudy suspension had formed. Cell suspensions were heated for 30 min at 30°C to allow viable sperm to swim up. The ‘swim-up’ sperm were carefully removed from the top, leaving dead sperm and tissue behind, put in a
Chapter 2  General Materials and Methods

fresh tube and made up to 1ml with BWW. Before use the aliquots were defrosted at room temperature and inverted to redistribute spermatozoa through the sample.

2.5.3 Sperm Counts

For sperm counts a 100µl aliquot was diluted (1:10) in 4% paraformaldehyde (PFA). 10µl of diluted sample was placed onto the chamber of an improved Neubauer hemocytometer (BDH). The total number of sperm located within 4 subdivided squares of the hemocytometer grid was counted. Two counts were made for each sample and the mean taken. From this the sperm concentration was determined according to the following equation:

\[
\text{Concentration} = \frac{\text{no. sperm counted}}{4 \times 10^3 \times 10} \text{ (dilution factor)}
\]

2.5.4 Morphology of sperm

Sperm samples (2.5.2) were diluted 1:10 with ‘sperm diluting fluid’ (SDF; 50g NaHCO₃, 10 ml Formalin, dH₂O to 1L). For each sample 2 microscope slides were prepared by washing in 100% ethanol. 10µl of sample was pipetted onto the end of the first slide and smeared across the surface with the other slide. The smear was allowed to air-dry and repeated with the same slides 3 times. The slides were fixed in ether/alcohol fixative (30% ether/70% ethanol) for 30 min.

The slides were stained using a Reastain Quick-Diff staining kit (Oy Reagena Ltd, Toivala, Finland) according to the manufacturers instructions. Briefly, each slide was dipped into Quick-Diff fixative 10 times and excess liquid drained using absorbent tissue paper. The slide was then dipped into Quick-diff I solution 20 times and drained of excess liquid before being dipped 20 times into Quick-diff II solution. The slide was drained as before and rinsed in tap water. Stained slides were allowed to air dry before mounting coverslips using Pertex.
2.5.5 Spermatozoa Chromatin Structure Assay (SCSA)

A modified version of the SCSA method described by Evenson et al. (1999) was used. The assay measures altered chromatin structure by the susceptibility of spermatozoa DNA to acid denaturation using the metachromatic dye acridine orange (AO). The dye fluoresces green when bound to double stranded DNA and red when bound to single stranded DNA. The sperm were adjusted to a concentration of 1-2 x 10^6 cells/ml with TNE (0.15M NaCl, 0.1M Tris, 1mM EDTA pH 7.4). 100µl of this sperm was mixed with 200µl acid detergent solution (0.1% TritonX-100, 0.15M NaCl, 0.08M HCl). After 30 sec, 600µl AO (Sigma) diluted in AO staining solution (37mM citric acid, 126mM Na_2HPO_4, 1mM EDTA, 0.15M NaCl, pH 7.4 with AO added fresh to a final concentration of 6µg/ml) was added to the spermatozoa mixed and left for 3 min at room temperature before analysing using a fluorescent assisted cell sorting (FACS) machine.

2.5.5.1 FACS analysis

The spermatozoa samples were analysed on a Coulter Epics XL Flow Cytometer (Beckman Coulter Ltd. High Wycombe, Bucks, UK) with a 480nm excitation laser. Fluorescence was detected using a green bandpass filter (530nm ± 15) and a red longpass filter (>650nm). The cytometer was calibrated for each run by adjusting the wild type control samples to give a mean fluorescence value (arbitrary units) of 145 ± 10 at 675nm and 445 ± 10 at 525nm. 10,000 events (sperm cells) were read for each sample. Raw data was analysed using Flowjo Software (Tree Star Inc., Ashland, Oregon, USA). Background contamination (cells other than spermatozoa) was removed by gating. The extent of DNA denaturation was determined by calculating the DNA fragmentation index (DFI), which represents the shift from green fluorescence to red fluorescence and is based on the ratio of denatured spermatozoa DNA (red) to total spermatozoa DNA (red/[red+green] fluorescence), expressed as percentage.
2.6 Meiotic spreads

2.6.1 Germ cell extraction from murine testes

Testes were dissected as described in section 2.1.6 into PBS. The testes were then moved into 200µl RPMI media (Sigma) warmed to 32°C where the tunica albicans was removed using forceps and discarded along with any large blood vessels. The remaining tubules were finely chopped using two scalpel blades to form a milky suspension. This was diluted to a final volume of 3ml RPMI. The resulting cell suspension was transferred to a 15ml falcon tube where the tubular remnants could settle to the bottom. The non-remnant fraction was transferred to a fresh 15ml falcon tube and centrifuged at 1000rpm for 5 min. The supernatant was discarded and the pellet containing germ cells was resuspended by gentle flicking in 2ml warm RPMI.

Glass slides (BDH) previously boiled in dH2O and air-dried were coated in 5 drops of 4.5% sucrose solution using a Pasteur pipette. A glass pipette was filled with the cell suspension and one drop was dropped onto the slides from 20-30cm above. Two drops of 0.05% TritonX-100 (in dH2O) were added to each slide and incubated at room temperature for 10 min. This was followed by 8 drops of fixative (2% formaldehyde, 0.02% SDS, pH 8.0) per slide and incubation in a humidified chamber for 1h. The slides were dipped briefly six times in dH2O and allowed to air dry for 5 min before storing at -70°C until use.

2.6.1.1 Immunostaining of meiotic spreads

Slides were defrosted by washing in PBS for 5 min. The slides were blocked with a non-immune block to prevent non-specific antibody binding. The blocking solution consisted of 5% goat serum, 0.15% BSA and 0.1% Tween-20. The slides were incubated in block for 1h at room temperature. Slides were subsequently dabbed on tissue paper to remove any excess liquid and the primary antibody added at the appropriate concentration (Table 2-4). The slides were incubated overnight in a humidified chamber at 4°C. The slides were washed 3 x 5 min in PBS and the secondary antibody added at the appropriate concentration (Table 2-5) and incubated
for 1h at room temperature. The slides were washed again 3 x 5 min in PBS but kept in darkness so as not to affect the fluorescence of the secondary antibody. The slides were subsequently incubated with DAPI nuclear stain (Sigma) for 10 min at a concentration of 1:1000 in PBS before being washed 2x 5min in PBS. Finally the slides were mounted in Permafluor aqueous mounting medium (Beckman Coulter).

Table 2-4 Summary of primary antibodies used in meiotic spreads

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCP3</td>
<td>Mouse mAB</td>
<td>Abcam</td>
<td>1:400</td>
</tr>
<tr>
<td>γH2AX</td>
<td>Rabbit IgG</td>
<td>Upstate</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2-5 Summary of secondary antibodies used in meiotic spreads

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Target species</th>
<th>Label</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Rabbit</td>
<td>Alexafluor 488</td>
<td>Molecular Probes</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat</td>
<td>Mouse</td>
<td>Alexafluor 546</td>
<td>Molecular Probes</td>
<td>1:500</td>
</tr>
</tbody>
</table>

2.6.2 Counting γH2AX foci as an indication of DNA damage

Pachytene spermatocytes were identified by the appearance of the homologous chromosomes stained with SCP3. A total of 50 pachytene spermatocytes were identified per mouse and the number of γH2AX foci per cell was counted. Any green γH2AX stain that crossed a pair of homologous chromosomes was counted as one focus. On a number of occasions large areas of γH2AX positive staining were observed which were most likely joined foci. These, however, could only be counted as one focus as it was impossible to distinguish the actual number. Thus, the counts recorded may be an under representation.
2.6.3 Fluorescent microscopy

Slides were examined on a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

2.7 Protein expression analysis

2.7.1 RIPA extraction from murine tissue

Mouse tissue previously dissected (2.1.6) and frozen (-70°C) was added to lysis buffer to disrupt the tissue thereby releasing the protein. Total protein was extracted from testes using RIPA buffer (2.12.1) containing protease inhibitor cocktail (Roche). The tissue was homogenised in 200µl RIPA buffer using a handheld motor driven grinder (Sigma) with a pestle (Sigma) designed to fit a 1.5ml microcentrifuge tube. The homogenate was incubated on ice for 1h prior to centrifugation at 2500rpm for 10 min. The protein rich supernatant was collected in a fresh 1.5ml tube and stored at -70°C until use.

2.7.2 Protein quantification

Protein samples were quantified in order to carry out comparative expression analysis. A Biorad DC protein assay kit (Biorad Laboratories, Hemel Hempstead, UK) was used to determine concentration according to the manufacturers instructions. Briefly, a standard curve was set up using solutions of known concentrations of BSA in RIPA buffer. These standards along with the samples of unknown concentration were subjected to a two-step assay based on the Lowry method of protein quantification. The first step involves a reaction between the protein and copper in an alkaline medium and the second step is the reduction of Folin reagent by the copper treated protein. The protein effects a reduction of the Folin reagent, which results in the production of one or more of several possible reduced species with a characteristic blue colour. The samples are then read on a spectrophotometer (Labsystems Miltiskan EX; VWR) at 690nm. The concentration of each unknown sample is determined using the standard curve produced from the protein standards.
2.7.3 Acrylamide gel electrophoresis

Protein samples were run on NuPage® Novex 4%-12% Bis-Tris ready-made polyacrylamide gels (Invitrogen, Paisley, UK). Between 20-25µg of each protein sample was loaded per well. The samples were diluted in PBS to the desired concentration and 5µl of bromophenol blue ‘loading buffer’ and 2µl ‘reducing reagent’ (both Invitrogen) added. The samples were boiled for 5 min immediately before loading each protein sample into the wells on the gel. For each gel, 5µl of SeeBlue® Plus-2 pre-stained multicoloured molecular weight marker (Invitrogen) was loaded into at least one lane. This marker contains proteins of known molecular weight thus allowing identification of the target protein at the correct molecular weight and allows visualisation of the transfer of protein from gel to membrane. The gel containing the samples was run in an electrophoresis tank, containing 1X NuPage® MOPS SDS Running Buffer (Invitrogen), at 100V to resolve the proteins according to their molecular weight. Electrophoresis was stopped when the bromophenol blue dye reached the bottom of the gel.

2.7.4 Transfer of proteins

The protein gel plates were carefully separated allowing access to the gel. The blotting apparatus included two porous pads, and 6 pieces of 3mm Whatman paper which were soaked in 1X NuPage® Transfer Buffer (Invitrogen) supplemented with methanol as per manufacturers instructions. The nitrocellulose membrane (Immobilin-P; Millipore, Bedford, UK) was rehydated in methanol for 30 sec then washed in transfer buffer. The above components including the gel were used to set up a gel-membrane sandwich as shown in Figure 2.3 and placed in the transfer tank with the membrane on the side of the positive electrode. This arrangement allows the separated proteins to transfer from the gel towards the anode until they reach the solid support membrane. The tank was filled with the above transfer buffer and attached to a power pack. The blot was transferred for 4h at 40V or overnight at 20V.
2.7.5 Probing the membrane

Odyssey® blocking buffer (LiCor, Nebraska, USA) was diluted 1:1 in PBS and used to block non-specific binding sites on the membrane by incubating for 1h at room temperature. The membrane was incubated overnight at 4ºC with the primary antibody diluted in Odyssey® blocking buffer/PBS-Tween (PBST). The antibodies and dilutions used are detailed in Table 2-6. An anti β-actin antibody was used as a loading control.

Table 2-6 Summary of primary antibodies used in Western blots

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO1</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>Stip1</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1:500</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Mouse</td>
<td>Cell Signaling</td>
<td>1:500</td>
</tr>
<tr>
<td>Hsp105</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
<tr>
<td>Bax</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
</tbody>
</table>
The secondary antibodies (Table 2-7) were fluorescently labelled and directed against the host species of the primary antibody as described in section 2.4.2.4. Any residual primary antibody was washed from the membrane with 4 x 5 min washes in PBST. The appropriate secondary antibody was diluted 1:5000 in the blocking buffer and incubated with the membrane for 1h at room temperature and protected from light due to the fluorescent label. The membrane was washed in PBST (4 x 5 min).

**Table 2-7** Summary of secondary antibodies used on Western blots for LiCor detection

<table>
<thead>
<tr>
<th>Fluorescent antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Excitation wavelength (nm)/ colour emitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR Dye 800</td>
<td>Goat anti rabbit</td>
<td>Rockland</td>
<td>800</td>
</tr>
<tr>
<td>Alexafluor®680</td>
<td>Goat anti mouse</td>
<td>Molecular Probes</td>
<td>680</td>
</tr>
</tbody>
</table>

2.7.6 Protein expression analysis – LiCor technology

Protein expression was analysed on a LiCor fluorescence detection system (LiCor). The LiCor allows detection of two proteins simultaneously using different coloured fluorescent markers and fluorescent filters. Membranes were washed in PBS alone to remove any residual Tween-20 prior to analysis. Protein expression was corrected for loading using β-actin.

2.8 RNA analysis

2.8.1 RNA extraction from murine tissue

RNA was isolated from tissue that had been frozen on dry ice at the time of dissection and kept at -70°C prior to use. An RNeasy Mini extraction kit (Qiagen, Crawley, UK) was used to extract the RNA according to the manufacturers’ instructions. Briefly, the testes were ground up using a RNase free, chilled pestle and
mortar kept on dry ice at all times. The resultant powder was added to the buffer provided in the kit and the solution passed through a QIAshredder column (Qiagen) in order to further homogenise and disrupt the tissue. Treating the sample, on the spin column provided, with RNase free Dnase according to manufacturers’ instructions, eliminated any contamination with genomic DNA. The extraction involves a multistep process, which results in a final elution of RNA in RNase free water.

### 2.8.2 RNA quantification

RNA concentration was determined using a Nanodrop-1000 (Labtech International, East Sussex, UK) spectrophotometer, which enables highly accurate and reproducible quantification. The RNA samples were then corrected to 100ng/ml using RNase free water to correct for differences between samples and facilitate further RNA analysis.

### 2.8.3 Reverse transcription of RNA (RT-PCR)

Reverse transcription of RNA to cDNA was performed using random hexamers to amplify total RNA including the 18S ribosomal RNA, which is used as an endogenous control in Quantitative Real Time PCR (Q-RT-PCR; 2.9.1). This was performed using the Applied Biosystems’ Taqman Reverse Transcription Reagents kit according to the following reaction:

- PCR Buffer II: 2µl
- MgCl₂: 4µl
- DNTPs: 2µl
- RNase Inhibitor: 1µl
- Mutiscribe RT: 1µl
- Random Hexamers: 1µl
- RNase free water: 5µl
RNA (100ng/µl) 4µl

The reagents were added together in a sterile PCR tube and placed in a thermocycler with the following cycle conditions:

- 25°C 10 min
- 42°C 1h
- 99°C 5 min

2.9 Taqman-quantitative PCR

Quantitative PCR was performed essentially as described in (Welsh et al., 2006) using either the Assay-On-Demand Gene Expression™ system (Applied Biosystems) or the Roche Universal Human Probe library (see) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The Taqman® Assay-on-Demand probes have a FAM™ reporter dye at the 5’ end of the Taqman MGB (minor groove binding) probe and a non-fluorescent quencher dye at the 3’ end. During the PCR reaction, cleavage of the probe separates the reporter dye from the quencher dye, which results in increased fluorescence of the reporter. This only takes place if the probe has hybridised to the target sequence of cDNA. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The expression level of the gene of interest was related to an endogenous control, 18S ribosomal RNA. Positive controls (human RNA; Applied Biosystems) and negative controls were included in each run.

All reagents were obtained from Applied Biosystems and samples run in triplicate on 96 well MicroAmp Fast Optical reaction plates (Applied Biosystems). For each sample the PCR reaction (2.9.1) was prepared to give a total volume of 15µl in each well and the plate was sealed with an adhesive cover (Applied Biosystems). This system was used to detect mRNA for Hif1 alpha (Cat no. Mm00468878).
2.9.1 Taqman Q-RT-PCR reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase free water</td>
<td>5.025µl</td>
</tr>
<tr>
<td>Assay on Demand primer/probe</td>
<td>0.75µl</td>
</tr>
<tr>
<td>Endogenous control (18S) primer/probe</td>
<td>0.225µl</td>
</tr>
<tr>
<td>Universal PCR Master Mix</td>
<td>7.5µl</td>
</tr>
<tr>
<td>CDNA</td>
<td>1.5µl</td>
</tr>
</tbody>
</table>

Total Volume 15µl

2.9.2 Roche Universal Probe Library™ – Q-RT-PCR

The Roche Universal Probe Library™ was also used for Q-RT-PCR and consists of 165 pre-validated real time PCR probes which are only 8-9 nucleotides in length. They are labelled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye as in the Taqman® Assay-on-Demand probes. The reaction which takes place with the dyes and the probe is described in section 2.9.

Using the Roche on-line ‘probe finder’ assay design system, primers were designed against the desired genes. This system provides the sequence of the required forward and reverse primers and also indicates which probe from the library to be used. Primers were obtained from MWG (London, UK), see Table 2-8 for sequences. Primers were first diluted to a stock concentration of 100µM and then to a working solution of 20µM in dH2O. The PCR was performed according to the reaction set out in section 2.9.2.1 on cDNA prepared with random hexamers as detailed in section 2.8.3. Each reaction was run in triplicate twice in a 96 well MicroAmp Fast Optical reaction plates (Applied Biosystems) on the ABI Prism 7900 Sequence Detection System (Applied Biosystems).
Table 2-8 Primer and probe sequences for transcripts studied using the Roche Universal Probe Library™

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe sequence (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO1</td>
<td>AGGCTAAGACCGCTCTTCT</td>
<td>TGTGTTCTCTGTACGATCA</td>
<td>AGGAGCTG (17)</td>
</tr>
</tbody>
</table>

2.9.2.1 PCR reaction for Roche Universal Probe Library™

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc&quot;</th>
<th>Vol for 20ul reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Faststart master mix</td>
<td>1x</td>
<td>10µl</td>
</tr>
<tr>
<td>Rox dye (6uM)</td>
<td>510nM*</td>
<td>1.5µl</td>
</tr>
<tr>
<td>Forward Primer 20µM</td>
<td>200nM</td>
<td>0.2µl</td>
</tr>
<tr>
<td>Reverse Primer 20µM</td>
<td>200nM</td>
<td>0.2µl</td>
</tr>
<tr>
<td>Probe 10µM</td>
<td>50nM</td>
<td>0.1µl</td>
</tr>
<tr>
<td>18S (0.08µM probe, 0.02µM primer)</td>
<td>0.3µl</td>
<td>5.7µl</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>2µl</td>
</tr>
</tbody>
</table>

cDNA                   |             |                       |

2.9.3 Analysis of results

Absolute quantification was performed on the ABI Prism sequence detection system using Real Time PCR. Real Time PCR reactions are characterised by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end. The earlier the target is detected the more cDNA was present.

The Taqman PCR results are presented as an amplification plot (Figure 2.4). This shows the amount of reporter dye generated during amplification, which is directly related to the amount of PCR product formed, which in turn is related to the expression level of the target gene. The FAM Ct (threshold cycle) value corresponds
to the cycle number at which the fluorescence generated crosses the threshold level. The threshold level is defined as the point at which an increase in signal is associated with an exponential increase of PCR product. The Ct value is directly related to the amount of PCR product. A change in Ct value of one equates to a two-fold difference in initial cDNA concentration. The 18S ribosomal RNA control is a measure of total RNA content in the sample and is used as an endogenous control for variation between samples.

Analysis of the results was carried out using the comparative Ct method. This is used to analyse changes in expression of a target gene relative to another reference sample (e.g. a control or untreated sample). For each sample, the ΔCt was calculated which refers to the difference between the FAM Ct and the 18S Ct which normalises the amplified signal against the total mRNA content. The mean ΔCt for the triplicates was calculated and used to determine the ΔΔCt, which is the difference between the ΔCt of the experimental sample compared to that of the control or untreated sample. The amount of amplified target is given the value of $2^{-\Delta\Delta C_t}$ which is based on the mathematical equation that describes the exponential amplification of the PCR reaction: $X_n = X_o \times (1+E_x)^n$ where $X_n$ is the number of target molecules at the threshold at cycle n, $X_o$ is the initial number of target molecules, $(1+E_x)$ is the efficiency of the target gene amplification and n equalling the number of cycles. Assuming that the efficiencies of the target and endogenous control reactions are equal, the $2^{-\Delta\Delta C_t}$ value is a measure of relative quantification and is used here to show the fold difference in mRNA expression of the samples relative to the control sample, where the control is given a $2^{-\Delta\Delta C_t}$ of 1.
2.9.3.1 **Statistical analysis**

Data was analysed for statistical significance using GraphPad Prism software. Significance was determined by using a students t-test or one-way analysis of variance (ANOVA). Results were deemed significant when P<0.05.
2.10 Mating studies

2.10.1 Mice
C57BL/6 males aged approximately 8 weeks were mated with C57BL/6 virgin females aged approximately 10 weeks. All mice were purchased from Harlan (Harlan Sprague-Dawley Inc, Oxford, UK).

2.10.2 Treatments
Male mice were anaesthetised and subjected to scrotal heating as described in section 2.2. Briefly, the lower third of the body was passed through a hole in a polystyrene raft, which was carefully placed in the water bath. Experiments were carried out at the following temperatures: 38ºC, 40ºC and 42ºC. Control animals received anaesthetic and were left at room temperature. All animals were administered the reversal reagent (2.2.1) and their cages placed on a warm mat to maintain body temperature and facilitate recovery.

2.10.3 Timed matings
Using the males from the above experiment, timed matings were set up to allow calculation of stage of gestation. Six control males and six heated males (singly housed) were set up to mate with three females each. The matings were set up for 5 days 23 – 28 days after heat treatment. This time point was chosen to reflect the insult on the spermatocytes at the time of heating. The time taken for spermatocytes to mature and reach the point of ejaculation is 19 – 32 days (Oakberg, 1956; Russell et al., 1990a), which includes a 5-day epididymal transit time.

Females were paired together with their mate at 2pm and monitored at 9 am for the subsequent 5 days. Detection of a postcoital plug was taken as evidence of mating and defined as embryonic day (e) 0.5. Plugged females were separated from their mate and date of plug noted. Embryos/fetuses were taken at two stages of gestation: e3.5 and e14.5. On day 5 of the mating period males were killed. Both testes were removed and one fixed (2.3.1), the other split in half with one half frozen on dry ice.
and the other placed in PBS for germ cell extraction (2.6.1). Their epididymides were used for sperm retrieval (2.5).

2.10.4 Collection of blastocysts from mated females

Females that had been mated to heated males or controls were sacrificed at e3.5. The uterus and ovaries were dissected intact into RPMI media (Sigma) in a sterile petri dish. Using iridectomy scissors the uterus was cut below the oviducts and at the bottom of the uterine horn. A sterile needle and syringe containing RPMI was inserted into one end of the uterine horn and the media was slowly released pushing the contents of the uterus out of the other end and into the media. The media in the dish was examined under a dissecting microscope (Leica MZ6) and blastocysts were recovered and transferred through a series of PBS and PBS/PVP washes using a fine-drawn glass pipette before being fixed in 4% PFA for 20 min (2.11.12).

2.11 In vitro fertilisation (IVF)

2.11.1 Mice

The animals used in the IVF experiments were sexually mature (approximately 8 weeks) C57BL/6 males and adult F1 (CBA male x C57b/1 female) females. The mice were killed by cervical dislocation. All dissections were carried out within a laminar flow hood (Astecair, Weston-Super-Mare, UK).

2.11.2 Dissection media

For dissections, Leibovitz media (Gibco, Paisley, UK) was used. The osmolarity was checked and adjusted to 285 +/- 5mmOsm/kg H2O. The media was supplemented with 3mg/ml BSA (Fraction V, TC grade, Sigma) and filter sterilised using a 22µm cellulose acetate syringe filter (syringes – Becton Dickinson, filters – Iwaki). This media was stored at 37°C overnight in an oven. On the day of IVF 1ml was placed
into enough embryo dishes (VWR) to allow for one dish per animal and placed back in the oven to warm the dishes to 37°C.

2.11.3 T6 preparation

Aliquots of each of the four components for T6 fertilisation media (Quinn et al., 1982) had been pre-prepared and stored at -70°C. The composition of which is detailed in section 2.12.2. The aliquots were defrosted and mixed before adding to 7.8ml sterile hospital grade water (IVEX) to the total volume of 10ml. The osmolarity of the media was checked and adjusted to 287 +/- 5mmOsm/kg H2O and supplemented with 10mg/ml BSA (Fraction V, TC grade, Sigma) and filtered as above. 0.5ml drops were placed gently on the bottom of 6-well tissue culture plates (Iwaki) by pipette and covered with silicon fluid (Merck, Lutterworth, UK). Enough drops were prepared for sperm collection and oocyte fertilisation. In addition extra drops were set up for washes. The plates were allowed to equilibrate overnight in a 37°C, 5% CO2 humidified incubator (Forma Scientific, Marietta, OH, USA) before IVF was carried out.

2.11.4 KSOM preparation

As described above pre-prepared aliquots of KSOM media (2.12.3) (Devreker and Hardy, 1997) were defrosted and mixed before adding to 7.8ml sterile hospital grade water (IVEX) to the total volume of 10ml. The osmolarity of the media was checked and adjusted to 254 +/- 5mmOsm/kg H2O and supplemented with 1mg/ml BSA (Fraction V, fatty acid free, TC grade, Sigma) and filtered as before. 100µl drops were placed carefully onto the bottom of 6-well tissue culture plates (Iwaki) and covered with silicon fluid. Enough drops were prepared for washing and holding fertilised oocytes from each experimental group overnight. The plates were prepared the day prior to the day of IVF as above and placed in an incubator (as in 2.11.3) to equilibrate. The remainder of the prepared KSOM was also kept in the incubator to allow for preparation of fresh plates for the 2-cell embryos the following day (see below).
2.11.5 Sperm preparation

C57BL/6 males (controls and those subjected to scrotal heat stress) aged approximately 8 weeks were used for sperm collection. The animals were killed by cervical dislocation and the dissections were carried out within a laminar flow hood (Astecair). From each male the testes, vas deferens and epididymides were removed and placed in embryo dishes containing dissection media (see 2.11.2). The tissue was further dissected under an inverted dissecting microscope fitted with a heated stage (microscope – Nikon, Tokyo, Japan/heated stage – Linkam, Tadworth, UK). Each testis was removed leaving the vas deferens and caudis epididymis. Using irredectomy scissors and watchmakers forceps any fat and blood vessels were removed. Both the vas deferens and caudis epididymis were placed in to a pre-equilibrated 0.5ml drop of fertilisation media (see 2.11.3). Under the microscope the cauda was teased apart and vas deferens squeezed to release sperm into the media. The tissue was then removed and discarded. The sperm preparation was placed in an incubator (Astecair) and left undisturbed for 2 hours.

2.11.6 Superovulation of females

The oocytes used in each IVF experiment were collected from superovulated adult F1 females aged 6-8 weeks. 68 hours before the day of IVF the females were injected into the peritoneum with 5IU Pregnant Mare Serum Gonadotrophin (PMSG; Intervet, Milton Keynes, UK) contained within 100µl of PBS. 48h later the same mice were injected with 5IU Human chorionic gonadotrophin (hCG; Intervet)/100µl PBS.

2.11.7 Oocyte collection

The females were killed by cervical dislocation near the end of the 2h sperm incubation period. The dissections were carried out within a laminar flow hood where the ovaries and oviducts were removed to embryo dishes containing dissection media (see 2.11.2) warmed to 37°C. Using insulin needles (Sherwood-Davis, Gosport, UK) the oocyte-cumulus complexes (OCCs) were released from the
ampulla region of the oviduct under an inverted microscope (Nikon) on a heated stage (Linkam). Using fine-drawn, BSA (0.1%) coated glass pipettes, the OCCs were transferred through two wash drops of pre-equilibrated fertilisation media and then into a final drop to await fertilisation.

2.11.8 Assessment of sperm

20µl of the sperm preparation was added to an equal volume of 4% paraformaldehyde. The total number of sperm per ml was calculated by counting 10µl of the fixed sperm using a haemocytometer chamber.

2.11.9 Fertilisation

Based on the assessment of the sperm preparation (2.11.8) 1-2 x10^6 sperm was added to the fertilisation drops containing the superovulated oocytes. Sperm was added at the same time for both controls and ‘heated’ males. The plates were returned to the incubator and left undisturbed for approximately 5 h.

2.11.10 Embryo development

2.11.10.1 Development to 2-cell stage

After the fertilisation period, the oocytes were transferred by glass pipette through two wash drops of pre-equilibrated KSOM (2.11.4) before being placed in the final drops of media. Counts were made of the number of oocytes in each group and the plates were returned to the incubator and left undisturbed for 17h. Following this period, the number of fertilised oocytes that had cleaved to the 2-cell stage were counted.
2.11.10.2 Development to blastocyst stage

The development media that had been left in the incubator from 2d prior was used to make up fresh drops of KSOM covered in silicon oil. The sizes of the drops were calculated based on the number of 2-cell embryos obtained in the previous stage. A strict one embryo to 2µl of media ratio was used. Where possible the embryos were kept in groups of 10 (in 20µl). This was to ensure that the effects of any endogenous products, produced by the embryos, were present at the same concentration in all drops. The drops were covered in silicon oil and returned to the incubator to equilibrate for 2-3h. Following this the 2-cell embryos were transferred into the fresh drops of development media, 24-26h after the beginning of the fertilisation period. The plates were returned to the incubator until the end of the culture period.

2.11.11 Assesment of developing embryos

The embryos were examined daily using an inverted microscope (Nikon) on a heated stage (Linkam). The number of embryos at each stage was counted daily and recorded for both the control group and experimental/heated group.

2.11.12 Fixation of embryos

The embryos were moved through three washes of PBS containing 3mg/ml polyvinylpirrolidone (PVP: Sigma) using fine-drawn glass pipettes before being placed in 4% paraformaldehyde (PFA). The embryos were fixed for 20 min at 4°C and washed in PBS/PVP before being subjected to immunostaining (2.11.13).

2.11.13 Immunostaining of embryos

Embryos were permeabilised for 20 min in 0.25% Triton-X in PBS. The embryos were washed 4 x 30 sec in PBS/0.5%-Tween 20 (PBT) using a fine-drawn glass pipette. The embryos were subjected to a non-immune block of 2% goat serum in PBT for 20 min. The primary antibody was applied at the appropriate concentration and incubated at room temperature for 1h followed by 4 x 30 sec washes in PBT. The
secondary antibody diluted 1:500 in block was applied for 30min. The embryos were subjected to 2 x 30 sec washes in PBT before being counterstained with DAPI. The embryo staining was visualised and images captured without delay on a Meta-Confocal microscope as in section 2.6.3.

2.12 Commonly used solutions

2.12.1 RIPA lysis buffer

5X RIPA buffer:
15ml 5M NaCl
25ml 1M Tris-HCl (pH 7.4)
5ml 0.5M EDTA
5g deoxycholate sodium
0.5g SDS
Protease inhibitor cocktail (Roche) was added to 1X RIPA at 100µl/ml.

2.12.2 T6 media stock solutions

Stock A (100ml)
Sodium Chloride 5.719g
Potassium Chloride 0.106g
Magnesium Chloride hexahydrate 0.096g
diSodium Hydrogen Phosphate 0.129g
Sodium Lactate (60% syrup) 4.652g
Glucose 1.0g

Stock B (100ml)
Sodium Hydrogen Carbonate 2.101g
Phenol Red 0.01g
Stock C (10ml)
Sodium Pyruvate 0.055g

Stock D (10ml)
Calcium Chloride dihydrate 0.262g

Stock A and B were aliquoted into 1ml per tube and C and D 100µl per tube. These are the volumes used of each stock when making this media for IVF (2.11.3).

2.12.3 KSOM media stock solutions

Stock A (100ml)
Sodium Chloride 5.55g
Potassium Chloride 0.186g
Potassium diHydrogen Phosphate 0.048g
Magnesium Sulphate Heptahydrate 0.049g
Sodium Lactate (60% syrup) 1.869g
Glucose 0.036g
EDTA 0.004g

Stock B (100ml)
Sodium Hydrogen Carbonate 2.101g
Phenol Red 0.01g

Stock C (10ml)
Sodium Pyruvate 0.022g

Stock D (10ml)
Calcium Chloride diHydrate 0.146g
Stock A and B were aliquoted into 1ml per tube. Stock C and D were aliquoted into 100µl per tube. These are the volumes of each used when making the media for IVF (2.11.4).

2.12.4 Phosphate buffered saline (PBS)

1x stock solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.29g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2g</td>
</tr>
</tbody>
</table>

Made to 1L in dH$_2$O
Chapter 3  Differential testicular function in mice with impaired DNA integrity

3  Differential testicular function in mice with impaired DNA integrity

3.1  Introduction

Assisted reproductive technologies such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Campbell and Irvine, 2000) are widely used to treat male infertility and although sperm from infertile men often have elevated levels of DNA damage this appears to have little effect on their ability to fertilize the oocyte following ICSI (Twigg et al., 1998). As a result the use of sperm from subfertile men could result in the introduction of damaged DNA of paternal origin into the embryo.

Spermatogenesis is a complex, multi-step process that involves DNA replication, meiosis and DNA packaging (Cooke and Saunders, 2002). During meiosis recombination events play an important role in creating genetic diversity among individuals within a population (Baarends et al., 2001). Recombination involves the induction of double strand breaks (DSBs), followed by crossing over between homologues and ligation of DNA molecules. Failure to introduce DSBs results in recombination and synapsis failure, leading to meiotic failure and infertility e.g. in Spo11 knockout mice (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000). Likewise failure to repair the DNA breaks is also deleterious and male infertility occurs in mice with knockouts of genes involved in recombination repair pathways (Cohen and Pollard, 2001; Marcon and Moens, 2005). Proteins involved in nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), single strand break repair (SSBR) and double strand break repair (DSB) are all expressed in the testis (Jaroudi and SenGupta, 2007). For example, histone variant 2AX (H2AX) is involved in the DNA damage response to DSBs (Bassing et al., 2002; Celeste et al., 2002; Downs et al., 2000; Sedelnikova et al., 2002) and acts by recruiting DNA repair factors to sites of DNA damage where it is rapidly phosphorylated resulting in formation of γH2AX foci (Rogakou et al., 1999).
Chapter 3  Differential testicular function in mice with impaired DNA integrity

3.1.1 Mouse models for DNA damage

In this chapter three different strains of mice were studied. These mice had targeted deletions in genes encoding Ercc1 (excision repair cross-complementing gene 1), Msh2 (MutS homolog 2, involved in mismatch repair pathway), or p53 (the tumour suppressor gene implicated in elimination of germ cells with DNA damage). All of these proteins are expressed in the testis and are involved in protecting DNA integrity by either DNA damage repair (ERCC1 and MSH2) or by acting as a checkpoint protein (p53).

3.1.1.1 The NER pathway and Ercc1

The NER pathway is responsible for the repair of UV-induced damage and bulky DNA lesions. The pathway consists of an initial lesion recognition step, followed by dual incision at the sites flanking the lesion and release of a lesion-containing oligonucleotide. Repair synthesis then takes place followed by ligation (reviewed by Wood, 1996). ERCC1 (excision repair cross-complementing gene) is essential for the NER pathway, where it acts in a complex with Xpf (ERCC4). It is also involved in DSB repair and repair of interstrand crosslinks (Hsia et al., 2003; Schiestl and Prakash, 1990). In the homologous repair (HR) pathway ERCC1 is involved in, but not essential for, the removal of protruding single-stranded ends adjacent to regions of homology (Davies et al., 1995). Ercc1 knockout male mice (McWhir et al., 1993) die before the first wave of spermatogenesis due to liver failure. However, the introduction of an Ercc1 transgene under the control of a liver specific promoter allows mice to survive to adulthood (Selfridge et al., 2001). The transgene positive mice are infertile and display germ cell depletion (Hsia et al., 2003). Analysis of sperm from these mice using the alkaline comet assay demonstrated increased levels of DNA damage with the TG-Ercc1−/− sperm showing consistently larger comet tails than control littermates. They also exhibit oxidative damage with a three-fold increase in the level of the oxidised base 7,8-dihydro-8-oxoguanine (8-oxoG) in the testis thus suggesting a role for ERCC1 in the repair of 8-oxoG (Hsia et al., 2003).
3.1.1.2 **The MMR pathway and Msh2**

MMR proteins are involved in the removal of errors made in DNA replication that have escaped the proof reading activity of DNA polymerase (reviewed in Stojic et al., 2004). In the mouse MMR pathway, five proteins (MSH2 to MSH6) function as heterodimers to initiate repair activity; MSH2/MSH6 and MSH2/MSH3 are involved in repairing replicative mismatches whereas MSH4/MSH5 is a meiosis specific complex essential for processing recombination intermediates (reviewed in Kunkel and Erie, 2005). MSH2 is highly expressed in mouse spermatogonia and leptotene/zygotene spermatocytes (Richardson et al., 2000). Msh2 knockout mice manifest an enhanced predisposition to skin cancer/tumorigenesis associated with UVB exposure (Meira et al., 2002; Yoshino et al., 2002) but no abnormalities in spermatogenesis have been reported.

3.1.1.3 **Tumor suppressor gene p53**

If DNA damage is not repaired or tolerated then the cells that harbour the damage are usually removed by apoptosis. During spermatogenesis most DNA damage-induced apoptosis is p53 dependent though elimination of spermatocytes with synaptic errors appears to be p53 independent (Odorisio et al., 1998). The p53 tumor suppressor protein is highly expressed in the testis and some studies have suggested a role for p53 in meiosis as it is expressed in both pachytene (Schwartz et al., 1993) and pre-leptotene (Sjoblom and Lahdetie, 1996) primary spermatocytes. p53 is also thought to play a role in repair of DNA strand breaks: it has been shown to bind single stranded DNA and promote strand transfer between complementary strands (Bakalkin et al., 1993) and plays a direct role in HR (Linke et al., 2003) and also in the non homologous end-joining of DSBs (Tang et al., 1999). p53 knockout mice have been shown to exhibit less mature motile spermatozoa than controls which would be consistent with compromised DNA repair (Schwartz et al., 1999; Yin et al., 1998a).
3.1.2 Aims of this chapter

The principal aim of the study described in this chapter was to determine the effects of impaired DNA integrity on testicular function in three different mouse models. This was achieved by analysing testicular histology and the incidence of apoptosis as well as the rates of DNA damage in spermatocytes and mature sperm.

3.2 Materials and Methods

3.2.1 Animals

The production of ERCC1 deficient mice with a liver specific Ercc1 transgene (TG-Ercc1) has been described previously (Selfridge et al., 2001); animals are on a segregating BALB/c X 129Ola X C57BL/6 background. The production of Msh2 mice (C57BL/6 background) and p53 mice (CBA background) have been described previously by de Wind et al (de Wind et al., 1995) and Clarke et al (Clarke et al., 1993) respectively. All mice used in this study were adult males aged 8-10 weeks. Animal sources, accommodation and welfare conditions are detailed in chapter 2, section 2.1. All procedures were carried out according to home office regulations under project licence 60/3544.

3.2.2 Preparation of Murine spermatozoa

Epididymides were carefully dissected from the body cavity using iridectomy scissors, any adherent fat removed and placed in 500μl BWW. Epididymides were finely minced using iridectomy scissors until a cloudy suspension had formed. Cell suspensions were kept at 32°C for 30 min to allow viable sperm to swim up. The ‘swim-up’ sperm were carefully removed from the supernatant, leaving dead sperm and tissue behind at the bottom of the tube. The supernatant was placed into a fresh tube and made up to 1ml with BWW and stored at -20°C. Before use the aliquots were defrosted at room temperature and inverted to redistribute spermatozoa throughout the sample.
3.2.3 Sperm counts

For sperm counts a 100µl aliquot was diluted (1:10) in 4% paraformaldehyde (PFA) and counted using a hemocytometer. Two counts were made for each sample and the mean taken. Further details can be found in chapter 2, section 2.5.3.

3.2.4 Study of morphology of sperm

Sperm samples were diluted 1:10 with sperm diluting fluid (SDF; 50g NaHCO₃, 10 ml formalin, dH₂O to 1L). For each sample 2 microscope slides were prepared by washing in 100% ethanol; 10µl of sample was pipetted onto the end of the first slide and smeared across the surface with the other slide. The smear was allowed to air-dry and repeated three times to obtain a higher concentration of sperm on each slide. The slides were fixed in ether/alcohol fixative (30% ether/70% ethanol) for 30 min.

Slides were stained using a Reastain Quick-Diff staining kit (Oy Reagena Ltd, Toivala, Finland) according to the manufacturer’s instructions. This process is detailed in chapter 2, section 2.5.4.

3.2.5 Spermatozoa chromatin structure assay (SCSA)

A modified version of the SCSA method described by Evenson et al. (1999) was used. The assay measures altered chromatin structure by measuring the susceptibility of spermatozoal DNA to acid denaturation using the metachromatic dye acridine orange (AO, Sigma). The dye fluoresces green when bound to double stranded DNA and red when bound to single stranded DNA. The sperm were adjusted to a concentration of 1-2 x 10^6 cells/ml with TNE. 100µl of this sperm was mixed with 200µl acid detergent solution. After 30 sec, 600µl AO diluted in AO staining solution (6µg/ml) was added to the spermatozoa mixed and left for 3 min at room temperature before analysing using a fluorescent assisted cell sorting (FACS) machine. Composition of solutions used here are detailed in chapter 2, section 2.5.5.
3.2.6 Histology and Immunohistochemistry

Testes were fixed in Bouins for 8h before cutting them in half; tissues were stored in 70% (vol/vol) ethanol and processed into paraffin wax using standard procedures. Tissue sections (5µm) were stained with haematoxylin and eosin as detailed in chapter 2, section 2.4.1. Immunodetection of mouse Vasa homolog (Mvh) and p63 was performed as described in chapter 2, section 2.4.2. Briefly, sections were dewaxed and rehydrated before being subjected to antigen retrieval using citrate buffer (section 2.4.2.1). Endogenous peroxide activity and non-specific binding sites were blocked with methanol peroxide and normal goat serum (NGS) respectively (section 2.4.2.2). The primary antibodies, Mvh (Abcam) and p63 (Santa Cruz), were incubated with sections overnight at 4°C both at 1:500 in NGS. Secondary antibodies were applied and detection was carried out using standard protocols as detailed in chapter 2, sections 2.4.2.4 and 2.4.2.5 respectively.

3.2.6.1 Measurement of tubule diameters

Images of H&E stained testes were captured from an Olympus microscope BH2 (Olympus, UK) under a x40 lens using a video camera (Hitachi HV-C20, Japan) and analyzed with Image Pro Plus™ software with a Stereology 5.0 plug-in (Media Cybernetics, Berkshire, UK). Briefly, the software was used to trace around each section, creating an area of interest (AOI). Twenty-five fields, randomly selected by the programme, within the AOI were then examined on one testis section from each animal. The software takes the average of three measured diameters per tubule. A minimum of 75 tubules per testis was measured and the frequency distribution of diameters determined.

3.2.6.2 Detection of apoptotic cells using immunolocalisation of Caspase 3

Testis sections (5µm) were dewaxed in xylene and rehydrated through a series of graded alcohols. Antigen retrieval was performed using citrate buffer (chapter 2,
section 2.4.2.1). Endogenous peroxidase activity was blocked and sections were incubated in normal goat serum (Chapter 2, section 2.4.2.2) to block non-specific binding sites. All washes between incubations comprised 2 x 5 min at room temperature in Tris-buffered saline (TBS; 0.05 M Tris-HCl (pH7.4), 0.85% NaCl). Sections were incubated overnight at 4°C with a primary rabbit antibody raised against cleaved caspase 3 (Cell Signalling Technology, Beverly, MA) diluted 1:200 in NGS/TBS/BSA. Negative control sections were incubated with blocking serum alone to confirm antibody specificity. After washing, sections were incubated with secondary antibody, biotinylated goat anti rabbit IgG diluted 1:500 in NGS/TBS/BSA for 30 min at room temperature. Bound antibodies were detected according to standard methods detailed in chapter 2, section 2.4.2.5. Counts of caspase 3 positive germ cells were made on a Provis AX70 microscope (Olympus Optical, London, UK). This was achieved by counting the total number of positive cells in four testis sections, taken at least 50µm apart. It is noted that the results presented for this analysis do not take into account that the tubules may be shrinking and therefore more positive cells may be observed in a cross section of the testis. In order to address this it may be useful to present the data as number of caspase-positive cells per Sertoli cell however this was not performed in this study.

3.2.7 Meiotic Spreads

Meiotic spreads were performed as detailed in chapter 2, section 2.6. Briefly, testes were dissected into 200µl RPMI media (Sigma) at 32°C and the tunica albuginea removed and discarded. The remaining tubules were finely chopped to form a milky suspension and diluted to a final volume of 3ml RPMI. The resulting cell suspension was subjected to centrifugation steps to obtain a pellet containing the germ cells, which was resuspended in 2ml warm RPMI.

Glass slides were coated in sucrose solution in dH2O and one drop of cell suspension was dropped onto the slides from a height of 20-30cm using a glass pipette. TritonX-100 (in dH2O) was added to each slide to induce permeabilisation of spermatocytes
and the slides were subsequently fixed. The slides were dipped briefly six times in dH2O and allowed to air dry for 5 min before storing at -70ºC until use.

### 3.2.7.1 Immunostaining of meiotic spreads

Slides were defrosted by washing in PBS and blocked with blocking buffer for 1h at room temperature (see Chapter 2, section 2.6.1.1). The primary antibodies (anti-SCP3 mouse monoclonal (1:400, Abcam, Cambridge UK) and anti-γH2AX rabbit polyclonal (1:200, Upstate Biotechnology, MA, USA)) were diluted in blocking buffer and incubated overnight in a humidified chamber at 4ºC. The secondary antibodies (goat anti-mouse Alexa-546, goat anti-rabbit Alexa-488 both 1:500, Molecular probes) were applied and incubated for 1h at room temperature. The slides were subsequently incubated with DAPI nuclear stain (Sigma) at 1:1000 in PBS for 10 min. Lastly, the slides were mounted in Permafluor aqueous mounting medium (Beckman Coulter). All washes between incubations consisted of 2 x 5 min in PBS.

### 3.2.7.2 γH2AX foci counts

For γH2AX quantification, 50 spread pachytene nuclei were analysed from each mouse and the number of foci occurring on the synaptonemal complexes coincident with SCP3 staining were counted. Microscope analysis was performed on a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

### 3.2.8 Statistical analysis

Results expressed as means and standard errors of the mean were analyzed using one-way ANOVA followed by the Bonferroni post hoc test, using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA).
3.3 Results

3.3.1 Testicular phenotypes of mice lacking Ercc1, Msh2 or p53

Examination of haematoxylin and eosin stained sections revealed disturbances in testicular architecture in TG-Ercc1−/− and Msh2−/− mice compared with control littermates (Figure 3.1 & Figure 3.2). Consistent with previous observations (Hsia et al., 2003) TG-Ercc1−/− testes had reduced numbers of germ cells within the seminiferous tubules and some tubules lacked germ cells altogether (Sertoli cell only, SCO) (Figure 3.1C, asterisks). All phases of germ cells were observed up to and including mature elongated spermatozoa. A similar, if less pronounced testicular phenotype was observed in the Msh2−/− testes (Figure 3.1F, asterisks). In both KOs the presence of tubules with gaps in the seminiferous epithelium were observed (Figure 3.1C & F arrows and Figure 3.2C, arrowhead). Mice heterozygous for Msh2 and Ercc1 were comparable to their wild type littermates. On closer examination of the TG-Ercc1−/− and Msh2−/− (adult and 30 day old) testes under a higher power of magnification it was evident that both lines of KO mice had varying degrees of germ cell loss between tubules within the same testis, with normal spermatogenesis in some tubules (Figure 3.2A, E & I highlighted ‘N’) and extensive germ cell loss in others (Figure 3.2B-D, F, G & J highlighted with X) including SCO tubules (Figure 3.2K & L, stars). In addition, abnormal-staining cells could be seen in the Msh2−/− 30d testes, which were assumed to be apoptotic due to their appearance (Figure 3.2F & G, arrows). It is assumed that the fact that some germ cells survive while others die is due to stochastic germ cell death as in the male the rapidly cycling cells are particularly susceptible to damage acquired during the meiotic stages. It is possible that the low level of transgene-derived Ercc1 may be sufficient to partially rescue the phenotype of some cells or tubules but not others (Hsia et al., 2003). This may also be true for Msh2 however analysis would be required to determine if there was any residual Msh2 in the testes of the KO mice. Testes from p53−/− mice appeared normal (Figure 3.1I).
3.3.2 Tubule diameter distribution

In TG-Ercc1\textsuperscript{−/−} and Msh2\textsuperscript{−/−} testes, changes in germ cell complement resulted in alterations in tubule diameters when compared to their littermate controls. In the TG-Ercc\textsuperscript{−/−} mice 99.5% of tubules were less than 200µm in diameter whereas there was an equal distribution of tubules > and < 200µm in +/− and ++/++ mice (Figure 3.3A). In the Msh2\textsuperscript{−/−} testes the majority of tubules were <200µm (Figure 3.3B). Consistent with the testicular histology there was no difference in tubule diameters between testes from p53\textsuperscript{−/−}, p53\textsuperscript{+/−} and p53\textsuperscript{+/+} mice (Figure 3.3C).

\textbf{Figure 3.1 Histological evaluation of testes from adult mice.} Haematoxylin and eosin staining of Ercc1 (A-C), Msh2 (D-F), and p53 (G-I) testes. Sertoli cell only tubules (SCO) are highlighted with asterisks and gaps in testicular epithelium with arrows in TG-Ercc1\textsuperscript{−/−} (C) and Msh2\textsuperscript{−/−} (F). Bar=100µm.
Figure 3.2 High power histological analysis of knock out testis tubular architecture. Haematoxylin and eosin staining of TG- Ercc1 KO (A-D), Msh2 30day KO (E-H), and Msh2 adult KO (I-L) testes. Tubules with normal appearance are highlighted with N. Gaps in seminiferous epithelium are highlighted with arrowheads. Abnormal/apoptotic cells are highlighted with arrows. Tubules with extensive germ cell deficiencies are highlighted with X. SCO tubules are highlighted with *. Bar = 50µm.
Chapter 3  Differential testicular function in mice with impaired DNA integrity

Figure 3.3 Distribution of seminiferous tubule diameters in A) TG-Ercc1, B) Msh2 and C) p53 lines. White bars represent wild type, solid bars heterozygotes and shaded bars knockouts. Data presented as mean number of tubules in each group per mouse. In TG-Ercc1 n=3, in Msh2 and p53 n=4.
3.3.3 Immunodetection of MVH and p63: characterisation of GC loss

Testicular sections from the three mutant lines were immunostained with mouse Vasa homolog (Mvh) and also with p63 to further characterise the germ cell loss seen in the H&E stained sections. These proteins were chosen to try to determine the type of germ cells that were being lost to result in the gaps seen in the seminiferous epithelium as they are both markers of germ cells. Mvh is localised to the cytoplasm of germ cells from the spermatogonia to the round spermatid stage but with the strongest expression in the pachytene spermatocytes and with elongating spermatids being Mvh-negative (Toyooka et al., 2000). As expected this expression pattern was seen in the testes from wild type and heterozygous mice from all three mutant lines and also in the $p53^{-/-}$ testes (Figure 3.4). However in the $TG-Ercc1^{-/-}$ and $Msh2^{-/-}$ (adult and 30d) testes, expression appeared patchy (Figure 3.4C, F and I) exhibiting the clonal loss of germ cells in these KOs and also confirming that these are seminiferous tubules that have experienced germ cell loss and not the rete testis which can appear similar to SCOs. In the majority of samples there appeared to be a darker stain at the border of the lumen around the round spermatids; it is assumed that this is cytoplasmic condensation occurring during the remodelling of the spermatids before cytoplasmic loss. Germ cell loss in these mutants was further confirmed with p63 immunodetection. p63 is thought to be involved in the proliferation and differentiation processes in spermatogenesis and is strongly expressed in pachytene spermatocytes along with weaker staining in round spermatids; spermatogonia and elongates are negative (Nakamuta and Kobayashi, 2004). In this study the expression of p63 in the testes of wild type, heterozygous and KO mice from all three mutant lines (Figure 3.5) was as described by Nakamuta and Kobayashi (2004). However, on looking closely at the extent of p63 positive staining, there appears to be less p63-positive pachytene cells within the testes from $TG-Ercc1^{-/-}$ and $Msh2^{-/-}$ (adult and 30d) mice (Figure 3.5C, F and I, highlighted with black stars), this reduction may in part be responsible for the reduction in tubule diameter. P53 $^{-/-}$ testes appear to be unaffected (Figure 3.5L).
Figure 3.4 MVH immunoexpression in the testis. Immunodetection of MVH in the adult testis of TG-Ercc1 (A-C), Msh2 (D-F), p53 (J-L) lines and also in Msh2 30d testes (G-I). Tubules with reduced germ cell complement are highlighted with a star and those with no germ cells or SCOs are highlighted with a diamond. Bar = 100µm.
Figure 3.5 p63 immunoexpression in the testis. Immunodetection of p63 in the adult testis of TG-Ercc1 (A-C), Msh2 (D-F), p53 (J-L) and also in Msh2 30d testes (G-I). Tubules which appear to have reduced numbers of p63 positive germ cells are highlighted with a star. Bar = 100\(\mu\)m.

3.3.4 Apoptosis

Testicular sections were stained for cleaved caspase-3 to detect any cells that were undergoing apoptosis (Figure 3.6). In testes from TG-Ercc1\(^{−/−}\) mice a 3.5 fold increase in apoptotic germ cells was observed compared to controls (Figure 3.7A). A similar increase (3 fold) was evident in the p53\(^{−/−}\) mice (Figure 3.7B). The majority of cells in these mutants undergoing apoptosis appeared to be spermatogonia (Figure 3.6). No increase in caspase 3-positive cells was observed in the Msh2\(^{−/−}\) adults. In order to determine whether an earlier wave of apoptosis might account for the
presence of SCO tubules in the adult $\textit{Msh2}^{-/-}$ testes, testes from mice during the first wave of spermatogenesis (day 30) were also examined and in these a slight but non-significant increase in the number of caspase-3 positive germ cells was detected (Figure 3.7D). It should be noted, however, that the group sizes were relatively small so the experiment may not be sufficiently powerful to test whether 30d $\textit{Msh2}^{-/-}$ mice have significantly higher levels of apoptosis. It is also noted that the lack of significance may be affected by variation in the developmental rate of the juvenile mice. The testes from d30 $\textit{Msh2}^{+/+}$ mice had increased apoptosis in comparison the adult wild types. This is presumably due to the fact that the first wave of spermatogenesis is characterised by a wave of apoptosis to adjust germ cell numbers to that which the Sertoli cell can support.

These results do not take into account that the tubules may be shrinking which may result in more positive cells being observed in a testis cross section. However this possible source of error is unlikely due to the large increase in apoptosis in $\textit{Ercc1}^{-/-}$ mice and cannot explain the increase in the $\textit{p53}^{-/-}$ because tubule diameter was unchanged (Figure 3.3C). TUNEL staining was also performed on these mice, however no variation was seen between the caspase-3 results and those for TUNEL and so these data were not included.
Figure 3.6 Detection of apoptotic cells. Immunodetection of caspase-3 positive germ cells in TG-Ercc1 (A-C), Msh2-adult (D-F), Msh2-30 day (G-I) and p53 (J-L) testes. Positive cells undergoing apoptosis are highlighted with arrows. Bar = 50µm.
Figure 3.7 Quantification of caspase-3 positive germ cells. Counts of caspase positive germ cells in four testis sections per animal ($n=\geq 3$). An * ($P<0.05$) indicates significant variation compared with wild type littermates.

### 3.3.5 DNA damage in spermatocytes

Germ cell spreads prepared from the testes of all three mutant lines were stained for γH2AX to detect unsynapsed DNA or double strand breaks. Co-staining with an antibody directed against the synaptonemal complex protein SCP3 was used to identify those spermatocytes at the pachytene stage of meiosis (Lammers et al., 1994). Consistent with previous publications, in pachytene spermatocytes from the testes of all three lines of mice intense, immunopositive staining for γH2AX was localized to the sex body (Mahadevaiah et al., 2001), which contains unpaired regions of the X and Y chromosomes (Figure 3.8, white arrows). Variability in the
numbers of unrepaired DNA lesions (γH2AX-stained foci) in pachytene spermatocytes from \textit{TG-Ercc1}^{-/-} mice was observed. For example, in some spermatocytes a 600% increase in the number of γH2AX foci on the autosomes was detected (Figure 3.8C, white arrowheads) whereas in others only a very slight, or no, increase in staining was observed (Figure 3.8D) compared with those from +/- or +/- animals where autosomes were on the whole γH2AX-negative. However although some spermatocytes from \textit{TG-Ercc1}^{-/-} were γH2AX-negative, when the mean number of foci in 50 cells were compared the number in cells from -/- males was found to be significantly increased (Figure 3.9). Quantification also revealed that spermatocytes from both \textit{p53}^{+/+} and -/- mice had a significant increase in the number of γH2AX positive foci (Figure 3.8K & L, summarised in Figure 3.9). In some \textit{p53}^{-/-} and +/- spermatocytes γH2AX staining was not detected on the sex body (X and Y chromosomes) (Figure 3.8L showing +/- spermatocyte). There was no difference in γH2AX expression observed in the \textit{Msh2}^{-/-} mice compared to the +/- and +/- littermates.
Figure 3.8 Double strand breaks in pachytene spermatocytes detected using γH2AX immunohistochemistry on germ cell spreads. Immunodetection of SCP3 (red) and γH2AX (green) in TG-Ercc1 (A-D), Msh2 (E-H) and p53 (I-L) spermatocytes. The sex body is highlighted with white arrows and in some nuclei foci are highlighted with arrowheads.
Figure 3.9 Quantification of double strand breaks in pachytene spermatocytes detected using γH2AX Immunohistochemistry on germ cell spreads. Quantification of γH2AX foci per 50 spermatocytes per mouse (n≥4) in Ercc1, Msh2 and p53 lines. ** (P<0.01) and *** (P<0.001) indicates significant variation from control littermates.

3.3.6 Sperm counts

On performing epididymal sperm counts it was evident that the TG-Ercc1−/− mice had a significant reduction in sperm concentration with a 97% decrease compared with wild type littermates (Figure 3.10). In contrast, both the Msh2 and p53 lines showed no significant changes in sperm counts between genotypes (although group sizes may be too small to detect small reductions e.g. in Msh2−/− KO mice).
Figure 3.10 Edidymal sperm counts in Ercc1, Msh2 and p53 lines. *** (P<0.001) indicates significant variation from Ercc1 wild type littermates. M/ml=millions sperm per ml, n ≥ 4.

3.3.7 Sperm morphologies

The majority of sperm from wild type mice in all three lines appeared morphologically normal (Figure 3.11A, Figure 3.12A, Figure 3.13A & B) although there were a few sperm present with abnormal head and hook shapes. The morphology of TG-Ercc1 +/-, Msh2 +/- and p53 +/- sperm were identical to their wild type littermates (Figure 3.11B, Figure 3.12B and Figure 3.13C & D). Additional abnormalities were detected in sperm from TG-Ercc1 -/- males including malformed apical hooks (Figure 3.11C & D), rounded heads or loss of hook (Figure 3.11 E and F). Msh2 +/- sperm appeared similar to controls, although some had slightly shorter and blunted apical hooks (Figure 3.12C). Sperm similar to this were also occasionally found in wild type animals. In contrast, many of the sperm from p53 +/-
males appeared strikingly different to those from wild type littermates with severely abnormal head shapes (Figure 3.13 F and G), which were comparable to the TG-Ercc1−/− sperm.

**Figure 3.11 Morphology of epididymal sperm from TG-Ercc1 wild type (A), heterozygous (B) and TG-Ercc1 KO (C-F) mice.** A and B) Normal head morphology with apical hook (arrows). C and D) abnormal hook formation (arrowheads). E and F) Rounded head and loss of hook (arrows). Sperm heads were approximately 7-10µm in length.

**Figure 3.12 Morphology of epididymal sperm from Msh2 wild type (A), heterozygous (B) and KO (C) mice.** A and B) Normal head morphology with apical hook (arrow). C) Normal head (arrow) and blunted hook (arrowhead). Sperm heads were approximately 7-10µm in length.
3.3.8 Sperm chromatin abnormalities

To investigate whether there was altered chromatin structure in the sperm from the mutant lines the SCSA was used. The sperm from the TG-\textit{Ercc1}\(^{-/-}\) mice exhibited a significant increase (3 fold) in the percentage of sperm with DNA damage compared to controls (Figure 3.14). The p53 deficient mice also showed altered sperm chromatin structure with a 2.5 fold increase (not significant) in the percentage of \(p53^{-/-}\) sperm with DNA damage over the wild type (Figure 3.14). However, the group sizes are small and thus the experiment may not be sufficiently powerful to test whether \(p53^{-/-}\) sperm have significantly more breaks and are therefore susceptible to acid denaturation. Sperm from the \textit{Msh2} line exhibited low percentages of sperm with DNA damage or abnormal chromatin irrespective of genotype (Figure 3.14).
Figure 3.14 Susceptibility of epididymal sperm to acid denaturation detected by the SCSA in TG-Ercc1, Msh2 and p53 lines. %DFI represents ratio of sperm with denatured DNA (red fluorescence) to total sperm (red/[red+green]) fluorescence. ** (P<0.01) indicates significant variation compared to wild type littermates. n ≥ 4.
3.4 Discussion

The impact of damaged DNA originating in the male germ line is poorly understood, but may contribute to early pregnancy loss (recurrent miscarriage), placental problems and have an impact on the health of the offspring. A number of studies have shown a link between DNA damage in sperm and early embryonic failure. Spermatogenesis is a complex process that cannot, as yet, be modelled in vitro (Cooke and Saunders, 2002). Germ cell maturation involves mitotic proliferation, meiotic division and spermiogenesis, followed by release of mature sperm and taking 35 days in mice (75 days in human). Studies using infertile and subfertile mouse strains, have identified a number of genes that are essential for DNA repair, meiosis and germ cell-somatic cell interactions (Edelmann et al., 1996; Griswold, 1995; Matzuk et al., 1995; Xu et al., 1996; Yuan et al., 2000). The present study has evaluated testicular function in three different lines of transgenic mice with deletions in different genes (Ercc1, Msh2 and p53) all of which are expressed in the testis. The main hypothesis was that deficiency in the DNA repair proteins (Ercc1 and Msh2) would result in the disruption of spermatogenesis due to an increase in unrepaired DNA in germ cells and that deficiency in the checkpoint protein p53 would result in an increase in the proportion of damaged germ cells progressing to mature spermatozoa but that these spermatozoa would be abnormal.

Hsia and colleagues (2003) have previously reported on the testicular phenotype of TG-Ercc1−/− mice. In order to expand on the previous observations the current study used quantitative methods to determine the extent of tubule diameter reduction and apoptosis in addition to performing meiotic spreads from the testes of these mice. In accordance with the previous findings this study demonstrates that the TG-Ercc1−/− males exhibit a reduced number of germ cells and mature sperm in the seminiferous tubules with a proportion lacking germ cells altogether (SCO tubules). The SCO phenotype is also seen in another such NER mutant, the mHR23B−/− mice (Ng et al., 2002) though this is atypical for mutations in the NER pathway with other NER mutants (xpa and xpc−/−) being fertile (Nakane et al., 1995; Sands et al., 1995). Here,
the p63 and Mvh immunostaining emphasised the reduced numbers of pachytene spermatocytes and the clonal loss of germ cells respectively. Hsia et al. (2003) also provided evidence of a loss of DNA integrity using the COMET assay, which only allows analysis of a single sperm cell. This data has been confirmed and extended here using the SCSA, which measures damage in many thousands of sperm simultaneously and therefore gives a much better estimate of the proportion of sperm with damaged DNA than the COMET assay. According to the SCSA there was a 3-fold increase in the percentage of sperm exhibiting chromatin abnormalities. In addition, a 3.5-fold increase in apoptosis was observed as shown by activated caspase 3 immunostaining, however it is clear that elimination of germ cells with damaged DNA was not 100% efficient as a population of mature sperm with chromatin abnormalities were detected using the SCSA. To gain new insight into the origins of this DNA damage this study examined the nature of the DNA lesions in the testicular germ cells using cell spreads and γH2AX immunostaining. Phosphorylation of histone (H) 2AX is known to occur following formation of DSBs (Rogakou et al., 1998) and at sites of unsynapsed chromosomes such as those found in the XY (sex) body (Hamer et al., 2003). Phosphorylated H2AX (γH2AX) appears to recruit repair proteins to sites of damage (Paull et al., 2000). The present study detected increased levels of DSBs persisting in pachytene spermatocytes recovered from TG-Erccl \(^{-/-}\) testes compared to TG-Erccl \(^{+/-}\) and \(^{++}\). This would be consistent with an important role for Erccl in the pachytene spermatocytes of prophase I where the highest levels of expression of the protein occur (Hsia et al., 2003). The observed increase in DSBs may also explain the increase in apoptosis, the reduction in germ cell numbers and the presence of SCOs. Furthermore the detection of DSBs in pachytene spermatocytes and the abnormal chromatin structure in mature sperm confirm that the repair functions of Erccl are essential for normal germ cell maturation and suggest that none of the other NER proteins can compensate for its ablation.

The mouse MMR pathway is involved in repairing a variety of mismatches and also participates in recombination during meiosis (Kirkpatrick, 1999; Kolodner, 1996;
Chapter 3  Differential testicular function in mice with impaired DNA integrity

Modrich, 1997). In mammals, five MutS homolog proteins (Msh2 to Msh6) function as heterodimers to initiate repair activity; Msh2/Msh6 and Msh2/Msh3 are involved in repairing replicative mismatches whereas Msh4/Msh5 is a meiosis specific complex essential for processing recombination intermediates. There are also four MutL homologs: Mlh1, Mlh3, Pms1 and Pms2 (Cohen and Pollard, 2001; Svetlanov and Cohen, 2004). MLH proteins are also involved in meiosis, for example, Mlh1 is found at sites of meiotic crossing over (Moens et al., 2002). Consistent with the need to correctly repair recombination events mice deficient in Msh5 or Mlh1 exhibit disrupted spermatogenesis and are infertile (Baker et al., 1996; Edelmann et al., 1999).

In the Msh2 mutant line there have been no reports of reproductive phenotypes to date and it was assumed that the mismatch repair function required for germ cell integrity was carried out by other members of the gene family such as Msh4. However the present study demonstrates, for the first time, that targeted ablation of Msh2 does compromise germ cell complement in the male. Similar to the TG-Ercc1^-/- testes, the Msh2^-/- testes exhibited the presence of SCOs, and also seminiferous tubules with gaps in the epithelium indicative of an intermittent defect in spermatogenesis. This was further confirmed with the immunodetection of p63, which suggested reduced numbers of pachytene spermatocytes in the Msh2^-/-, though this would need to be confirmed by cell counts to be conclusive. However, the Msh2^-/- males are fertile whereas the TG-Ercc1^-/- males are not which is consistent with the normal sperm counts and appearance of sperm heads in the former when compared with the latter. The reduced germ cell complement was reflected in tubule diameters. For example, dividing the tubules into two groups based on diameter (those <200μm and those >200μm) it was evident that the majority of tubules fell into the smaller diameter group compared with an even distribution in controls. This disturbance in tubule diameter was also seen in the TG-Ercc1^-/- testes. Although germ cell loss was observed in Msh2^-/- testes this was surprisingly not associated with an increase in apoptosis (cleaved caspase 3 immunostaining) in the adults. However, on examining testes from 30 day old Msh2^-/- males a slight increase (not
significant) was observed in caspase 3-positive germ cells suggesting that the presence of MSH2 may be more critical during the first wave of spermatogenesis when the process is less efficient and thus may in turn account for reduced germ cell complement/tubule diameter in adults. It has been reported that functional MMR is required for the induction of apoptosis in response to DNA damage in cancer (Zhang et al., 1999) although it would appear that other proteins in the MMR family, of which many are expressed during spermatogenesis, may substitute for this function in the testes of the adult Msh2 mutants since apoptosis still occurred in the Msh2−/− mice. On the other hand it is also possible that the pathway that responds to damage in cancer is not utilised in germ cells. The SCSA did not detect an increased frequency of sperm with abnormal chromatin structure in Msh2−/− adults. However, as this assay detects altered chromatin structure arising from DNA breaks it would not be expected to reveal sperm with persistent DNA mismatches arising from Msh2 deficiency although lesions of this type could have a significant effect on early embryonic development. Pachytene spermatocytes from the Msh2 line did not show elevated levels of damage in terms of γH2AX immunostaining irrespective of genotype which was expected as mismatches do not lead to DNA breaks but could however cause replication errors and mutation.

The effect of p53 deficiency on fertility appears to be mouse strain dependant. On most backgrounds p53−− mice are fertile, however male infertility has been reported in mice on the 129 genetic background (Rotter et al., 1993). In another study, spermatogenesis and fertility appeared to be unaffected by the loss of p53 on a C57BL/6 x C3H background (Embree-Ku and Boekelheide, 2002). However, other papers have reported increased levels of sperm abnormalities, decreased numbers of motile sperm and reduced fertility, on a C57BL/6 background (Schwartz et al., 1999; Yin et al., 1998a). In the present study mice were on a CBA background, which has not been studied before in terms of p53 deficiency. This study presents new data in these mice regarding an increase in the level of damaged sperm using the SCSA and the associated increase in γH2AX foci in the p53+− and the p53−− spermatocytes. The SCSA results contradict previous studies (Marty et al., 1999), showing a
decrease in sperm DNA damage using the COMET assay, this was however on a 129 x C57BL/6 background. p53 has previously been implicated in the NER pathway (reviewed in Seo and Jung, 2004). It would therefore be of interest to investigate whether there were any changes in the activity of the pathway in these mutant mice. p53 has also been shown to play a part in DNA repair via direct roles in HR and the non homologous end-joining of DSBs (Bakalkin et al., 1993; Linke et al., 2003; Tang et al., 1999). It is therefore not surprising that an increase in DSBs were observed as an increase in γH2AX foci. Given that an increase in foci was observed in the p53 +/- it is surprising that a similar increase was not observed in the TG-Erec1 +/- spermatocytes. In addition to this a small number of p53-/- and +/- pachytene spermatocytes appeared to have no sex body, a stage at which the X and Y chromosomes should produce a strong γH2AX signal. This could possibly be due to precocious XY dissociation, which has been reported in studies investigating the effects of heat stress on mice (van Zelst et al., 1995; Waldbieser and Chrisman, 1986b). It may also be explained, however, by the mouse background: CBA mice have been found previously to have an increased incidence of univalent chromosomes in spermatocytes compared with other mouse strains (Krzanowska, 1989; Polanski, 2000). If this were the case however, similar numbers of cells with dissociated chromosomes would be present in the +/- mice, which was not observed in this study but could be clarified by increasing the n number. The level of apoptosis was also increased in the p53 -/-, indicating that loss of p53 did not prevent apoptosis. A number of studies have reported that the apoptotic mechanism that responds to disruption of meiosis in spermatocytes is p53-independent and that FAS (CD95) is responsible for this p53-independent pathway for germ cell elimination (Yin et al., 2002). FAS is a transmembrane receptor protein that is capable of initiating apoptosis in response to binding its ligand, FASL (CD95L) (Nagata and Golstein, 1995). Although DNA damage-induced apoptotic elimination of spermatocytes is reported to be p53 independent, spermatogonial apoptosis, at least following irradiation treatments, is thought to be p53 dependent (Odorisio et al., 1998). In the present study, the majority of dying cells in the p53 +/- appeared to be spermatogonia suggesting that apoptosis in this case does not depend solely on p53 and points
towards the activation of a p53-independent pathway in spermatogonia following heat stress. The increase in caspase-3 staining we observed supports this as caspase-3 mediates apoptosis in both the intrinsic (p53-dependent) and the extrinsic (p53-independent) pathways (Zimmerman et al., 2001; Orrenius, 2004).

In summary this study has confirmed our hypothesis that loss of Ercc1 and Msh2 would disrupt spermatogenesis and that loss of p53 would result in the production of abnormal sperm. The current study has provided new evidence that p53 and Ercc1 play an essential role in DNA damage repair during spermatogenesis and further confirms that p53 is more than just a ‘checkpoint’ protein (on a CBA background) and its role in damage repair, for example in NER/HR. In addition, the study has shown that lack of Msh2 affects germ cell complement in the testis and that a deficiency in the former two genes results in the production of abnormal sperm. This may compromise embryo development and survival or possibly result in problems in offspring later in life both of which should be further investigated. It would also be of interest to investigate whether deficiencies in these genes make the testes more susceptible to insults such as heat/oxidative stress and the effect this has on their offspring.
Mild scrotal heat stress causes DNA damage and subfertility

4.1 Introduction

The impact of damaged DNA originating in the male germ line is poorly understood, but may contribute to early pregnancy loss (recurrent miscarriage), birth and developmental defects and/or have consequences for the offspring later in life. For example, a number of studies have shown that DNA damage in sperm caused, for example, by oxidative stress (in smoking fathers) can be passed from the father to the offspring (Zenzes, 2000; Zenzes et al., 1999) and that this is associated with an increased incidence of childhood cancer in their offspring (Ji et al., 1997; Sorahan et al., 1997). There are thus concerns over the risks of using sperm with DNA damage from subfertile men that could lead to increased incidence of early embryonic failure, birth defects and health problems in their children (Gosden et al., 2003).

4.1.1 Temperature, the testis and spermatogenesis

In the male, problems with spermatogenesis remain the most common cause of infertility (Irvine, 1998). Spermatogenesis and normal testicular function are both temperature dependant and in most mammals the testes are kept between 2 and 8°C below core body temperature (Harrison and Weiner, 1948). Testicular temperature is regulated by a counter-current heat exchange system between incoming arterial blood and outgoing venous blood, the temperature of which is lower than that of arterial blood due to the loss of heat through the skin of the scrotum. The fact that the scrotum has no subcutaneous fat helps to dissipate heat to the exterior (Glad Sorensen et al., 1991).

In man, raised scrotal temperature may occur as a result of occupational exposure, lifestyle or a clinical disorder (Mieusset et al., 1987). Occupational exposure can occur in men who work in high temperature environments such as bakers and welders (reviewed in Thonneau et al., 1998) and also occupations that involve long
periods in a sedentary position such as professional drivers. Recent studies have also reported that posture and clothing can cause increased scrotal temperature (Mieusset et al., 2007). Clinical disorders including cryptorchidism, where one or both testes fail to descend into the scrotum and remain in the abdominal cavity, can also result in the exposure of the testes to abdominal temperatures. Men with scrotal temperatures above the normal range often exhibit fertility problems and their ejaculates have been shown to contain an increased incidence of abnormal and immature sperm (Mieusset et al., 1987).

A number of animal models of heat stress have been generated either by a transient exposure of the testes to heat or by surgically inducing cryptorchidism. Both methods have been reported to cause a variety of problems including a decrease in testicular weight, increased apoptosis and germ cell loss and altered fertility (Lue et al., 1999; McLaren et al., 1994; Setchell et al., 1998; Setchell et al., 1996). The majority of previous studies have looked at the effects of a single temperature and some for long periods of heat stress (12h and 24h). However, there are few studies looking at the effects of a mild, transient, heat stress over a range of temperatures and the effects these different temperatures have on fertility and embryo development.

### 4.1.2 Aims of this chapter

The principal aim of this study was to complement and extend previous studies on the effects of increased scrotal temperature on testicular function and fertility using a mouse model system. This was carried out by looking at three different temperatures (38°C, 40°C and 42°C) to determine whether there was increasing disruption to testicular function with increasing temperature. Another aim was to examine the integrity of DNA in spermatocytes and determine the effect of sperm resulting from these spermatocytes on fertilisation, embryo development and survival using natural matings and IVF.
4.2 Materials and Methods

4.2.1 Animals and treatments

C57BL/6 males aged approximately 8 weeks were used for scrotal heat experiments. All mice were purchased from Harlan (Harlan Sprague-Dawley Inc, Oxford, UK). Female mice for natural matings were C57BL/6 aged approximately 10 weeks and were also purchased from Harlan. Female F1 (from CBA male x C57b/1 female) mice used for IVF/superovulation were aged 6-8 weeks and were bred in house at the University of Edinburgh animal facilities.

Male mice were anaesthetised and subjected to scrotal heating as described in chapter 2, sections 2.2.1 and 2.2.2. Briefly, the lower third of the body was passed through a hole in a polystyrene raft and placed in the water bath for 30 min. Experiments were carried out at the following temperatures: 38°C, 40°C and 42°C. Control animals received anaesthetic and remained in their cages. All animals were administered an anaesthetic reversal reagent according to their body weight (section 2.2.1) and their cages placed on a warm mat to maintain body temperature and facilitate recovery. For each temperature a minimum of 3 mice per group were studied at each time point.

4.2.2 Tissue recovery

Animals were sacrificed at the following time points after heating in order to analyse the effects on specific germ cells (shown in brackets) and also the testis as a whole: 3h (spermatozoa), 6h (spermatozoa), 24h (spermatozoa), 48h (spermatozoa), 7 days (spermatids), 14 days (spermatids) and 28 days (spermatocytes). At each time point one testis was removed and fixed in Bouins (section 2.3.1), the other testis removed and halved with one half frozen on dry ice and the other placed in PBS for germ cell extraction (section 2.6.1). The epididymides were used for sperm retrieval (section 2.5).
4.2.3 Terminal deoxynucleotidyltransferase mediated deoxy-UTP nick end labelling (TUNEL)

The in situ TUNEL method was used to identify cells with DNA fragmentation (Gavrieli et al., 1992) using a standard protocol, which is detailed in chapter 2, section 2.4.4. Assessments of TUNEL-positive cells were performed by counting the total number of positive cells in 100 tubule cross sections per animal.

4.2.4 Meiotic spreads and immunostaining of spermatocytes

Meiotic spreads were performed as detailed in chapter 2, section 2.6. Briefly, germ cell suspensions were prepared from heated and control testes and fixed on glass slides as described in the previous chapter and in more detail in chapter 2 section 2.6.1. For immunostaining of spread spermatocytes, slides were washed in PBS for 5 min, blocked with blocking buffer for 1h before being incubated overnight at 4°C with the primary antibodies (anti-SCP3 mouse monoclonal (1:400, Abcam, Cambridge UK) and anti-γH2AX rabbit polyclonal (1:200, Upstate Biotechnology, MA, USA)). The slides were incubated with the secondary antibodies (goat anti-mouse Alexa-546, goat anti-rabbit Alexa-488 both 1:500, Molecular probes) for 1h at room temperature followed with DAPI nuclear stain (Sigma) at 1:1000 in PBS for 10 min. All washes between incubations consisted of 2 x 5min in PBS. Lastly, the slides were mounted in Permafluor aqueous mounting medium and analysed using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). γH2AX quantification was performed as described in chapter 2, section 2.6.2.

4.2.5 Sperm preparation for counts, morphology and SCSA

Sperm samples from heated and control mice were prepared as detailed in chapter 2, section 2.5.2. For sperm counts (section 2.5.3) the samples were diluted in 4% paraformaldehyde (PFA) and counted using a hemocytometer. Two counts were made for each sample and the mean taken. Morphological analysis of sperm was
performed using the Reastain Quick-Diff staining kit (Oy Reagena Ltd, Toivala, Finland) according to the manufacturer’s instructions. This process is detailed in chapter 2, section 2.5.4. Sperm samples were also used for analysis of chromatin structure in the SCSA as detailed in chapter 2, section 2.5.5.

4.2.6 Fertility analysis

Six control males and six heated males (singly housed) were set up to mate with three females each. The matings were set up for 5 days, 23 to 28 days after heat treatment. This time point was chosen to reflect an insult to the spermatocytes at the time of heating. The time taken for spermatocytes to mature and reach the point of ejaculation is 19 to 32 days (Oakberg, 1956) (Russell et al., 1990a), which includes a 5-day epididymal transit time. Detection of a postcoital plug was taken as evidence of mating and defined as embryonic day (e) 0.5. Plugged females were separated from their mate and date of plug noted. Females were killed and fetuses were examined at embryonic day (e) 14.5. On day 5 of the mating period males were killed and tissue was recovered as described above in section 4.2.2.

4.2.7 IVF

IVF was carried out as detailed in chapter 2, section 2.11. Superovulated control female mice were used for oocyte collection and sperm were isolated from the cauda epididymides and vas deferens from control males and males subjected to a 30min heat stress at 42°C (section 4.2.1). The number of embryos that progressed to the 2-cell stage, 4-cell stage and blastocyst stage were scored. Blastocysts obtained from IVF were washed in PVP/PBS twice, fixed in 4% PFA (20 min) and stained for 10 min with DAPI nuclear stain.

4.2.8 Blastocyst retrieval from mated females

Timed matings were set up as described above (section 4.2.6) and females that had plugged were sacrificed at e3.5. The uterus and ovaries were dissected intact into RPMI media (Sigma). In a sterile petri dish containing RPMI the uterus was cut
below the oviduct and at the bottom of the uterine horn. A sterile needle and syringe containing RPMI was inserted into one end of the cut uterine horn and the media was slowly released pushing the contents of the uterus out into the media. The media in the dish was examined under a dissecting microscope (Leica MZ6) and blastocysts were recovered and transferred through a series of PBS and PBS/PVP washes using a fine-drawn glass pipette before being fixed in 4% PFA for 20 min.

### 4.2.9 Immunostaining of blastocysts

Full details of the blastocyst immunostaining protocol are provided in chapter 2, section 2.11.13. Briefly, blastocysts retrieved from females mated with heated or control males were fixed in 4% PFA for 20 min and permeabilised in 0.25% Triton-X/PBS. Blastocysts were washed thoroughly in PBT (PBS, 0.05% Tween-20) and blocked in 2% goat serum (30 min) before being incubated with primary antibodies directed against Oct3/4 (1:200; Santa Cruz) and ZO-1 (1:200; Zymed) for 1h at room temperature. After thoroughly washing in PBT, the blastocysts were incubated in fluorescently labelled secondary antibodies (goat anti rabbit Alexa-546 and goat anti mouse Alexa-488). Blastocysts were washed again and incubated in Dapi nuclear stain before four final washes in PBT. Blastocysts were mounted in a droplet of PBS on a glass-bottomed petri dish for immediate visualisation of immunostaining on a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd).

### 4.2.10 Statistical analysis

Results expressed as means and standard errors of the mean were analyzed using the student’s t-test or a one-way ANOVA followed by the Bonferroni post hoc test, using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA). Control values represented in each figure consist of the controls studied at each temperature and each time point pooled into one group. Analysis of variance was carried out to determine that there was no significant variance between each control temperature group or time group.
4.3 Results

4.3.1 Heat induced altered testicular architecture

Haematoxylin and eosin stained testes were examined for changes in histology at various time points after heating. This revealed disturbances in testicular architecture in the 40°C group and extensive changes in the 42°C group (Figure 4.1 & Figure 4.2). There were no obvious changes to testicular histology in the 38°C group (Figure 4.1B, F & J and Figure 4.2A & B). Testes from mice subjected to a single transient heat stress of 40°C exhibited some degree of germ cell loss by 48h (Figure 4.2F, star) and also the appearance of cells that were thought to be apoptotic due to the appearance of their nuclei, which had abnormal chromatin condensation (Figure 4.2E). An increase in temperature of only 2°C to 42°C induced much more striking disturbances. Only 6h after heat stress testes contained abnormal cells in the seminiferous epithelium thought to be undergoing apoptosis due to the appearance of their nuclei (not shown). By 24h the seminiferous epithelium contained enlarged degenerating germ cells thought to have been spermatocytes (Figure 4.1D, arrows and Figure 4.2H, arrows). These testes were also characterised by the presence of multinucleated giant cells within the lumen of the testis (Figure 4.2 G, highlighted by ●). By 48h vacuoles or gaps in the epithelium had appeared and this was associated with the loss of ‘giant’ cells (Figure 4.1H, arrows and Figure 4.2H, stars) resulting in extensive germ cell loss. This was visible to a greater extent by 14d after heat stress where many tubules within the testis were severely depleted of germ cells (Figure 4.1L, stars and Figure 4.2I & J, star). It appeared as though most of the tubules with depleted germ cell numbers had lost an entire generation of round spermatids leaving only spermatocytes and elongating spermatids with a narrow linear gap where the epithelium had collapsed (Figure 4.2I, arrowheads in box). The disordered architecture described above was seen in all of the 42°C testes and in the 40°C group the majority of testes exhibited the disturbances described for this temperature.
Figure 4.1 Histological evaluation of testes from adult mice subjected to scrotal heating and their controls. Haematoxylin and eosin staining of testes from control mice (A, E, I) and those subjected to scrotal heating at 38°C (B, F, J), 40°C (C, G, K) and 42°C (D, H, L). Bar = 100μm. Tubules with depleted germ cell numbers are highlighted with stars. Germ cell loss also highlighted with arrowheads in panel K. Giant degenerating cells are highlighted with arrows in panel D. Vacuolisation of seminiferous epithelium highlighted with arrows in panel H.
Figure 4.2 Histological evaluation of higher powered magnification (x100) of testes from adult mice subjected to scrotal heating. Haematoxylin and eosin staining of testes subjected to scrotal heating at 38°C (A & B 24h), 40°C (C, D, E (7d) & F (14d)) and 42°C (G (24h), H (48h), I (24h) & J (7d)). Bar = 50μm. Tubules with normal architecture are highlighted with N (panels A-D). Abnormal degenerating cells are highlighted with arrows (panels E, H & I) and multinucleated giant cells with ✱ (panel G). Germ cell loss is highlighted with stars (panels F, H & J) and epithelial collapse with arrowheads in box (panel I).
4.3.2 TUNEL

TUNEL staining is a marker of cellular DNA fragmentation and is commonly used as a marker of cell death. As expected there were few TUNEL positive germ cells within control testes and the majority were found in stage XII tubules (Figure 4.3A-C). Testes from the 38°C group showed similar levels of positive cells to controls although a slight increase (2x) was observed 24h after heat stress, this was not significantly different from control levels (Figure 4.4). Testes from the 40°C group, however, exhibited a significant increase in TUNEL positive cells by 24h after heating to around 6-fold over control levels. This had returned to control levels by the 48h time point (Figure 4.3G-I and Figure 4.4). A further temperature increase of only 2°C to 42°C resulted in increases in TUNEL positive cells by 6h after heat stress and by 24h a significant increase of around 38-fold over controls was observed and this only returned to control levels 28d after heat (Figure 4.3J-L and Figure 4.4).
Figure 4.3 TUNEL stained testicular cross sections from adult mice subjected to transient scrotal heat stress and harvested 24h, 48h and 14d later. TUNEL positive cells are highlighted with arrows in testes from control mice (A-C), those heated to 38°C (D-F), 40°C (G-I) and 42°C (J-L). Bar=100µm.
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

Figure 4.4 Quantification of cells with fragmented DNA using TUNEL staining. The total number of TUNEL positive cells per 100 tubules were counted per mouse (n≥3). ***(P<0.001) and * (P<0.05) indicates significant variation from control mice.

4.3.3 Impaired DNA repair in spermatocytes

Germ cell spreads prepared from testes exposed to heat stress (38°C, 40°C or 42°C) at various time points after exposure (3h, 6h, 24h, 48h, 7d, 14d, and 28d) were stained for γH2AX to detect unsynapsed DNA or double strand breaks (DSBs). In order to identify those spermatocytes at the pachytene stage of meiosis, the spreads were co-stained with an antibody directed against the synaptonemal complex protein SCP3 (Lammers et al., 1994). As expected the pachytene spermatocytes demonstrated strong, immunopositive staining of γH2AX localised to the sex body (Mahadevaiah et al., 2001), which contained the unpaired regions of the non-homologous X and Y chromosomes (Figure 4.5, arrows) at a stage of meiosis when the autosomes should be fully paired i.e. γH2AX negative.
\( \gamma \text{H2AX} \) immunostaining in controls was low/negative with never more than 2-3 foci on the autosomes of each spermatocyte (Figure 4.5A, B & C). This was quantified by counting the number of \( \gamma \text{H2AX} \) foci occurring on the synaptonemal complexes coincident with SCP3 staining in 50 spermatocytes per mouse. Results from the 38°C group were comparable to controls (Figure 4.5D, E & F) though a slight increase in the number of \( \gamma \text{H2AX} \) foci could be seen at the 6h and 24h time points, this was not significantly different from controls (Figure 4.6). Spermatocytes from the 40°C group exhibited an increase in the \( \gamma \text{H2AX} \) immunostaining of the autosomes (Figure 4.5G, H & I). This was also reflected in the quantification of the number of foci. There appeared to be a clear association between increasing DSBs and temperature (Figure 4.6): in spermatocytes from testes heated to 42°C there was a more pronounced increase in \( \gamma \text{H2AX} \) immunostaining of the autosomes (Figure 4.5J, K & L) with a significant increase in the number of \( \gamma \text{H2AX} \) foci by only 3h after heat stress (Figure 4.6). This increase continued to be significantly different from controls until 14d after heat stress and had only returned to control levels by the 28d time point. Also, in a minority of the spermatocytes from the 40°C group and in several from the 42°C group there appeared to be a dissociation of the X and Y chromosomes with no distinct XY body of intense \( \gamma \text{H2AX} \) immunostaining (Figure 4.5J, inset & K).
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

Figure 4.5 Double strand breaks in pachytene spermatocytes detected using γH2AX immunohistochemistry on germ cell spreads. Immunodetection of SCP3 (red) and γH2AX (green) 24h, 48h and 14d after experiment in controls (A – C), the 38°C group (D – F), the 40°C group (G – I) and the 42°C group (J – L). The sex body is highlighted with white arrows and γH2AX foci with arrowheads. Spermatocyte with dissociated X and Y chromosomes depicted in panel J inset.
Figure 4.6 Quantification of double strand breaks in pachytene spermatocytes detected using γH2AX Immunohistochemistry on meiotic germ cell spreads. Quantification of γH2AX foci per 50 spermatocytes per mouse from testes heated to 38°C, 40°C and 42°C. *** (P< 0.001), ** (P< 0.01) and * (P<0.05) indicates significant variation from control mice, (n≥3).

4.3.4 Sperm counts

Epididymal sperm counts were performed on all three groups of heat-stressed mice. This revealed variability both between mice within the same group and also at different time points after heating (Figure 4.7). There appeared to be a decrease in sperm concentration in all groups by 24h after heat stress however this had recovered back to around control levels by 7 days after heat stress in the 38°C and 40°C groups. The decrease seen at the 24h time point represents the effect on the epididymis, which is very sensitive to disturbances in temperature. Only in the 42°C group did
there appear to be a trend towards decreasing sperm concentration with time after heating. By 14 days after heat stress these mice exhibited a significant decrease compared to controls (Figure 4.7, P<0.01), which is consistent with the long-term effect on testicular function.

![Epididymal sperm counts in 38°C, 40°C and 42°C groups.](image)

*Figure 4.7 Epididymal sperm counts in 38°C, 40°C and 42°C groups.** (P< 0.01) and * (P<0.05) indicates significant variation from control mice, (n≥3). M/ml=millions sperm per ml.

### 4.3.5 Sperm morphologies

The majority of sperm retrieved from control mice and the 38°C group appeared morphologically normal (Figure 4.8) and in these groups the occurrence of sperm with abnormal head or tail morphologies was rare. However samples from the 40°C group (Figure 4.9) had a higher number of sperm with abnormalities and these were even more prevalent in the 42°C group (Figure 4.10). Abnormal sperm from mice subjected to a 40°C scrotal heat had malformed heads with either a shorter and
blunted apical hook (Figure 4.9C & E) or loss of hook and a rounded head (Figure 4.9F & G). The abnormal sperm from epididymides of mice heated to 42°C also had malformed heads with blunted apical hooks (Figure 4.10B, E, F &H). In addition to this some of these sperm had abnormal tail formations including thicker (Figure 4.10C, red arrow) and coiled tails (Figure 4.10D & H, arrowheads). The abnormal sperm seen in these mice backs up the sperm count data.

**Figure 4.8 Morphology of epididymal sperm from 38°C heated males.** Sperm with normal head and tail formation in the 38°C group. Sperm heads were approximately 7-10µm in length.

**Figure 4.9 Morphology of epididymal sperm from 40°C heated males.** Normal head morphology with apical hook (arrowheads). Abnormal head morphology with blunted or no apical hook (arrows). Sperm heads were approximately 7-10µm in length.
4.3.6 Heat stress induced chromatin abnormalities in sperm

The sperm chromatin structure assay (SCSA) was performed on sperm from control and heat stressed mice. This is a population assay that determines the occurrence of acid-induced chromatin damage within a population of 10,000 sperm per sample. The control mice exhibited sperm with DNA damage in low numbers of around 10-15%. After a single transient heat stress of 38°C there was a small increase in the number of sperm exhibiting damage 24h later to 25% representing a slight epididymal effect. This had decreased at 48h and by 7d post heat the numbers had returned to control levels (Figure 4.11). However 24h after a 40°C heat stress the percentage of sperm with damage had increased significantly (P<0.05) to around 30%. Again this appeared to decrease by 48h however by the 7d time point this had again increased significantly compared to controls (representing the effect on the testis; Figure 4.11) but had recovered to control levels by 28d after heat stress. A similar trend could be seen in the 42°C group although at more striking levels. By 24h after heat stress the percentage of sperm exhibiting chromatin abnormalities was around 3 fold over that of controls at 42% and 15% respectively. This had declined by around 10% by 48h though still significantly increased (P<0.05). As in the 40°C...
group the percentage of sperm with DNA damage had increased again by 7d to around 6.5 fold higher than that of control mice. This represents an epididymal and a testis effect of temperature. By 28d this had decreased considerably though not quite to control levels.

![Graph showing susceptibility of epididymal sperm to acid denaturation detected by the SCSA in 38°C, 40°C and 42°C groups. %DFI represents ratio of sperm with denatured DNA (red fluorescence) to total sperm (red/[red+green] fluorescence). *** (P< 0.001), ** (P< 0.01) and * (P<0.05) indicates significant variation from control mice, (n≥3).]

**Figure 4.11 Susceptibility of epididymal sperm to acid denaturation detected by the SCSA in 38°C, 40°C and 42°C groups.** %DFI represents ratio of sperm with denatured DNA (red fluorescence) to total sperm (red/[red+green] fluorescence). *** (P< 0.001), ** (P< 0.01) and * (P<0.05) indicates significant variation from control mice, (n≥3).

### 4.3.7 Reduced fertility in heat stressed males

Females mated to heat stressed (40°C or 42°C) males were assessed for fertility by determining pregnancy rate and fetus numbers at e14.5. The matings were set up for 5 days, 23 to 28 days after a single 30 min heat stress to reflect an insult to the spermatocytes at the time of heating (see section 4.2.6). The pregnancy rate in the
40°C group was comparable to controls (Figure 4.12 and Figure 4.13) however the number of fetuses produced by these females appeared slightly reduced though this was not statistically significant (Figure 4.14). On examining the effects of a 42°C heat stress the females exhibited a drastic reduction in pregnancy rate with a 7-fold reduction to only 14% of the control rate (88%). The females in this group that did achieve pregnancy had reduced numbers of fetuses, which was on average one fetus per female (Figure 4.12, arrowhead and Figure 4.14).

Figure 4.12 Pregnant females (e14.5) previously mated to control males or those that have been subjected to a transient scrotal heat stress (40°C or 42°C). White arrows highlight the uterus void of fetuses. The white arrowhead highlights a single fetus and all other fetuses are numbered.
Mild scrotal heat stress causes DNA damage and subfertility

Figure 4.13 Pregnancy rate of females mated to control males or males that have been subjected to a transient scrotal heat stress (40°C or 42°C). Rates are expressed as percentage of females pregnant per male. *** (P< 0.001) indicates significant variation from control mice. n=6 males and n=3 females per male i.e. n=18 females per group.

Figure 4.14 Effect of transient scrotal heat stress (40°C or 42°C) on number of viable fetuses per female on embryonic day (e) 14.5. *** (P< 0.001) indicates significant variation from control mice, n=18 females per group.
In these females the numbers of resorptions or resorbed fetuses was also counted. This is displayed as the average number of resorptions per female (Figure 4.14, blue bars) and appears only slightly increased in the 40°C group. However on analysing this further by determining the percentage of females per group that had resorption sites, it became clear that this was, in fact, increased to a great extent (Figure 4.15). Suggesting that although the fetuses have survived long enough to implant, they are still failing in the 40°C group.

![Figure 4.15](image)

*Figure 4.15 Occurrence of fetal resorptions in female mice mated to control males or males that have been subjected to a transient scrotal heat stress (40°C or 42°C). Results expressed as percentage of females per group that exhibited resorptions, n=18 females per group.*

### 4.3.8 IVF

In order to examine further at which stage the embryos were failing to progress in the 42°C group, *in vitro* fertilisation was carried out. This was performed using sperm retrieved from males that had been subjected to a single transient heat stress either 16h previously (Figure 4.16) or 23 days previously (Figure 4.17) in order to
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

determine the effects of an epididymal insult and a spermatocyte insult respectively. Embryo development was examined for the 2-cell, 4-cell and blastocyst stages. The results for both the 16h and 23d groups were similar. Both groups exhibited no change in the numbers of embryos developing to the 2-cell or the 4-cell stage (Figure 4.16 and Figure 4.17). The number of embryos developing to the blastocyst stage was greatly reduced in the 16h group (i.e. epididymal insult) by around 40% (Figure 4.16). In the group representing the spermatocyte insult, however, the results were more striking in that all of the embryos that had progressed to 4-cell stage failed to develop into blastocysts (Figure 4.17).

Figure 4.16 Embryo development following in vitro fertilisation (IVF) with sperm recovered from control mice and mice subjected to a single transient scrotal heat stress 16h previously. % 2 cell embryos represents % of total oocytes retrieved. % 4 cell and blastocyst embryos represents the percentage of 2 cell that developed to these stages. n=4 IVF cycles. ** (P<0.01) indicates significant variation from control mice using the Mann-Whitney non-parametric test.
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

4.3.9 Embryo development

The embryos obtained from the flushing of the uterus of mated females, were also assessed for developmental abnormalities. The embryos were flushed at e3.5, at which stage they should have developed into blastocysts. The retrieved embryos were immunostained with antibodies directed against OCT 3/4 and ZO-1. OCT 3/4 is a transcription factor that is expressed in early embryonic development and plays a role in pluripotency. Maternal OCT 3/4 is expressed until the 4-8 cell stage when it switches to embryonic OCT 3/4. When the blastocyst forms expression of OCT 3/4 is downregulated in the trophectoderm cells and expression becomes restricted to the inner cell mass (ICM) (Pesce et al., 1998; Yeom et al., 1996). Zonula occludens-1 (ZO-1) is a tight junction protein and its expression in trophectoderm cells

Figure 4.17 Embryo development following in vitro fertilisation (IVF) with sperm recovered from control mice and mice subjected to a single transient scrotal heat stress 23 days previously. % 2 cell embryos represents % of total oocytes retrieved. % 4 cell and blastocyst embryos represents the percentage of 2 cell that developed to these stages, n=4 IVF cycles. * (P<0.05) indicates significant variation from control mice using the Mann-Whitney non-parametric test.
contributes to the formation of the blastocoelic cavity. This in turn helps protect and control the environment of the ICM, which is found within the blastocoelic cavity and from which the entire fetus develops after implantation (Gardner and Papaioannou, 1975). The blastocysts obtained from females mated with control mice appeared to have a normal structure with OCT 3/4 positive ICM and normal blastocoelic cavity formation with a ZO-1 positive trophectoderm (Figure 4.18). The majority of embryos obtained from the 40°C group appeared morphologically normal however a small number of embryos appeared to be at earlier stages than blastocyst, for example, the 32-cell stage embryo when recovered at e3.5 (Figure 4.18, white arrowhead). It was assumed that these embryos might be normal but were developing at a slower rate. This is, however, based on the assumption that all oocytes were fertilised at a similar time. The embryos obtained from the 42°C group appeared grossly abnormal when compared to the control blastocysts (Figure 4.18 and Figure 4.19). These embryos appeared to have fragmented nuclei, abnormal OCT 3/4 staining and an amorphous appearance (Figure 4.18 and Figure 4.19). No normal blastocysts were observed in the media resulting from the flushing of the uteri of females mated to males heated to 42°C.
Figure 4.18 Blastocyst stage embryos immunostained with OCT3/4 and ZO-1 from female mice mated to control and heated (40°C & 42°C) males. In normal blastocysts (controls) OCT 3/4 is localised to the cells of the inner cell mass (ICM) and ZO-1 is localised to the tight junctions. White arrow shows blastocoelic cavity. Blastocysts measured between 95 and 115 µm.
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

Figure 4.19 Z-stack images of blastocyst stage embryos immunostained with Oct3/4 and ZO-1 from female mice mated to control and heated (40°C & 42°C) males. Oct 3/4 is localised to the cells of the inner cell mass (ICM) and ZO-1 is localised to the tight junctions.
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

Discussion

In most mammals the temperature of the testis is kept between 2°C and 8°C below core body temperature (Harrison and Weiner, 1948). This lower temperature is required for normal spermatogenesis. In man raised scrotal temperature can occur as a result of occupational exposure, lifestyle or a clinical disorder such as cryptorchidism (Mieusset et al., 1987). Men with scrotal temperatures above the normal range are often sub- or infertile and their ejaculates contain an increased incidence of abnormal and immature spermatozoa (Mieusset et al., 1987). In mice, a number of studies have reported that heat stress results in germ cell loss, poor quality sperm with altered DNA integrity and chromatin packaging as well as early embryo loss (Banks et al., 2005; Jannes et al., 1998; Rockett et al., 2001; Zhu and Setchell, 2004). The principal aim of the studies in this chapter was to complement and extend those studies by investigating the impact of a mild transient scrotal heat stress on germ cell survival, germ cell DNA integrity and fertility in mice. Mice were subjected to scrotal heating at three different temperatures: 38°C, 40°C and 42°C and several parameters were evaluated at various time points after heat stress: 3, 6, 24 and 48h and 7d, 14d and 28d.

Previous studies have used a variety of techniques for studying the effects of heat on the testis, including: 1) surgically inducing cryptorchidism (Vigodner et al., 2003; Yin et al., 1997; Yin et al., 2002; Yin et al., 1998b); 2) exposing the testis to microwave radiation (Saunders et al., 1983; Saunders and Kowalczuk, 1981); 3) exposure of the whole body of the animal to a hot environment (Zhu et al., 2004; Zhu and Setchell, 2004); 4) insulation of the scrotum for either intermittent periods (Arman et al., 2006; Mieusset et al., 1992a) or continuously (Fleming et al., 2004; Vogler et al., 1991; Walters et al., 2006); 5) immersion of the scrotum in a water bath (Banks et al., 2005; Lue et al., 2000; Lue et al., 1999; Lue et al., 2002; Rockett et al., 2001; Setchell et al., 2001; Setchell et al., 2002; Zhang et al., 2005).
Chapter 4  Mild scrotal heat stress causes DNA damage and subfertility

It was well documented in earlier studies that pachytene spermatocytes and early spermatids are the cell types that are most susceptible to heat (reviewed in Setchell, 1998). In the present study the disturbances observed in testicular architecture and TUNEL staining are consistent with these reports. For example, during this study the effects of a 38°C, 40°C and 42°C heat stress on the histology of the testis were evaluated and impact on germ cell loss was shown to be temperature related with the highest temperature having the most drastic effects. Heat stress at 42°C induced the formation of giant degenerating germ cells, multinucleated giant cells, and was accompanied by germ cell loss and vacuolisation of Sertoli cell cytoplasm. During the first 48h after heat stress the cells that started to deteriorate and die were predominantly spermatocytes. By 7d many tubules had lost an entire generation of round spermatids leaving only spermatogonia, early spermatocytes and a reduced number of sperm. These changes were associated with an increase in TUNEL-positive staining of spermatocytes suggesting that germ cell elimination occurred via an apoptotic mechanism. The formation of multinucleated giant cells has been reported previously in cases of induced cryptorchidism (Chaki et al., 2005; Yin et al., 1997) and also localised scrotal heating (Chowdhury and Steinburger, 1970; Waldbieser and Chrisman, 1986a). These cells may be the result of sloughing of Sertoli cells or round spermatids, however, these structures did not appear positive after TUNEL staining. This aggregation of abnormal and apparently degenerating cells appears to be unique to the testis and may be related to the phagocytic properties of the Sertoli cells (Mizuno et al., 1996). In contrast the numbers of TUNEL-positive spermatogonia did not increase markedly after heat stress and these cells were therefore able to participate in the repopulation of the testis with germ cells allowing recovery of full spermatogenesis. To a lesser extent than the 42°C group, effects were also seen in the 40°C group exemplified by the detection of degenerating cells and some germ cell loss but at a much lower level. A minor, non significant, increase in TUNEL positive cells was detected 24h after heat stress at 38°C. There notably, seems to be a cell-dependent threshold for heat-induced expression of specific stress response proteins, for example heat shock factor 1 (HSF1) is activated in isolated male germ cells at 38°C whereas in the Sertoli cells
activation does not occur unless temperatures are above 42 °C (Sarge, 1995). This is paralleled by activation of certain heat shock proteins (Hsps) above specific temperatures, for example Hsp70 is expressed in germ cells including pachytenes around 42°C (reviewed in Sarge and Cullen, 1997). Activated heat-dependent genes may be involved in the elimination process of germ cells beyond a specific temperature threshold, for example activation of HSF1 can induce apoptosis in pachytene spermatocytes (Nakai et al., 2000).

Further investigations on the impact of heat stress on the spermatocytes revealed that heat stress resulted in an increased incidence of DNA stand breaks i.e. γH2AX foci, in pachytene spermatocytes. Phosphorylation of histone (H) 2AX occurs following formation of DSBs (Rogakou et al., 1998) and a dramatic increase in DSBs was detected in germ cells in the 42°C group, which was consistent with the observed increase in cell death in this group compared with those subjected to lower temperatures. No increase was observed in the 38°C group but a significant increase in DSBs was evident 24h and 48h after a 40°C heat stress. It is unclear whether the heat stress applied in the current study is inducing chromosome aberrations or impairing their repair. Studies by Rockett et al. (2001) have shown that a number of DNA repair genes are downregulated after a 20 min heat stress at 43°C, including XPG, RAD54 and MSH6 suggesting that inhibition of repair functions could be an important factor. γH2AX is thought to play a direct role in the induction of apoptosis by interacting with p53 (Hamer et al., 2003) causing p53 to translocate to the mitochondria triggering a wave of caspase-3 activation (Erster et al., 2004). There are however conflicting reports on the induction of apoptosis following heat stress in the mouse testis. For example, some studies suggest it is the intrinsic mitochondrial pathway that is induced after heat resulting in the activation of the initiator caspase-9 and the executioner caspase-3 and that the Fas-FasL pathway is redundant (Hikim et al., 2003; Vera et al., 2004). However, others have suggested it is mainly the Fas signalling pathway that is induced upon heat stress in the mouse testis (Miura et al., 2002). It is therefore possible that both pathways play a role in the elimination of germ cells after heat stress.
In the current study distinct staining of an XY body was absent in some of the pachytene spermatocytes although γH2AX were present. Previous studies have reported heat-induced XY chromosome dissociation in diakinesis-metaphase I primary spermatocytes (van Zelst et al., 1995; Waldbieser and Chrisman, 1986b), however, no one has reported that this can occur at the (earlier) pachytene stage. The cause of this phenomenon remains unclear, though Waldbieser and Chrisman (1986) postulated that heat stress interferes with X inactivation which in turn interferes with X and Y chromosome pairing.

The disruption of testicular function induced by heating was mirrored by changes in a number of sperm parameters consistent with an impact on epididymal as well as testicular function. Sperm concentrations varied widely between individual mice and although there appeared to be a trend towards a decrease at 24h and return to control levels by 7d it was only in the 42°C group that had a continued decline from 24h onwards with significant decreases observed at 48h, 7d, 14d and 28d time points. The heat stress also appeared to have an effect on the morphology of sperm. There was a slight increased incidence of head abnormalities in the 40°C group by 24h. These abnormalities were also evident in the 42°C group at all time points between 24h and 28d along with abnormal tail coiling. Abnormalities observed in mature spermatozoa retrieved from the epididymis 24h and 48h after heat reflect an epididymal insult, those retrieved at 7d and 14d after heat reflect an insult on spermatids and those retrieved at 28d reflect an insult on spermatocytes. These timings are based on a 5d epididymal transit time (Oakberg, 1956).

Whilst many germ cells appeared to be eliminated by apoptosis especially in the 42°C group, this process was not 100% efficient as sperm with increased DNA fragmentation as assessed by the SCSA, were recovered from the epididymis and therefore had undergone meiosis I and II, spermiogenesis and release. Studies comparing the pregnancy outcome from patients whose partner had a high SCSA value reported that this assay can identify the likelihood of achieving a pregnancy and that this has proven to be more reliable than conventional measurements of
sperm parameters (Larson-Cook et al., 2003; Virro et al., 2004). In this study, it was apparent that testicular heat stress resulted in the release of sperm with DNA damage with the highest occurrence being in the 42°C group at 7d and 14d after heating. This agrees with results in a previous study by Sailer et al. (1997), which reported that epididymal sperm were more susceptible to acid denaturation in the SCSA on days 3, 7, 11 and 14 after heat stress. This is also consistent with the suggestion that spermatocytes and spermatids are the germ cell types most susceptible to heat stress as the mature sperm retrieved from the epididymis at day 14 would have been late pachytenes/early spermatids when thermal stress occurred. In addition it suggests that germ cells are only capable of limited DNA repair and prompts the question: do pre-meiotic (i.e. spermatogonia) germ cells have a greater capacity for repair, making them less susceptible to stress-induced abnormalities? A significant increase in the number of sperm with DNA damage was also observed at the 24h time point (42°C and 40°C). This would reflect the status of the DNA in sperm that were present in the epididymis at the time of the insult and may be due to an increased level of oxidative stress and thus a less favourable epididymal environment as has been suggested in previous studies (Banks et al., 2005). Previous investigators have also reported that increased temperature causes changes in oxygen levels, water and ion transport mechanisms, protein synthesis and secretion and also in the cell structure of the epididymis (Djakiew and Cardullo, 1986; Seiler et al., 2000). It is therefore likely that this disturbance in the environment of the epididymis causes the abnormalities detected in the morphology and integrity of the sperm. Spermatids, during the normal process of spermiogenesis, incur strand breaks to facilitate condensation of germ cell DNA and replacement of histones with protamines (Marcon and Boissonneault, 2004). It therefore logical that this stage of germ cell maturation would be one of those most vulnerable to heat-induced DNA damage. In the 38°C group there was an increase (not significant) in the percentage of sperm with DNA damage at 24h but not at the other time points indicating that this temperature is high enough to have an epididymal effect but not a testis effect.
The effects of heat stress on the testis discussed above, such as increased apoptosis and germ cell loss, increased incidence of DSBs and sperm chromatin damage at the early stages are all primary effects of heat stress and may not be the full story. The fact that we still detect disturbances in spermatogenesis days and weeks after the single heat stress begs the question, why does the testis take so long to recover? The occurrence of abnormal structures such as multinucleated giant cells that are TUNEL negative and the lengthy recovery suggests that testicular hyperthermia results not only in an acute effect but in long term secondary effects which appear to include disruption of the testicular environment. Studies on the rat have shown that increased testicular temperature caused by induced cryptorchidism can result in lowered levels of plasma testosterone, LH, FSH and inhibin B (Ren et al., 2006) and similar studies in rats subjected to scrotal heating in a water bath showed reduced testicular testosterone levels (Lue et al., 1999). Studies on cynomolgous monkeys have also reported heat-induced decreases in testosterone and inhibin B levels (Lue et al., 2002). Testosterone is known to act upon the Sertoli cells and PTM cells which express the androgen receptor (AR) (Bremner et al., 1994) and expression of AR in Sertoli cells is essential for fertility (De Gendt et al., 2004). As germ cell maturation is dependent on structural and functional support by Sertoli cells, it is not surprising that any effects of heat on Sertoli cell function could also have a longer-term effect on spermatogenesis. For example, decreased testosterone has been shown to alter the polymerisation of vimentin, which the Sertoli cell intermediate filament cytoskeleton is composed of (Show et al., 2003) and microarray studies of heat-stressed testes have shown upregulation of genes involved in cell adhesion such as laminin suggesting perturbation of the basal lamina-Sertoli cell contacts (Rockett et al., 2001). Interactions between the basal lamina and Sertoli cells are essential for maintaining barrier functions and normal spermatogenesis (Tung and Fritz, 1993). Any disruption to the supporting cells in the testis would most likely result in long term effects on the cells they are supporting i.e. the germ cells.

A number of studies have shown that DNA damage in sperm, caused by oxidative stress (in fathers who smoke), can be passed on from father to child causing an
increased incidence in childhood cancer in their offspring (Zenzes, 2000; Zenzes et al., 1999). This has raised concerns over the use of sperm from subfertile men in assisted reproduction (IVF and ICSI). Thus, the final aim of this part of the study was to determine whether any of the aforementioned heat induced aberrations resulted in problems with fertility and had any effect on embryo development. To test the fertility of the males, natural matings were set up for five days, 23 to 28 days after heat stress (40°C and 42°C) a time chosen to reflect an insult to the spermatocytes at the time of heating. No difference was observed in the pregnancy rate in the 40°C group as compared with controls, however the 42°C group exhibited a 7-fold reduction and those that did achieve pregnancy exhibited a reduction in the number of fetuses at e14.5, however an increase in resorption sites was not observed. Therefore it was concluded that either there was a reduction in fertilisation rate or embryos were failing to implant. This data is consistent with a recent study by Perez-Crespo et al. (2007), which found a decrease in the number of pregnancies and fetuses but no change in resorption rate. Fetus number in the 40°C group appeared very slightly reduced (not significant), however the percentage of females mated to the 40°C males with resorption sites was greatly increased: around 55% compared to less than 20% in the controls and in the 42°C group. This suggests that the embryos resulting from matings where the spermatocytes were subjected to a 40°C heat shock can develop past the ‘block’ that occurred in the 42°C group but then failed to progress so that the resorptions were detectable at e14.5. It has been suggested that the paternal genome is important for the development of extraembryonic tissues, the trophoblast and the general growth of the embryo and this may explain why the development of the fetuses in this study was not normal. For example, one of the first studies on genomic imprinting showed that embryos with two female pronuclei can implant but undergo resorption and exhibit poor extraembryonic membrane and trophoblast formation (Surani et al., 1984). In the current study the male genome appears to be compromised and fetal development impaired. Due to the minimal effects of testicular heating on fertility at 40°C, a further study was not conducted with the 38°C group. It is clear that the length of time the mice are exposed to heat is also important as a recent study found scrotal heating at only 36°C but for a period of
12h reduced pregnancy rate and litter size and impaired embryo development in IVF (Yaeram et al., 2006) whereas we saw no such difference when the heat stress at 40°C was for only 30 min. A number of different studies looking at the effect of heating for 24h at 36°C also reported abnormal development in embryos recovered from females mated to heat-stressed males including reduced numbers of embryos and blastomeres and altered gene expression at the 2-cell stage (Zhu and Maddocks, 2005; Zhu et al., 2004; Zhu and Setchell, 2004).

In order to analyse embryo development more closely, IVF was carried out with males subjected to a 42°C heat stress. This was carried out with males that had been heated either 16h or 23d before to reflect an insult on the epididymis or on the spermatocytes respectively. The latter time point also mirrors that of the natural matings. Although there was no real difference observed in the number of embryos developing to the 2-cell or 4-cell stages in both groups, there was a significant reduction in those that developed to blastocysts. In the 16h group the numbers of developed blastocysts were 40% less than controls and in the 23d group the embryos did not develop to the blastocyst stage. This confirms the data from the natural matings and clarifies at which stage the embryos were failing i.e. between 4-cell and blastocyst. This suggests that though the sperm with DNA damage are still capable of fertilisation, the DNA that is passed on is introducing genomic instability to the embryo and having fatal effects on development.

Embryos generated from the natural matings were immunostained with ZO-1, a tight junction protein, and OCT 3/4, a transcription factor and a marker of pluripotency. These proteins are localised to the trophoderm and inner cell mass (ICM) respectively. Embryos harvested from the control group appeared morphologically normal with distinct blastocoelic cavity and trophoderm formation. The majority of embryos from the 40°C group were also normal, however there were some, which appeared to be at an earlier stage of development although they were harvested at the same time as controls. This could be accounted for by the fact that not all oocytes will have been fertilised by the sperm at the same time or it may possibly be that the
Mild scrotal heat stress causes DNA damage and subfertility

embryos generated using sperm from the 40°C males developed at a slower rate than controls. The embryos produced from females mated to the 42°C group of males had a drastically different appearance from controls. The majority had obviously arrested, become fragmented and were grossly abnormal compared to control blastocysts. This coupled with the IVF results confirms that the embryos were failing before reaching blastocyst stage. Given that maternal OCT3/4 is expressed until the 4-8 cell stage at which point it switches to embryonic OCT3/4 (Pesce et al., 1998; Yeom et al., 1996) it would not be surprising if a number of other genes were to switch around the same time. It is thus logical that there would be an embryonic block around this time when the embryonic genes are switching on (Zeng et al., 2004). Reactivation of the paternal genome occurs during early cleavage from the one-cell stage onwards (Matsumoto et al., 1994) and therefore any mutations/deletions of DNA in the male genome could result in the disruption of regulatory networks in early embryos. For example, un-repaired damaged DNA could block transcription and lead to miscoding. The damage could also disrupt DNA replication during the rapid early cleavage divisions, triggering gene expression changes and genome instability in the embryos. The fact that the embryos resulting from heated males were able to undergo the first two cleavages as normal but fail after this point further suggests that the paternal genome is not involved in these cleavages but becomes more active after the 4 cell stage. This agrees with a recent study showing the effects of irradiation-induced sperm DNA damage on bovine embryonic development, which reported normal fertilisation but embryo arrest before blastocyst formation (Fatehi et al., 2006). Studies investigating gene expression in the preimplantation embryo have demonstrated a wave of reprogramming at the 4- to 8-cell stage (Hamatani et al., 2004; Wang et al., 2004) which is presumably when the embryos in this study died. While reports on the activation of paternal genes describes active transcripts as early as the 1-cell stage and paternally derived protein at the 2-cell stage (Matsumoto et al., 1994), others suggest that the paternal genome is not required before the 8-cell stage in mice (Renard et al., 1991).
4.4 Conclusions

This study has confirmed that spermatogenesis is susceptible to scrotal temperatures above the normal physiological range and shown that there may be a temperature threshold, which is also influenced by duration, above which germ cell degeneration is induced. The current studies also demonstrate that although the testis contains an active apoptotic machinery it is not 100% efficient at eliminating germ cells with abnormalities and that many of these abnormal cells go on to develop into mature sperm with DNA damage. These studies also show that increased scrotal temperature causes subfertility in male mice by affecting pregnancy rate, resorption rate and embryo development in normal control females. This provides further evidence of male-mediated effects on embryo survival. It would be of interest to determine any transgenerational effects on offspring produced from these ‘heated’ fathers. These findings should be taken into consideration when using sperm from infertile men in IVF/ICSI treatments where the normal quality control processes involved in fertilisation are bypassed. This does, however, still leave the question of what is the underlying cause of the primary effects seen here in the testis, such as increased DSBs and apoptosis seen after heat stress. One possibility is that the testis may be becoming hypoxic as a result of heat stress and inadequate blood flow into the testis and this topic of heat-induced hypoxia and the stress response is investigated and discussed in the next chapter (Chapter 5).
5 Mild Scrotal heat stress and the stress response

5.1 Introduction

Spermatogenesis can be adversely affected by exposure to environmental stresses such as hyperthermia. The testis displays a variety of mechanisms that are triggered on exposure to stress, including DNA repair (discussed in chapter 4), the heat shock response, the oxidative stress response and also apoptosis and cell death. The importance of the thermoregulation of the testis is illustrated by the fact that slight increases in temperature can disrupt spermatogenesis and cause infertility, as detailed in chapter 4.

Heat stress (among other stimuli) induces the expression of heat shock proteins (Hsps) via the activation of heat shock transcription factor 1 (HSF1) (Morimoto, 1998). Activation of heat shock genes is mediated by HSF1, which, under stress conditions, trimerises, acquires DNA binding activity and interacts with heat shock elements (HSEs) in the promoter regions of Hsp genes thereby promoting expression of Hsps (Baler et al., 1993). During heat shock, the Hsps function as molecular chaperones that bind to, and aid the folding of, damaged proteins, thereby preventing protein aggregation under stressful conditions (Hartl and Hayer-Hartl, 2002; Young et al., 2003). Hsp70 and Hsp90 are both expressed under normal and stress conditions and facilitate the correct folding of proteins (Mayer and Bukau, 2005; Wiech et al., 1992). They have also been implicated in the prevention of apoptosis via the suppression of caspase activation (Mosser et al., 2000). Hsp105 is a testis-specific Hsp90-related protein and has previously been shown to exhibit increased expression in the rat in response to experimental cryptorchidism (Kumagai et al., 2000). In addition to this, Rockett et al. (2001) showed, using microarrays, that Hsp40, Hsp60 and Hsp70 are all upregulated only a few hours after heat stress. Another stress response protein, Stress inducible protein (Stip)-1, that is expressed in the mouse testis (Mizrak et al., 2006), has been shown to directly regulate the individual functions of Hsp70 and Hsp90 (Song and Masison, 2005) and is involved
in the chaperoning of these Hsps (70 and 90) (Odunuga et al., 2004; van der Spuy et al., 2001).

As well as being involved in the normal maintenance of germ cell number in the testis (de Franca et al., 1993), cell death or apoptosis has been shown previously, by TUNEL or caspase expression, to be induced by hyperthermia. For example, testicular heat stress has been shown to cause apoptosis of spermatogonia, pachytene spermatocytes and early spermatids (Hikim et al., 2003). There are two main pathways of apoptosis: intrinsic and extrinsic (Hengartner, 2000; Reed, 2000). The intrinsic pathway involves the mitochondria, the initiator caspase-9 and the executioner caspases-3, 6 and 7 (Orrenius, 2004). The extrinsic pathway involves ligation of the death receptor Fas to its ligand (FasL), the initiator caspases 8 or 10 and the executioner caspases 3, 6 and 7 (Zimmermann et al., 2001). The apoptotic response to stress is usually mediated through the intrinsic pathway which is modulated by the Bcl2 family of proteins: Bcl2 itself is an apoptosis inhibitor and Bax is proapoptotic (Adams and Cory, 1998; Borner, 2003). It has also been reported that induced cryptorchidism causes the Hsp105 to bind to p53 and is a possible mechanism of inducing apoptosis (Kumagai et al., 2000) following an elevation in temperature. There are, however, conflicting views on the mechanisms that induce apoptosis following heat stress in the mouse testis. For example, some studies suggest that the intrinsic pathway is induced after heat resulting in the activation of the initiator caspase-9 and the executioner caspase-3 and that the Fas-FasL pathway is redundant (Hikim et al., 2003; Vera et al., 2004). However, others have suggested that hyperthermia induces the Fas signalling pathway (Miura et al., 2002).

Studies on blood flow into the testis have suggested that the increased metabolism in the testis after heat stress is not met by a sufficient increase in blood flow (Galil and Setchell, 1988) thus leading to the possibility of the testis becoming hypoxic (reviewed in Setchell, 1998). Hypoxia occurs when the oxygen tension drops below that required for normal cellular function in a particular tissue, which can be caused by inadequate blood flow or reduced oxygen transport capacity (Hockel and Vaupel,
2001). Hypoxia leads to cell cycle arrest and apoptosis (Carmeliet et al., 1998; Iida et al., 2002). Previous studies have shown that exposure of mice to hypobaric hypoxic conditions induces Hypoxia inducible factor1 (Hif1)-α expression in spermatocytes and Sertoli cells (Marti et al., 2002). Hif1 is a transcription regulator and is composed of α and β subunits, which dimerise under hypoxic conditions (Wang and Semenza, 1995). Upon Hif1 activation Hif1α binds to hypoxia response genes to restore oxygen homeostasis by activating genes involved in vasodilation, angiogenesis and glycolysis (Shweiki et al., 1992; Wenger, 2002). The increase in germ cell DNA damage following heat stress (chapter 4) could be linked to the testis becoming hypoxic. Although it is unclear whether hypoxia or the re-oxygenation of the tissue following hypoxia contribute to the disturbances in spermatogenesis.

The testis is also susceptible to oxidative stress and expresses a number of antioxidants to protect its cells from oxidative damage. These include superoxide dismutase (SOD), glutathione reductase (GR), peroxidase and heme oxygenase 1 (HO1). Oxidative stress can be caused by the production of reactive oxygen species (ROS) during the reoxygenation of hypoxic tissue. In response to oxidative stress the heme oxygenase system plays an important role in cell protection and consists of heme oxygenase (HO)-1 (inducible) and -2 (constitutive) (Maines, 1997). Expression of HO1 in the testis has been shown to increase in patients with varicocele (Shiraishi and Naito, 2005) and also under hyperthermic conditions (Ewing and Maines, 1995; Maines and Ewing, 1996).
5.1.1 Aims of this chapter

The principal aim of this chapter was to characterise the stress response that occurs in the testis following a mild transient scrotal heat stress. This was achieved by investigating expression of a selection of genes and proteins that fall under three different categories of stress response, namely the heat shock response (Hsp105 and Stip1), apoptosis (caspase-3, caspase-9 and Bax) and oxidative stress (HO1). In addition expression of Hif1α mRNA and protein was quantified as a marker of hypoxic stress.
5.2 Materials and Methods

5.2.1 Animals and tissue recovery

The mice used in this chapter were C57BL/6 males aged approximately 8 weeks that had been subjected to scrotal heating at 38°C, 40°C and 42°C (see chapter 2, section 2.2). All mice were purchased from Harlan (Harlan Sprague-Dawley Inc, Oxford, UK). All animals were administered an anaesthetic and a reversal reagent according to their body weight (section 2.2.1) and their cages placed on a warm mat after each experiment to maintain body temperature and facilitate recovery. Control animals received anaesthetic and were left at room temperature. Animals were sacrificed at various time points after heating: 3h, 6h, 24h, 48h, 7d, 14d and 28d. Testes were fixed in Bouins for 8h before cutting them in half; tissues were stored in 70% (vol/vol) ethanol and processed into paraffin wax using standard procedures (section 2.3.1).

5.2.2 Immunohistochemistry

Immunodetection of mouse cleaved caspase-3, Hif1α and Stip1 were performed using standard procedures. Briefly, sections were dewaxed and rehydrated before being subjected to antigen retrieval using citrate buffer (section 2.4.2.1). Endogenous peroxidase activity and non-specific binding sites were blocked with methanol peroxide and normal goat serum (NGS) respectively (section 2.4.2.2). The primary antibodies (Table 5-1) were incubated with sections overnight at 4°C. Secondary antibodies were applied and detection was carried out using standard protocols as detailed in chapter 2, sections 2.4.2.4 and 2.4.2.5 respectively. All washes between incubations comprised 2 x 5 min at room temperature in Tris-buffered saline (TBS; 0.05 M Tris-HCl (pH 7.4), 0.85% NaCl). Negative control sections were incubated with blocking serum alone to confirm antibody specificity. Counts of caspase 3 positive germ cells were made on a Provis AX70 microscope (Olympus Optical, London, UK). This was achieved by counting the total number of positive cells in four testis sections, taken at least 50µm apart.


**Table 5-1 Summary of primary antibodies used for immunohistochemistry**

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>Source</th>
<th>Dilution</th>
<th>Host species</th>
<th>Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved caspase-3</td>
<td>Cell Signaling</td>
<td>1:200</td>
<td>Rabbit</td>
<td>Citrate</td>
</tr>
<tr>
<td>Hif1 alpha</td>
<td>Abcam</td>
<td>1:200</td>
<td>Rabbit</td>
<td>Citrate</td>
</tr>
<tr>
<td>Stip1</td>
<td>BD Biosciences</td>
<td>1:100</td>
<td>Mouse</td>
<td>Citrate</td>
</tr>
</tbody>
</table>

**5.2.3 Western blotting**

Protein quantification analysis was carried out following SDS-PAGE and Western blotting (section 2.7). Briefly, protein was extracted from frozen testes using RIPA lysis buffer (section 2.7.1) and quantified using the Biorad protein assay (Biorad Laboratories, Hemel Hempstead, UK, section 2.7.2). 20µg of each protein was loaded onto each well of an acrylamide gel (Invitrogen) and subjected to electrophoresis at 200V for 1h. Proteins were electro-transferred from the gel and immobilised onto a PVDF membrane. Proteins were subsequently detected on the membranes using specific antibodies (Table 5-2) as described in section 2.7.5. As a standardisation loading control, β-actin was used. Time did not permit more than a preliminary analysis of a small number of samples by Western blots and thus limits the conclusions.

**Table 5-2 Summary of primary antibodies used for Western blotting**

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>Source</th>
<th>Dilution</th>
<th>Host species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>Santa Cruz</td>
<td>1:200</td>
<td>rabbit</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Cell Signaling</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>HO-1</td>
<td>Abcam</td>
<td>1:500</td>
<td>rabbit</td>
</tr>
<tr>
<td>Hsp 105</td>
<td>Santa Cruz</td>
<td>1:200</td>
<td>rabbit</td>
</tr>
<tr>
<td>Stip1</td>
<td>BD Biosciences</td>
<td>1:500</td>
<td>mouse</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sigma</td>
<td>1:750</td>
<td>mouse</td>
</tr>
<tr>
<td>β-actin</td>
<td>Abcam</td>
<td>1:500</td>
<td>rabbit</td>
</tr>
</tbody>
</table>
5.2.4 Taqman QRT-PCR

RNA was extracted from frozen testes using the RNeasy Mini extraction kit (Qiagen, Crawley, UK) and quantified using the Nanodrop-1000 (Labtech International, East Sussex, UK) spectrophotometer as detailed in section 2.8.2. Random hexamer primed cDNA was prepared using the Applied Biosystems Taqman® reverse transcription kit (Applied Biosystems, Foster City, CA) as detailed in section 2.8.3. Quantitative PCR was performed on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the Assay-on-Demand gene expression™ system for Hif1 alpha (Cat no. Mm00468878) and the Roche Universal Probe Library™ for HO-1 (Table 5-3).

### Table 5-3 Primer/probe details for HO-1 expression analysis using the Roche Universal Probe Library™

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe sequence (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO1</td>
<td>AGGCTAAGACCCCTTCCT</td>
<td>TGTGTTCCTCTGACATCA</td>
<td>AGGAGCTG (17)</td>
</tr>
</tbody>
</table>

5.2.5 Statistical analysis

Results expressed as means and standard errors of the mean were analyzed using one-way ANOVA followed by the Bonferroni post hoc test, using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA).
5.3 Results

5.3.1 Heat induced hypoxia

5.3.1.1 Mild transient heat stress induces hypoxia

Analysis of the impact of heat stress on Hif1α mRNA expression using quantitative RT-PCR suggested that there was no effect on gene transcription in the 38°C or 40°C groups compared to controls (Figure 5.1). However, localised scrotal heating at 42°C induced a significant (5 fold) increase in Hif1α mRNA expression at 6h, this then proceeded to decrease to around 2 x that of controls and subsequently increased to 4 fold that of controls at 7d (Figure 5.1).

Testicular sections from the heat stress mice were immunostained with Hif1α to examine the pattern of testicular protein expression. Hif1α is localised to the cytoplasm at normal oxygen levels and under hypoxic conditions translocates to the nucleus. There appeared to be cytoplasmic staining in the interstitium and seminiferous epithelium of testes from both control and heated animals (Figure 5.2) and no nuclear immunostaining was observed in the control or the 38°C group (Figure 5.2A – F). However, nuclear staining was present in some germ cells in the 40°C group at 24h (Figure 5.2G, arrows), but not at 48h and 14d (Figure 5.2H and I). In the 42°C group there was a substantial increase in nuclear Hif1α immunostaining at 24h (Figure 5.2J) and a few positive nuclei persisted at 48h, although substantial germ cell loss had occurred at this time point (Figure 5.2K). There also appeared to be stronger staining in the Leydig cells of the heated testes compared to controls (Figure 5.2). It is noted that a number of cells within the testis were undergoing apoptosis and although apoptotic cells are notoriously ‘sticky’ in immunohistochemistry applications, it was assumed here that the nuclear staining was true as there were many visibly apoptotic cells which were Hif1α negative. This could be confirmed using a blocking peptide for the antibody, however this was not available at the time.
Figure 5.1 Hif1 alpha mRNA expression in testes from adult mice subjected to transient scrotal heat stress and harvested between 3h and 28d later. ***(P<0.001) and *(P<0.05) indicates significant variation compared with wild type littermates (n≥3).
Chapter 5  
Mild scrotal heat stress and the stress response

Figure 5.2 Immunodetection of Hif1 alpha on testicular cross sections from adult mice subjected to transient scrotal heat stress and harvested 24h, 48h and 14d later. Nuclear staining of Hif1 alpha is highlighted with arrows in testes from control mice (A-C) and those heated to 38°C (D-F), 40°C (G-I) and 42°C (J-L). Bar=100µm.

5.3.1.2 Heme oxygenase 1 (HO1)

The impact of heat stress on expression of \( HO1 \) mRNA, which is reported to be expressed in Sertoli cells and Leydig cells after heat shock (Maines and Ewing, 1996) was measured using quantitative RT-PCR. A slight increase in expression was detected at 3h and 6h compared to controls (Figure 5.3). In the 42°C group
expression was still increased significantly at 24h but by 48h mRNA levels in all three groups had decreased to almost control levels (Figure 5.3). Western blot analysis was carried out to quantify expression of HO1 protein. The resulting western blot appeared to have some non-specific staining (extra green bands in Figure 5.4A) and a blocking peptide could be used to control for this however there was not one available at the time of this study for this antibody. In the 38°C group there was no real change in the amount of HO1 total protein at any of the time points investigated. However in the 40°C and 42°C groups there was a 120% and 80% increase in the amount of HO1 by 24h respectively (Figure 5.4B). It was unexpected that the lower of the two temperatures resulted in the measurement of a larger amount of total HO1 protein. Expression had returned to normal levels by 14d in all samples.

![Figure 5.3 HO1 mRNA expression in testes from adult mice subjected to transient scrotal heat stress and harvested between 3h and 28d later. * (<0.05) indicates significant variation compared with wild type littermates. For heated samples n=≥3 except for time points 14d and 28d where n=2.](image)
Figure 5.4 HO1 protein expression in adult mice subjected to transient scrotal heat stress and harvested 6h, 24h, 48h and 14d later. A) Western blot analysis of HO1 protein expression. B) Quantitative analysis of protein expression normalised to the loading control β-actin and expressed as fold difference relative to protein samples from control mice run on the same gel, each bar represents n=2.
5.3.2 Stress response in the testis

5.3.2.1 Stip1

Testicular sections were immunostained for Stip1 (Figure 5.5). This protein is known to be expressed in the mouse testis and has been localised to the cytoplasm of all types of spermatogonia and spermatocytes (Mizrak et al., 2006). In the current study immunopositive staining of Stip1 was found to be most intense in the spermatocytes and some low level staining in the spermatids was observed in control testes and those subjected to heat stress. However, it was unclear whether there was an increase in protein expression after heat stress when analysing the immunostaining alone and so Western blotting was performed to quantify this (Figure 5.6A). There appeared to be a pattern of change in expression level after heat stress with an increase at 6h, then gradually decreasing until 48h and an increase again by 14d, though unexpectedly the highest increase, of around 50%, was observed in the 38°C group at 6h (Figure 5.6B). All three groups had returned to control levels by 48h but exhibited a similar increase (between 40 and 50%) in Stip1 expression at 14d (Figure 5.6). The error bars however are very large and it is not possible to conclude any definite pattern as low numbers of animals were studied (n=2) in the quantification analysis and germ cell loss was most extensive in the 42°C group.
Figure 5.5 Immunodetection of Stip1 on testicular cross sections from adult mice subjected to transient scrotal heat stress and harvested 24h, 48h and 14d later. Control mice (A-C) and those heated to 38°C (D-F), 40°C (G-I) and 42°C (J-L). Bar=100µm.
5.3.2.2 Hsp105

Hsp105 has been localised to pachytene spermatocytes and early spermatids in control testes and is reported to translocate from the cytoplasm to the nucleus after heat stress (Kumagai et al., 2000). Western blotting was used to detect any changes in level of protein expression following heat stress. In all samples there were 2 bands at the correct molecular weight as this antibody also detects Apg-1 and 2. As the bands for the two proteins did not appear to differ in intensity and the fact that they were so close together made it difficult to distinguish between them and so they were pooled to give one value in the expression analysis. Scrotal heating at all three temperatures appeared to increase expression of Hsp105 in the mouse testis (Figure 5.7). No distinct pattern of increase was observed in each of the three temperature

Figure 5.6 Stip1 expression in testes from control and heated mice. A) Western blot analysis of Stip1 protein expression. B) Quantitative analysis of protein expression normalised to the loading control β-actin and expressed as fold difference relative to protein samples from control mice run on the same gel, each bar represents n=2.
groups, however they all showed an increase at 6h compared to controls and a return to control levels by 14d in the 38°C and 42°C groups (Figure 5.7B). Though there was a decrease by this stage in the 40°C group, levels had not quite reached that of the controls (Figure 5.7). This data was based on a small number of samples per time point (n=2). It is therefore, not possible, to make any definitive conclusions without further study.
**Figure 5.7 Expression of Hsp105.** Protein expression in testes from adult mice subjected to transient scrotal heat stress and harvested 6h, 24h, 48h and 14d later. A) Western blot analysis of Hsp105 protein expression. B) Quantitative analysis of protein expression normalised to the loading control β-actin and expressed as fold difference relative to protein samples from control mice run on the same gel, each bar represents n=2.

### 5.3.3 Heat induced apoptosis

#### 5.3.3.1 Bax

Testicular protein samples were analysed for changes in Bax expression after scrotal heating. Bax is a proapototic protein and is thought to modulate the intrinsic mitochondrial pathway (Jurgensmeier et al., 1998). Heating at all three temperatures appeared to induce Bax expression in the testis (Figure 5.8). Bax expression increased at 6h by 30%, 30% and 40% in the 38°C, 40°C and 42°C groups respectively. This was followed by a slight increase in the 38°C group at 24h, however the 40°C group exhibited decreased expression back to control levels and
42°C showed a decrease in expression of around 10% followed by a return to control levels by 48h (Figure 5.8B). The early changes in Bax expression are consistent with its role as an early response protein although additional samples are required to confirm this.
Figure 5.8 Bax protein expression in adult mice subjected to transient scrotal heat stress and harvested 6h, 24h, 48h and 14d later. A) Western blot analysis of Bax protein expression. B) Quantitative analysis of protein expression normalised to the loading control β-actin and expressed as fold difference relative to protein samples from control mice run on the same gel, each bar represents n=2.

5.3.3.2 Caspase-9

Caspase-9 is also involved in the apoptosis pathway. This protein is part of the intrinsic mitochondrial pathway that responds to stress stimuli. Western blotting was performed for caspase-9 to determine whether heat stress induced expression of this protein (Figure 5.9A). The antibody used detects both the procaspase-9 and the cleaved forms at 39 and 37 KDa. The two bands that appear in Figure 5.9A were assumed to be the cleaved or activated caspase-9 as they were approximately the above sizes. There appeared to be no distinct pattern for caspase-9 induction with respect to temperature though there was an increase in expression in all three groups compared to controls (Figure 5.9B). In the 38°C group expression increased by
around 30% at 6h, had increased by around 70% at 24 and 48h and by 14d had returned to control levels. In the 40°C group, again caspase-9 expression had increased at 6h but by around 90% and then returned to control levels at 24h only to gradually increase again (Figure 5.9). In the 42°C group a 35% and 45% increase were observed at 6h and 24h respectively followed by a decrease to control levels at 48h only to increase again at 14d (Figure 5.9). These results need to be validated by analysing more samples as the group sizes in this study were small (n=2).
Figure 5.9 Caspase-9 protein expression after heat stress in adult mice subjected to transient scrotal heat stress and harvested 6h, 24h, 48h and 14d later. A) Western blot analysis of caspase-9 protein expression. B) Quantitative analysis of protein expression normalised to the loading control β-actin and expressed as fold difference relative to protein samples from control mice run on the same gel, each bar represents n=2.

5.3.3.3 Cleaved caspase-3 immunostaining

Testicular sections were stained for cleaved caspase-3 to detect any cells that were undergoing apoptosis (Figure 5.10). The majority of cells seen to be undergoing apoptosis were spermatogonia. All three temperatures tested (38, 40 and 42°C) appeared to induce apoptosis via caspase-3. In testes harvested 24h after a 38°C heat stress there was a 14 fold increase in the number of positive cells and by 48h this had decreased to only 2 fold (Figure 5.11). This was also the case in the 40°C group, which exhibited an 11 fold increase at 24h and had decreased to 3 fold by 48h (Figure 5.11). In the 42 °C group there was already a 9 fold increase by 6h. At 24h there was a substantial 27 fold increase in the number of apoptotic cells. This was
still more than 10 times that of control at 48h (Figure 5.11). These results do not, however, take into account stage distribution of spermatogenesis. For example stages 7 and 8 in the mouse are longer and therefore a cross section of the testis will have a higher proportion of these than other stages. It would therefore be of use to determine the stages at which apoptosis was occurring, however, this was not carried out in this study.

Figure 5.10 Detection of apoptotic cells. Immunodetection of caspase-3 positive germ cells in control mice (A-C) and those heated to 38°C (D-F), 40°C (G-I) and 42°C (J-L). Positive cells undergoing apoptosis are highlighted with arrows. Bar = 50μm.
Figure 5.11 Quantification of caspase-3 positive germ cells. Counts of caspase positive germ cells in four testis sections per animal (n=4). An ***(P<0.001) indicates significant variation compared with wild type littermates.
5.4 Discussion

As discussed previously in this thesis, most mammals require that the temperature of the testis be kept between 2°C and 8°C below core body temperature (Harrison and Weiner, 1948) and this lower temperature is required for normal spermatogenesis. There have been several studies in mice looking at the effects of heat stress on the testis and these report germ cell loss, poor quality sperm with altered DNA integrity and chromatin packaging as well as early embryo loss (Banks et al., 2005; Jannes et al., 1998; Rockett et al., 2001; Zhu and Setchell, 2004). Results reported in the previous chapter, revealed that heat stress results in DNA damage specifically in spermatocytes (γH2AX positive DSB foci) and confirmed previous reports of germ cell loss and altered DNA integrity in sperm (SCSA results). However it is still unclear what the underlying causes of these effects on testicular function are. One possibility is that the testis may be becoming hypoxic as a result of heat stress and inadequate blood flow into the testis. The principal aim of this chapter was to gain a better understanding of the stress response to testicular/scrotal hyperthermia and to determine whether the testis was becoming hypoxic. More specifically, a number of different genes and proteins were investigated that are involved in the response to hypoxia (Hif1α), the stress response (Stip1, Hsp105 and HO1) and the intrinsic pathway of apoptosis (Bax, Caspase-9 and Caspase-3).

Hypoxia is a well-characterised cellular stress that occurs when oxygen tension drops below that required for normal cellular function (Hockel and Vaupel, 2001) and can result both from inadequate blood flow to tissues or disrupted oxygen transport capacity. Part of the response to hypoxia in mammalian cells is the upregulation of a number of genes that encode both tissue specific and ubiquitous proteins (Helfman and Falanga, 1993). The hypoxia-inducible factor (Hif)-1 is a ubiquitously expressed transcriptional regulator of many genes in response to hypoxia. Hif1 is a heterodimer and consists of an oxygen-regulated α subunit and an oxygen-independent β subunit. Under hypoxic conditions Hif1α translocates from the cytoplasm to the nucleus and dimerises with Hif1β (Chilov et al., 1999; Hofer et al., 2001). Thus nuclear
localisation of Hif1α can be used as a marker of hypoxia. In this study immunolocalisation of Hif1α was analysed in testes heated previously to 38°C, 40°C and 42°C. Immunoreexpression in the control was in the cytoplasm as expected. This was also the case for the 38°C group, however there were a very small number of positive nuclei in the 40°C group. This was in contrast to the localisation in the 42°C group, which showed a dramatic increase in nuclear immunostaining at 24h suggesting that this temperature caused some degree of hypoxia. This data was accompanied by quantitative mRNA analysis of Hif1α expression, which was significantly increased by 6h after heat stress but again only in the 42°C group. It is thought that the reason that the testicular tissue becomes hypoxic is the heat-induced increase in metabolism is not being matched by sufficient increase in blood flow to maintain an adequate level of oxygen and thus can cause heat-induced hypoxia. As the lower temperatures did not result in a similar increase in the amount of Hif1α mRNA it suggests that the lower temperatures are not sufficient to cause hypoxia possibly because they do not enhance metabolism to the same degree as the higher temperatures. A study on the effects of local heating on testicular blood flow published around 20 years ago demonstrated that exposure to temperatures of 42°C and above resulted in the significant reduction in testicular blood flow in the rat (Galil and Setchell, 1988) though other studies on the ram showed an increase in blood flow with temperature (Mieusset et al., 1992b) and therefore further investigations are required to confirm whether the current experimental protocol had an impact on blood flow or not.

It is unclear whether hypoxia itself causes DNA damage or the reoxygenation of the tissue, as previous reports have suggested reoxygenation can cause a significant amount of DNA damage (Hammond et al., 2003). Although hypoxia is not thought to cause DNA damage directly (Koumenis et al., 2001) it is possible that it has an indirect role here. It has been shown previously that under hypoxic conditions H2AX is phosphorylated by ATR to form the distinct nuclear foci (Hammond et al., 2003) comparable to those reported in chapter 4, which is suggestive of some kind of
hypoxia-induced chromatin damage. In addition to this, recent studies have reported that hypoxia suppresses DNA repair pathways such as the mismatch repair pathway (MMR) and the homologous repair pathway HR (Francia et al., 2005; Mihaylova et al., 2003) and reviewed in Bindra et al., 2007). Hypoxia has been shown to downregulate the expression of the MMR genes, MLH1 (Mihaylova et al., 2003) and MSH2 (Koshiji et al., 2005). Koshiji et al. (2005) demonstrated that HIF-1α inhibits production of MSH2 and MSH6, thereby decreasing levels of the MSH2-MSH6 complex, which is involved in repair of replicative mismatches, resulting in genetic instability and this study has already shown that MSH2 deficiency can have an impact on testicular function (chapter 3). In addition to this, a study by Rockett et al. (2001) demonstrated a down regulation of further DNA repair genes after heat stress, for example heat stress of 43°C caused decreased expression of XPG and XPC, which are involved in nucleotide excision repair (NER). This impaired DNA damage repair could account for the increase in DSBs seen in spermatocytes in chapter 4. It is also the subject of debate as to whether hypoxia induces the production of reactive oxygen species (ROS) or not (Waypa and Schumacker, 2002; Waypa and Schumacker, 2005) this is relevant because it is known that ROS can cause DNA damage both in somatic cells (Halliwell and Aruoma, 1991) and sperm (Lopes et al., 1998b). It may however be the reoxygenation of the tissue that results in the production of ROS (Li et al., 2002).

Hif1 has also been reported to mediate the activation of heme oxygenase (HO)-1 in response to hypoxia as HO1 contains specific sequences, which can bind Hif1 and mutation of these sequences abolishes Hif1 binding and hypoxia-induced gene activation (Lee et al., 1997). HO1 is known to be part of the response to oxidative stress and plays a protective role during these conditions (Abraham et al., 1995). Previous studies have shown increased expression of HO1 in Sertoli and Leydig cells in the heat stressed rat (Ewing and Maines, 1995; Maines and Ewing, 1996) and also in patients with varicocele (Shiraishi and Naito, 2005). In the current study heat stress at 40°C and 42°C resulted in an increased protein expression compared to controls though it was surprising that the lower of the two temperatures gave the
higher induction. This might be consistent with changes in protein turnover at the higher temperature and might suggest that positive cells (Sertoli and Leydig) were lost through apoptosis. However these results may be an artefact of the small groups used in this study and further experiments should be carried out to confirm these findings. Quantitative mRNA analysis of HO1 expression was also performed and this showed increased expression not only in the 40°C and 42°C groups but also slightly in the 38°C group. However these results were surprising as Hif1α is described as upregulating HO1 and no increase in Hif1α expression was observed at 38°C and in the higher temperatures HO1 seems to increase prior to Hif1α. This may imply that the upregulation of HO1 in the testis is independent of Hif1α (hypoxia) but could also occur in response to the production of ROS or some other free radicals. HO1 is a known antioxidant and previous studies have reported an increase in ROS after heat stress (Ikeda et al., 1999).

Programmed cell death or apoptosis occurs normally during spermatogenesis in order to maintain a balance between germ cells and Sertoli cells. However, heat stress has been shown previously to increase apoptosis and cause germ cell loss (Hikim et al., 2003; Miura et al., 2002). In the previous chapter an increase in TUNEL positive, presumptive apoptotic germ cells was recorded. This study demonstrates an increase in caspase-3 immunostaining as determined by counts of caspase-3 positive germ cells confirming that there is a heat-induced increase in apoptosis. However as caspase-3 is involved in more than one apoptosis pathway this data does not clarify which pathway is involved. In order to further characterise which pathway is being induced upon heat stress in the testis the expression of two further proteins was determined (Caspase-9 and Bax).

There are two main pathways of apoptosis, intrinsic and extrinsic (Hengartner, 2000; Reed, 2000). The intrinsic pathway involves the mitochondria and the release of cytochrome c into the cytoplasm where it binds apoptotic protease activating factor 1 (Apaf 1). This activates the initiator caspase-9 which subsequently activates the executioner caspases 3, 6 and 7 (Orrenius, 2004). The extrinsic pathway involves
the ligation of the death receptor Fas to its ligand FasL, which recruits the Fas-associated death domain (FADD). This complex then binds to the initiator caspase-8 or 10 which then go on to activate the executioner caspases-3, 6 and 7 (Zimmermann et al., 2001). Thus both pathways have caspase-3 activation in common. There are, however conflicting views on the induction of apoptosis following heat stress in the mouse testis. For example, some studies suggest it is the intrinsic mitochondrial pathway that is induced after heat resulting in the activation of the initiator caspase-9 and the executioner caspase-3 and that the Fas-FasL pathway is redundant (Hikim et al., 2003; Vera et al., 2004). However, others have suggested it is primarily the Fas signalling pathway that is induced upon heat stress in the mouse testis (Miura et al., 2002). It is possible that both pathways play a role in the elimination of germ cells after heat stress. The apoptotic response to stress is usually mediated through the mitochondrial or intrinsic pathway, which is modulated by proteins from the Bcl2 family. Bcl2 inhibits apoptosis whereas Bax, another member of the Bcl2 family, induces apoptosis (Adams and Cory, 1998; Borner, 2003). Also p53, which is highly expressed in the testis, is thought to mediate apoptosis in response to diverse stimuli including hypoxia, oxidative stress, and DNA damage (Fridman and Lowe, 2003). It has been shown that p53 can induce Bax expression and downregulate Bcl2 thereby promoting apoptosis (Miyashita et al., 1994). In this study changes in protein expression of Bax following heat stress was found to be very slightly increased compared to controls. This does not preclude a role for Bax as heat stress may not induce an increase in total protein concentrations but could have an impact on the distribution of Bax as this is believed to be important (Yamamoto et al., 2000). Previous studies have shown that a redistribution of Bax upon heat stress is accompanied by translocation of cytochrome c and activation of caspase-9 and this merits further investigation in the current model system.

This study has also looked at the expression of caspase-9, which is also involved in the intrinsic mitochondrial pathway. Preliminary analysis (n=2) of total caspase-9 protein in testes subjected to 38°C, 40°C and 42°C heat stress showed a slight increase in amounts compared to controls though there was no distinct time or
Chapter 5 Mild scrotal heat stress and the stress response

Temperature dependent pattern and therefore it is necessary to increase the numbers of samples studied. The apparent increase in Bax and caspase-9 (around 6h) and the increase in caspase-3 at 24h are consistent with the order of activation of each protein in this pathway. These results point to the involvement of the intrinsic pathway of apoptosis but this does not rule out the involvement of other pathways. Complementary studies need to be performed on the same data set to examine expression of FAS and caspase-8 in order to determine whether one or both pathways were activated by heat stress.

Heat stress induces the expression of heat shock proteins (Hsps), which function as molecular chaperones that bind to, and aid, the folding of damaged proteins, thereby preventing protein misfolding and aggregation (Hartl and Hayer-Hartl, 2002; Young et al., 2003). Hsp105 is a testis specific Hsp90-related protein and has previously been shown to exhibit increased expression in the rat in response to experimental cryptorchidism (Kumagai et al., 2000). This protein is localised to the cytoplasm under normal conditions but translocates to the nucleus following heat stress (Kumagai et al., 2000). In the current study Hsp105 protein expression was increased in the mouse in comparison with controls after heat stress at all three temperatures investigated. It has been shown previously that heat stress caused by induced cryptorchidism causes Hsp105 to bind to p53 and is a possible mechanism of inducing apoptosis (Kumagai et al., 2000). In addition to this, Rockett et al. (2001) showed, using microarrays, that Hsp40, Hsp60 and Hsp70 are upregulated only a few hours after heat stress. Another stress response protein, Stress inducible protein (Stip)-1, is expressed in the mouse testis (Mizrak et al., 2006) and has been shown to directly regulate the function of two Hsps (Hsp70, Hsp90) (Song and Masison, 2005) in part by acting as a chaperone of the same proteins (Odunuga et al., 2004; van der Spuy et al., 2001). In the current study immunoexpression of Stip1 on testis sections from heated mice showed the expected localisation as detailed by Mizrak et al. (2007). This group had suggested that it played a role in heat sensitivity and therefore protein quantification after heat stress was carried out. The results surprisingly suggested that Stip1 was more abundant in the 38°C group compared with the testes
obtained from the other groups. This may be explained in part by the fact that many of the germ cells that express Stip1 are undergoing apoptosis and being lost at the higher temperatures but this does not explain why there is not a comparable increase at 6h (after stress at 40°C and 42°C) as the Stip1 positive germ cells do not appear to be dying until 24h after heat stress. Further studies are required to validate the preliminary findings.

5.5 Conclusions

This study has already shown that spermatogenesis is susceptible to increased scrotal temperatures and results in apoptosis, spermatocytes displaying DSBs (γH2AX foci) and the production of mature sperm with impaired DNA integrity (chapter 4). However, it was unclear what mechanism was causing the DNA damage and via which mechanism the germ cells were dying. This chapter has presented data that would support previous suggestions that the testis may be becoming hypoxic following heat stress by demonstrating an increase in the hypoxia-induced gene, Hif1α. The results from this study also demonstrate that one pathway involved in heat-induced apoptosis is the mitochondrial intrinsic pathway and is associated with increased expression of proteins (Caspase-9 and Bax) specific to this pathway. However, due to time constraints, a general weakness of the Western blot study was the small group sizes which did not permit more than a preliminary analysis of the chosen proteins.
6 Final Discussion

Infertility represents a major clinical problem and the contribution of the male partner is significant. Although there is no cure for men who produce no sperm (azoospermic), if some sperm or post-meiotic germ cells are present, ART such as ICSI is commonly used. As mentioned in chapter 1 there are a number of concerns over using sperm from infertile men as they often contain high levels of damage (Irvine et al., 2000), which could result in the introduction of damaged DNA into the embryo and cause problems for the offspring later in life, for example an increased incidence of cancer (Ji et al., 1997).

In developing germ cells DNA repair is required for both meiotic recombination and the correction of DNA damage (Baarends et al., 2001). A number of studies have used mouse models to investigate the effects of deficiencies in DNA repair pathways though not many have demonstrated a comprehensive characterisation of spermatogenesis in these models. In addition there have been various studies of the impact of stresses such as heat on spermatogenesis. However, few previous studies have undertaken a detailed examination of the effects of a range of different temperatures on testicular function and fertility and most have used either a single temperature or a series of temperatures but only one end-point. Therefore the main objective of this study was to provide a better understanding of the origins and consequences of DNA damage in the male germ line by studying a panel of mouse models defective in key DNA repair pathways and by using transient heat stress to exacerbate DNA damage. In order to achieve this the following aims were set: 1) to characterise testicular function in three contrasting mouse models with targeted ablation of genes involved in DNA repair; 2) to characterise the effects of increased scrotal temperature on testicular function, DNA integrity and fertility; 3) to determine the mechanisms of heat induced DNA damage by examining the stress response in the testis including hypoxia and apoptosis related pathways.
6.1 DNA repair pathways and testicular function

Proteins involved in mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), single strand break repair (SSBR) and double strand break repair (DSB) are all expressed in the testis (Jaroudi and SenGupta, 2007). The present study compared the integrity of DNA in germ cells and sperm from mice with defects in three different genes the products of which are involved in DNA repair or DNA damage response pathways. These had deletions in *Ercc1*, *Msh2* and *p53*. One of the main hypotheses of this study was that deficiency in the DNA repair proteins (*Ercc1* and *Msh2*) would result in a significant disruption of spermatogenesis resulting in infertility. Though this was true for the *Ercc1*-deficient mice, it was not the case for those that were *Msh2* deficient. Although a relatively detailed study of the testicular phenotype in *TG-Ercc1*+/- mice had previously been performed (Hsia et al., 2003), the current study has expanded on, and provided new insight into, this transgenic model by demonstrating for the first time that depletion of *Ercc1* from germ cells results in an increased incidence of un-repaired DSBs in pachytene spermatocytes (as determined by γH2AX immunostaining) and this was associated with an increase in the number of apoptotic germ cells. The detection of DSBs in pachytene spermatocytes and abnormal chromatin structure in mature sperm confirmed that the repair functions of *Ercc1* are essential for normal germ cell maturation and suggest that none of the other NER proteins can compensate for its ablation. In contrast, the *Msh2*-deficient testes did not contain spermatocytes with increased numbers of DSBs nor was there an obvious increase in apoptotic germ cells in adulthood though some evidence of disrupted spermatogenesis (SCOs and gaps in seminiferous epithelium) was documented, these results are entirely consistent with the reported function of *Msh2* as it is involved in repairing small base mismatches and not DNA breaks. Prior to the data presented in the current study there had been no indication that *Msh2*+/- male mice had any disturbance in testicular function. In addition, due the fact that a slight increase in caspase-3 positive germ cells in pre-pubertal mice was observed, it appears that the presence of *Msh2* may be more critical during the first wave of spermatogenesis when the process is less
efficient. Therefore it would be of interest to investigate the role of Msh2 starting with analysis of protein levels during the first wave of spermatogenesis.

In relation to the p53-deficient mice, the hypothesis of this study was that deficiency in the checkpoint protein p53 could result in an increase in the proportion of damaged germ cells progressing to mature spermatozoa with the result that more spermatozoa with DNA abnormalities would be detected. Previous studies have reported a range of testicular phenotypes in p53<sup>−/−</sup> mice that appeared to be strain dependent (see chapter 3). The present study is the first to report the phenotype of p53<sup>−/−</sup> male mice on a CBA background and to demonstrate an increased incidence of DSBs in pachytene spermatocytes, an increase in the level of damaged sperm using the SCSA and an increase in apoptosis in these mutant mice. These studies have further confirmed that p53 is more than just a ‘checkpoint’ gene, on this genetic background, and confirms previous data suggesting a role in HR/NER (Linke et al., 2003; Seo and Jung, 2004; Sjoblom and Lahdetie, 1996; Tang et al., 1999). It would therefore be of interest to investigate whether there were any changes in the activity of these pathways in these mutant mice. Although these studies have shown an increase in DNA damage in spermatocytes in the TG-Ercc1<sup>−/−</sup> and p53<sup>−/−</sup> mice using γH2AX immunostaining, it would be worthwhile investigating the expression of other proteins involved in the DNA repair pathways as an indicator of DNA damage, for example, Rad51 and DMC1 (see chapter 1, section 1.3.3). During these studies attempts were made to localise these proteins to sites of damage on meiotic spreads prepared from the testes of these mouse lines, however this was unsuccessful due to technical difficulties and had to be abandoned due to lack of time.

The present studies demonstrated a slight increase in caspase-3 positive germ cells in p53<sup>−/−</sup> mice, however this disagrees with previous studies, which have reported that caspase-3-mediated apoptosis is entirely p53 dependant (Erster et al., 2004) especially in spermatogonia (Odorisio et al., 1998). In the current study, the majority of cells in the p53<sup>−/−</sup> that were caspase-3 positive appeared to be spermatogonia suggesting that in our mice apoptosis does not depend solely on p53 and points
towards the existence of a p53 independent pathway in spermatogonia. Follow up studies could investigate whether other components of the apoptotic pathway are upregulated in these mice. It may also be of interest to further confirm the caspase-3 immunohistochemistry results by performing Western blot analysis to determine any changes in protein levels.

6.2 Scrotal heat stress, DNA damage and male fertility

The principal aim of this part of the study was to complement and extend previous studies on the effects of increased scrotal temperature on testicular function and fertility using a mouse model system. This was carried out by looking at three different temperatures (38°C, 40°C and 42°C) to determine whether there was increasing disruption to testicular function with increasing temperature. Another aim was to examine the integrity of DNA in spermatocytes and determine the effect of sperm resulting from these spermatocytes on fertilisation, embryo development and survival using natural matings and IVF. These aims were fulfilled and results demonstrated increasing adverse effects on the testis with increasing temperature. The main findings were that increased scrotal temperature caused germ cell loss and increased apoptosis, impaired DNA repair in pachytene spermatocytes, altered sperm parameters such as abnormal morphologies and increased incidence of sperm with DNA damage, reduced fertility and abnormal development of embryos (see chapter 4). It is acknowledged that some of the effects observed within the testis may not be the result of the direct action of heat on the testis as a third of the body was heated as opposed to only the testes and this would obviously result in changes to respiration throughout the body. However the purpose of this study was to mimic a warm bath for humans, which does indeed involve exposing a significant area of the body to the increased temperature.

These studies demonstrated that the testis, despite only being heated for 30 min, required a lengthy period of recovery (more than 4 weeks). As discussed in chapter 4, this highlights the possibility that there are not only primary acute effects but also
long term secondary effects of heat on the testicular environment. It would therefore be of interest to investigate the effect of heat on the supporting cells within the testis i.e. the Sertoli cells and the Leydig cells and also the products of these cells. For example, following the acute stress there may be long-term changes in production of hormones such as testosterone. Notably, whilst the present study was underway a paper was published stating that increased testicular temperature caused by induced cryptorchidism can result in lowered levels of plasma testosterone, LH, FSH and inhibin B (Ren et al., 2006) and other earlier studies in rats subjected to scrotal heating in a water bath recorded reduced testicular testosterone levels (Lue et al., 1999). Although the current studies revealed heat-induced germ cell loss using Mvh and p63 immunostaining as well as H&E staining (see chapter 3), with hindsight it would have been beneficial to record testis weights in these mice as this would not only have provided further verification of germ cell complement but would also have allowed calculations of changes in numbers of Sertoli and Leydig cells after heat stress, which may have provided some evidence of an impact on somatic cells. One gene known to be androgen regulated in Sertoli cells is rhox5 (Pem) (Rao et al., 2003; Sutton et al., 1998) and therefore in future experiments measurement of this mRNA could be used as a way of assessing whether intratesticular androgen action is impaired.

Although the current studies showed slight epididymal effects at 38°C and further effects at higher temperatures i.e. on sperm counts and sperm with DNA damage, the effect of heat on the epididymis was not investigated in great detail. It would be worthwhile investigating epididymal protein changes by immunohistochemistry or Western blotting and also changes in candidate genes which are known to be induced by heat such as Hsps. It may also be worth carrying out a more global, microarray analysis to detect any changes in expression of genes that may not be obvious candidates. There are some previous studies, which have shown expression of some epididymal genes to be temperature-dependent. For example changes in epididymal gene products, such as CD52, have been demonstrated following induced cryptorchidism (Gebhardt et al., 1999; Kirchhoff et al., 2000; Pera et al., 1996).
As with the study on the mice deficient in DNA repair proteins (section 6.1) scrotal heat stress resulted in an increase in γH2AX foci (presumptive unrepaired DSBs) in pachytene spermatocytes. However attempts to localise other markers of DNA damage such as Rad51, DMC1 and members of the MRN complex (Mre11-Rad50-Nbs1), which is involved in strand resection (Dolganov et al., 1996; Dong et al., 1999; Petrini, 1999) were unsuccessful and therefore further analysis is required to establish when/where they are expressed.

One of the more puzzling aspects of this study were the results obtained using the SCSA to analyse sperm from the 42°C group. Rockett et al. (2001) had reported the most drastic effect on fertility was obtained when matings were carried out between 23-28 days after heat shock. In agreement with this we also showed reduced fertility when the males were used at this time point, however, our SCSA results suggest that at this time point sperm DNA damage was only raised a little above control levels (see chapter 4, Figure 4.11) and had apparently almost recovered compared with earlier time points. However, this may be explained by the fact that the SCSA detects alterations in chromatin condensation and packaging rather than DSBs.

Our studies investigated the impact of a very brief scrotal heat stress (30 min) on male fertility and successfully demonstrated that heating at 42°C not only reduced pregnancy rate, but in those females that became pregnant the litter size was significantly reduced and embryos developed abnormally. Other recent studies have examined the impact of a slight increase in temperature over a longer duration (36°C for 12h or 24h). For example, in a study where the entire body was exposed to 36°C for 24h, a reduction in the number embryos sired by heated males was recorded. IVF using sperm from males heated 7d (an epididymal or late spermatid effect) earlier showed reduced numbers of embryos developing from the 4-cell stage onwards and those from mice heated 21d (a spermatocyte effect) earlier resulted in a reduction from the 2-cell stage onwards (Zhu and Setchell, 2004). A further study looking at the effects of increased whole body temperature by exposing male mice twice to
36°C for 12h on each occasion demonstrated reduced sperm number, pregnancy rate and litter size with maximum effects seen 10 or 14d after heat stress (Yaeram et al., 2006). In the current study where heat stress was of limited duration and restricted to the lower third of the body, the only effect after exposure to 40°C was an increased rate of fetal resorptions. This increase was not observed at 42°C, where pregnancy rate was drastically reduced, suggesting that the embryos resulting from matings where the spermatocytes were subjected to a 40°C heat shock could develop past the ‘block’ that occurred in the 42°C group but then failed at a later stage so that the resorptions were detectable at e14.5.

IVF was also carried out to further investigate when the block in embryo development occurred. Using sperm from males stressed 16h and 23d earlier there was no change in fertilisation rate or the number of embryos that progressed to the 4-cell stage compared with controls. However there was a significant difference in the number of blastocysts recovered demonstrating that the embryos derived from ‘heated’ sperm failed between the 4-cell and blastocyst stages of development. It would therefore be of interest to scrutinise the different stages of embryo development more closely to determine the exact stage of failure e.g. 8-cell stage, 16-cell stage or morula. This could be achieved by increasing the number of observation times. It would also be of value to carry out microarrays using these embryos to determine if there are any differences in gene expression between these stages and if possible to identify any paternally derived genes with disturbances in timing/expression to determine which gene regulatory networks are disrupted in the embryos resulting from DNA-damaged sperm. It has been suggested that the paternal genome is important for the development of extraembryonic tissues. For example, one of the first studies on genomic imprinting showed that embryos with two female pronuclei could implant but underwent resorption and exhibited poor extraembryonic membrane and trophoblast formation (Surani et al., 1984). With reference to the present studies it would be interesting to examine placental development in females mated to 40°C and 42°C heat stressed males especially following the detection of increased resorption rate in the 40°C group. It may be
worth performing gene expression analysis on the placentae to determine any discrepancies between the heated and control mice. Our IVF studies also show that the deficits seen in testis function that were induced by heat stress did not have an impact on spermiogenesis or the ability to fertilise i.e. sperm capacitation was normal (or at least sufficient) as embryonic failure was after the fertilisation event. This further verifies the concerns of using sperm from infertile men in ICSI/IVF as sperm with DNA damage are obviously capable of fertilisation but could introduce errors into the embryo that would cause gene expression changes and genomic instability. A number of studies published during the course of this work have demonstrated differential expression of genes at different stages of mouse embryo development. For example, studies investigating gene expression in the preimplantation embryo have demonstrated a wave of reprogramming at the 4- to 8-cell stage (Hamatani et al., 2004; Wang et al., 2004). Further microarray analyses have shown specific changes in gene expression related to the stage of embryo development and have identified an upregulation of genes for cytoskeletal, cell adhesion and junction proteins. For example increased expression of Cadherin 1 and Claudin 7 occurred in morulae compared with 4-cell stage embryos. In addition genes involved in ion channels, membrane traffic and lipid metabolism were higher in blastocysts compared to morulae (Cui et al., 2007). Results from the current studies suggest that embryonic failure may occur following reactivation of the DNA-damaged paternal genome as they can progress at least to the 4-cell stage. However it is not clear cut when this activation occurs as previous studies have shown that paternal gene expression starts as early as the 1-cell fertilised embryo, however paternally derived protein is not synthesised until the 2-cell stage (Matsumoto et al., 1994) whereas others suggest that the paternal genome is not required before the 8-cell stage in mice (Renard et al., 1991). Additionally it is possible to speculate that there is differential expression of paternally derived genes at different stages and thus the reactivation of only few critical genes could cause the embryonic loss observed in the current studies.

In addition as a complement to the studies using induced testicular hyperthermia in wild type mice, it would also be of interest to determine the impact of heat stress on
the mice with deficiencies in DNA repair proteins described above (section 6.1). Although the frequency of individuals in the human population homozygous for any of these mutations will be very low, the frequency of heterozygotes could be much higher. Thus, it would be interesting to determine if heterozygotes that do not show any obvious impact on testicular morphology would be more susceptible to heat-induced DNA damage than wild types and if this would result in more drastic effects of heat-induced stress than on the control mice seen here. An obvious starting point for these experiments would be to study the $TG$-$Ercc1$ +/- as they are fertile with apparently normal testes but comet assays have detected increased DNA damage in sperm compared with that from wild type littermates suggesting that sperm maturation is sub-optimal (Banks et al., 2005).

### 6.3 Stress response in the testis

The results discussed above revealed that heat stress resulted in both short and long term effects on testicular function including DNA damage and cell death. However after these studies were performed it was still unclear what the underlying cause of these effects on testicular function was. As discussed previously, one possibility is that the testis is becoming hypoxic as a result of heat stress and inadequate blood flow into the testis. Thus, the principal aim of the final part of the study was to gain a better understanding of the stress response to testicular/scrotal hyperthermia and to determine whether the testis was becoming hypoxic. This was achieved by analysing protein and/or gene expression of a number of factors relating to hypoxia, stress response and apoptosis. The results obtained succeeded in demonstrating for the first time that the testis was indeed becoming hypoxic during a single acute heat stress. This was supported by the alterations in the expression/localisation of the hypoxia-sensitive protein Hif1α. A direct link between hypoxia and DNA damage was not investigated in this study. However, recent studies, some of which were published during the course of our investigations, have reported that hypoxia suppresses DNA repair by the mismatch repair (MMR) and homologous repair pathways (Francia et al., 2005; Mihaylova et al., 2003) and reviewed in Bindra et al, (2007). In addition,
hypoxia is reported to downregulate the expression of the MMR genes, *MLH1* (Mihaylova et al., 2003) and also *MSH2* (Koshiji et al., 2005). Koshiji et al. (2005) demonstrated that HIF-1α inhibits synthesis of MSH2 and MSH6 in cancer cells, thereby decreasing levels of the MSH2-MSH6 complex, which is involved in repair of replicative mismatches, resulting in genetic instability and the current study has already shown the effect of MSH2 deficiency in the testis (chapter 3). The studies by Koshiji et al, however, were not performed on the testis. It would thus be of value to investigate whether there are any changes in levels of MSH2 as a result of heat-induced hypoxia in the mice generated in our study.

The current results highlighted the possibility that the intrinsic pathway of apoptosis is involved in the removal of damaged germ cells following heat stress. However, due to the low animal numbers used (n=2) it will be necessary to repeat these studies with more animals to confirm whether changes in caspase-9 and 3 are significant and time/temperature dependent. Also, these results do not discount the involvement of other pathways of apoptosis in the heat-stressed testis. Thus further investigations should include expression analysis of factors involved in these other pathways such as caspase-8, FAS and FAS ligand and with higher numbers of animals. Furthermore as a recent study proposed that both the intrinsic pathway and the death receptor-dependent pathway are involved in heat-induced apoptosis by way of HSF1 activation (Vydra et al., 2006), expression of this factor should be determined. As it has been postulated that FAS-mediated pathways are involved in germ cell apoptosis induced by a paracrine mechanism via Sertoli cells (Francavilla et al., 2000) it is also important to investigate the effects of heat stress on the somatic cells within the testis something that has received little attention to date.

It is well known that heat stress induces stress-related proteins such as Hsps in a variety of tissues. This study looked at the expression of three stress related proteins: HO1, Hsp105 and Stip1. HO1 is expressed in the testis (Bauche et al., 1994) and has the capacity to act as an ROS scavenger. Expression analysis of HO1 suggested that the testis may be subjected to oxidative stress as a result of hyperthermia although
HO1 may also be induced by Hif1α (see chapter 5 discussion). Although ROS are involved in some processes of spermatogenesis such as maturation and capacitation (de Lamirande and Gagnon, 1993) (see chapter 1, section 1.3.1) if the balance between ROS generation and scavenging is disrupted i.e. the amount of ROS exceeds that of the ROS scavenging capacity, testicular function may be disturbed and consistent with this oxidative stress has recently been shown to cause depletion of germ cells (Jedlinska-Krakowska et al., 2006). Our studies also showed that total Hsp105 protein was increased in the testis in comparison with controls after heat stress at all three temperatures investigated. It has been shown previously that heat shock by way of induced cryptorchidism causes HSP105 to bind to p53 and is a possible mechanism of inducing apoptosis (Kumagai et al., 2000). It is thus possible that the expression of both HO1 and Hsp105 are contributing to the increased apoptosis seen in the testes in the current study though it would be worthwhile increasing animal numbers in the case of Hsp105 as these conclusions were based on n=2. Another puzzling aspect of this study was the data obtained from the Stip1 Westerns as the results surprisingly showed a higher Stip1 level in the 38°C group compared with the higher temperatures. However, differences in absolute amounts of protein between samples may be influenced by germ cell survival especially if the protein is expressed in those that are being lost. This would result in an underestimation at 42°C where germ cell loss is greatest.

It is interesting to note that for more than 75 years it has been reported that heat has an adverse effect on the testis. For example in 1922 it was suggested by Crew (Crew, 1922) that cryptorchidism caused disrupted spermatogenesis because it was at a higher temperature. Despite the numerous studies since, the reason that the testes function best at a lower temperature is still not fully understood.

### 6.4 General conclusions

In conclusion, DNA damage in the male germ line caused either by induced stress, or by targeted ablation of DNA repair genes, can disrupt testicular architecture, function
and therefore fertility. These data have demonstrated that deletion of \textit{Ercc1}, \textit{Msh2} and \textit{p53} can have differential but overlapping affects on germ cell function and sperm production and that increased scrotal temperature can cause subfertility in male mice. A summary diagram linking possible routes via which the observed changes in testicular function occur is shown in Figure 6.1. Our results have demonstrated that fertility is critically dependent on DNA integrity and point to a role for both the epididymis and the testis in maintaining favourable environments for production of normal mature sperm. This study has provided further confirmation of possible male-mediated effects on embryo survival and these findings should be taken into consideration when using sperm from infertile men in IVF/ICSI treatments where the normal quality control processes involved in fertilisation are bypassed.
Figure 6.1 Summary diagram of possible pathways of heat-induced disruption of spermatogenesis and subfertility.
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